290 P THE IMPORTANCE OF THE AMINO ACID PHENYLALANINE 107 FOR FUNCTION AND LIGAND RECOGNITION AT THE 5-HT3 RECEPTOR

L.J. Steward, F.G. Boess, J.A. Steele, B. P. Phipps, D. Liu & I.L. Martin, Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7.

The 5-HT_3 receptor is a member of the ligand-gated ion channel family. In the present study, we have characterised mutants of the 5-HT_3 receptor which were constructed to alter the phenylalanine residue at position 107. This complements our previous studies with the adjacent glutamate residue at position 106 (Steward et al., 1996).

The 5-HT_3A_L cDNA from NG108-15 cells was subjected to individual point mutations using the Altered Sites *in vitro* mutagenesis system (Promega). The altered cDNA was subcloned into the eukaryotic expression vector pRC/CMV and transiently expressed in HEK 293 cells by the calcium phosphate-DNA coprecipitation method. Radioligand binding and whole-cell patch clamp were used to determine the effect of the mutations on selective agonist and antagonist affinities.

Radioligand binding studies were performed on membrane homogenates in Hepes (10 mM, pH 7.5) from transiently transfected HEK-293 cells. Saturation analysis with [^{3}H]GR65630 (0.04 - 12 nM), in which metoclopramide (300 µM) was used to define specific binding, indicated that neither F107Y (0.23 ± 0.04 nM) or F107N (0.35 ± 0.08 nM) produced a significant change in Kd value compared to wildtype (WT, F107; 0.27 ± 0.03 nM; mean ± SEM, n = 3-10). Compared to WT, competition for [^{3}H]GR65630 (0.5-2 nM) binding demonstrated that 5-HT affinity decreased for F107Y (13-fold), while it increased 10-fold for F107N (Table 1). These changes were similar in direction and magnitude to those found in whole cell patch clamp determinations of mean EC_{50} values (95% confidence intervals; n = 5-11) were as follows: WT 1.16 (1.05-1.29) µM, F107Y 10.55 (9.09-12.25) µM and F107N 0.19 (0.15-0.25) µM. However it would appear that the Hill coefficient for F107N was decreased (0.98) compared to WT (1.94). This was not the case for F107Y (1.57), but both activation and deactivation were markedly slowed compared to WT. F107N showed activation characteristics similar to WT but both desensitisation and deactivation were considerably slower. [^{3}H]GR65630 competition studies with 5-HT_3 receptor antagonists showed no affinity changes for either mutant except for a 9-fold decrease in granisetron affinity for F107N (Table 1).

These data suggest that F107 is important for recognition and gating.

	WT	F107Y	F107N
5-HT	15.6 ± 3.2 (8)	203 ± 59 (5)	1.62 ± 0.41 (4)
2-Me-5-HT	173 ± 18 (9)	610 ± 81 (5)	41.66 ± 8.43(5)
mCPBG	5.01 ± 1.09 (6)	11.0 ± 1.2 (3)	2.36 ± 0.79(4)
Granisetron	0.28 ± 0.03(6)	0.35 ± 0.07(4)	2.43 ± 0.69 (5)
Ondansetron	0.58 ± 0.10 (5)	0.74 ± 0.14 (5)	0.74 ± 0.08 (5)
Renzapride	1.29 ± 0.17 (4)	0.85 ± 0.14 (3)	1.68 ± 0.17 (3)

Table 1. The affinities (Ki, nM; mean ± SEM (n)) of various compounds competing for [^{3}H]GR65630 binding WT data has been previously presented and is used for comparison (Steward et al., 1996).

This work was supported by MRC (Canada) and Glaxo (Canada). L.J.S. holds a MRC (Canada) Fellowship. We are grateful to Dr T.P. Blackburn for the gifts of drugs.

Steward, L.J., Boess, F.G., Steele, J.A. and Martin, I.L. (1996) *Br. J. Pharmacol.* 117, 96P.

---

291 P FUNCTIONAL AND BINDING STUDIES OF GLYCOSYLATION SITE MUTANTS OF 5-HT3 RECEPTORS

S.C.R. Lummis & E.J. Fletcher, Division of Neurobiology, LMB, Hills Road, Cambridge CB2 2QH and Dept of Zoology, University of Cambridge, Cambridge CB2 3EJ

5-HT_3 receptors, which are members of the family of ligand-gated ion channels, possess four consensus sequences for glycosylation. We have shown that glycosylation is important for the correct processing of 5-HT_3 receptors (Green et al., 1995) and other studies have demonstrated the importance of glycosylation for the proper maturation and function of nicotinic acetylcholine receptors (e.g. Sumikawa et al.,1988). In this study we examine the importance of the various putative glycosylation sites by removing them using site-directed mutagenesis, followed by radioligand binding and whole cell patch clamp of cells expressing the mutant receptors.

Full length 5-HT_3 receptor DNA was obtained from N1E-115 mRNA using PCR. Consensus glycosylation sequences were removed using site-directed mutagenesis. The sequences were inserted into the eukaryotic expression vector pRc/CMV and transfected into HEK 293 cells using calcium phosphate precipitation. Expression of receptors was confirmed using 5-HT_3 specific antibodies, and the mutants examined using radioligand binding studies and whole cell patch clamp.

Antibody labelling demonstrated that all the mutant receptors were expressed. Radioligand binding with the 5-HT_3 antagonist [^{3}H]granisetron revealed that only one of the glycosylation sites, N191, was essential for binding. The K_d values obtained from the other mutants were not significantly different, although B_{max} values were lower in cells expressing mutants N107D and N175S (Table 1).

Table 1.

	WT	N107D	N175S	N191S	N378S
K_d (nM)	0.70±0.21	0.43±0.14	0.52±0.18	-	0.58±0.22
B_{max} (% WT)	100	4.8±2.1*	5.0±1.0*	-	53.7±27.1

Values are mean ± s.e.mean, n = 4. * sig diff from WT, $p<0.05$.

Whole cell patch clamp revealed that all of the mutants except N191S were functional. The EC_{50} for 5-HT for mutant N175S was lower than WT, and maximal currents evoked were lower in mutants N107D and N175S (Table 2).

Table 2.

	WT	N107D	N175S	N191S	N378S
EC_{50} (µM)	1.6±0.02	1.5±0.2	3.2±0.6*	no response	1.2±0.2
R_{max}	953±145	531±64*	359±43*		1011±184
n	16	9	8	10	9

Values are mean ± s.e.mean, * sig diff from WT, $p<0.05$.

The results show that N191 is essential for binding and function of 5-HT_3 receptors, although its removal does not prevent expression. Glycosylation at N107 and N175 may be required for efficient maturation. Removal of the potential glycosylation site N378 has no apparent effect on receptor expression or function.

Green, T., Stauffer, K.A. & Lummis, S.C.R. (1995) *J. Biol. Chem.* 270, 6056-6061
Sumikawa, K., Parker, I. & Miledi, R. (1988) *Mol. Brain. Res.* 4, 191-199.

SCRL is a Royal Society University Research Fellow. We also acknowledge support from the Wellcome Trust.

292 P STRUCTURAL DETERMINANTS OF THE ANTAGONIST POTENCY OF (+)-TUBOCURARINE AT RECOMBINANT 5-HT$_3$ RECEPTOR SUBUNITS

A.G. Hope, D. Belelli, J.J. Lambert & J.A. Peters. Neurosciences Institute, Dept. Pharmacology & Clinical Pharmacology, Ninewells Hospital and Medical School, Dundee University, Dundee DD1 9SY.

The 5-HT$_3$ receptor displays a species dependent pharmacology that is revealed by compounds that include (+) tubocurarine ((+)-Tc) Despite an 85% sequence identity, human (h) and mouse (m) recombinant 5-HT$_3$R-A$_s$ subunits exhibit an 1800-fold difference in the antagonist potency of (+)-Tc (Table 1; Belelli *et al.*,1995). By analogy to nicotinic receptors (nAChR), divergence in primary amino acid sequence within an N-terminal region homologous to 'Loop C'(Galzi & Changeux, 1995) may influence the potency of (+)-Tc. The present study has examined this possibility

A chimaeric receptor consisting of the extracellular amino terminal domain (residues 1 - 228) of m5-HT$_3$R-A$_S$ and the transmembrane domains, connecting loops and carboxy terminal (residues 224 - 455) of h5-HT$_3$R-A$_S$ was constructed using the polymerase chain reaction (Higuchi *et al.*, 1988). Site-directed mutagenesis (Kunkel *et al.*, 1991) was employed to convert selected extracellular regions of m5-HT$_3$R-A$_S$ to h5-HT$_3$R-A$_S$ sequence as detailed below.

```
m5-HT3R-As  PQFKEFSIDISNSYAEMKFYVIIRR 222
mutant 1    PYFREFSMESSNSYAEMKFYVIIRR 222
mutant 2    PYFREFSMESSNYYAEMKFYVVIRR 222
```

The pharmacological profile of chimaeric and mutant subunits was examined by two electrode voltage-clamp applied to *Xenopus laevis* oocytes previously injected with appropriate cRNAs or cDNAs. Experiments were conducted at -60 mV at room temperature (18-23°C). Results are expressed as the mean ± s.e.m. (n= 3-4)

Recombinant chimaeric and mutant receptors responded to bath applied 5-HT (0.6-100μM) with a transient inward current response. The agonist potency of 5-HT (EC_{50}) and associated interaction coefficient (nH) varied only slightly between the homo-oligomeric constructs (Table 1). The IC_{50} for (+)-Tc, (determined against the appropriate 5-HT EC_{50}) at the chimaeric construct was similar to that reported for m5-HT$_3$R-A$_S$ (Downie *et al.*, 1994; Table 1). Substitution of either 5 (mutant 1) or 7 (mutant 2) amino acid residues of m5-HT$_3$R-A$_S$ by the homlogous residues of h5-HT$_3$R-A$_S$ produced decrements in antagonist potency that increased with the degree of substitution.

Table 1. Potencies of 5-HT and (+)-Tc at 5-HT$_3$R-A$_S$ constructs

Receptor construct	5-HT EC_{50} & nH	(+)-Tc IC_{50} & nH
m5-HT$_3$R-A$_S$[1]	2.3±0.1μM; 2.2±0.2	1.4±0.2nM; 0.9±0.1
Chimaera	2.5±0.3μM; 2.0±0.2	1.2±0.1nM; 1.1±0.1
Mutant 1	5.8±0.6μM; 1.5±0.1	73±13nM; 1.3±0.01
Mutant 2	4.8±0.4μM; 1.9±0.1	226±18nM; 1.1±0.1
h5-HT$_3$R-A$_S$[2]	3.1±0.1μM; 1.9±0.1	2.6±0.2μM; 1.0±0.1

[1]From Downie *et al.* (1994) and [2]Belelli *et al* (1995)

In conclusion, the extracellular N-terminal domain dictates the potency of (+)-Tc as a 5-HT$_3$ receptor antagonist. Discrete regions homologous to loop C of nAChR influence, but do not wholly account for, the variable potency of (+)-Tc.

We thank the Wellcome Trust for financial support.

Belelli, D., Balcarek, J.M., Hope, A.G,. *et al.* (1995). *Mol Pharmacol.* 48, 1054-1062.
Downie, D.L., Hope, A.G., Lambert, J.J. *et al.* (1994). *Neuropharmacology* 33, 473-482.
Galzi, J.-L. & Changeux, J.-P. (1995). *Neuropharmacology* 34, 563-582.
Higuchi, R., Krummel, B. & Saiki, R.K. (1988). *Nucleic Acids Res.* 16, 7351-7367.
Kunkel, T.A., Bebenek, K. & McClary, J. (1991). In *Methods Enzymol.* 204, 125-139.

---

293 P PHARMACOLOGICAL CHARACTERIZATION OF A 5-HT$_3$ RECEPTOR SUBUNIT DERIVED FROM RAT SUPERIOR CERVICAL GANGLION

Ian D. Mair, Jeremy J. Lambert and John A. Peters. Neurosciences Institute, Department of Pharmacology and Clinical Pharmacology, University of Dundee, Ninewells Hospital and Medical School, Dundee, DD1 9SY., U.K.

The 5-HT$_3$ receptor is unique amongst 5-HT receptors in that it incorporates an integral cation selective ion-channel and may mediate fast excitatory neurotransmission within the CNS (Sugita *et al.*, 1992). Previous studies have demonstrated a marked interspecies variation in the pharmacology of the 5-HT$_3$ receptor. These observations have been reproduced in studies of heterologously expressed recombinant 5-HT$_3$ receptor subunits (5-HT$_3$R-A) cloned from mouse and human tissue (Downie *et al.*, 1994; Belelli *et al.*, 1995). The cloning and subsequent expression of other 5-HT$_3$R-A species homologues can be expected to facilitate the identification of amino acid residues involved in the binding of agonists and antagonists. Here, we describe some pharmacological properties of the 5-HT$_3$R-A cloned from rat superior cervical ganglion (Isenberg *et al.*, 1993) upon expression in *Xenopus laevis* oocytes.

Experiments were conducted upon stage VI *Xenopus laevis* oocytes previously injected (2-10 days) with cRNA (25-50 ng) transcribed *in vitro* from the rat 5-HT$_3$R-A cDNA. Transmembrane currents were recorded at room temperature (18-23°C) at holding potential of -60 mV using conventional two electrode voltage-clamp. Quantitative data are given as the mean ± s.e.m.

Injected oocytes responded to bath applied 5-HT (0.3-10μM) with a concentration-dependent inward current response which displayed desensitization in the continued presence of the agonist. Analysis of the concentration-effect relationship yielded an EC_{50} of 1.1 ± 0.1 μM and a Hill coefficient (nH) of 2.7 ± 0.3 (n = 4). The 5-HT$_3$ receptor selective agonists, 2-methyl-5-HT (EC_{50} = 4 ± 0.3μM, nH = 1.8 ± 0.3, n = 4) and 1-phenylbiguanide (PBG; EC_{50} = 3.3 ± 0.2 μM, nH = 2.2 ± 0.2, n=4) had potencies comparable to that of 5-HT, whereas *meta*-chlorophenylbiguanide (*m*CPBG EC_{50} = 130 ± 10 nM, nH = 1.5 ± 0.1, n = 4) was clearly more potent. The biguanide compounds acted as full agonists, but the maximal peak current response to 2-methyl-5-HT was only 61 ± 2 % (n=4) of that elicited by a saturating concentration of 5-HT, suggesting the compound to be a partial agonist. Current responses evoked by 5-HT at EC_{50} were reversibly inhibited by the selective 5-HT$_3$ receptor antagonist ondansetron (222 ± 16 pM, n = 4), and the non-selective antagonist, (+)-tubocurarine (32 ± 3 nM, n=4).

Heterogeneous properties of 5-HT$_3$ receptors have been demonstrated with compounds that include (+)-tubocurarine and biguanides (Belelli *et al.*, 1995). The present studies indicate the potencies of PBG and *m*CPBG to be higher than reported for mouse and human 5-HT$_3$R-A homologues, a finding which agrees with the results of radioligand binding studies performed on native 5-HT$_3$ receptors. In addition, the apparent affinity of tubocurarine was intermediate to the high and low potencies noted for mouse (Downie *et al.*, 1994) and human 5-HT$_3$R-A (Belelli *et al.*, 1995) respectively. Sequence comparisons between the aforementioned 5-HT$_3$R-A homologues will be instructive in identifying amino acid residues underlying interspecies variation in 5-HT$_3$ receptor properties.

We thank Dr. J. Yang for the gift of the rat 5-HT$_3$R-A cDNA and the Wellcome Trust for financial support. IDM holds a Dundee University Studentship.

Downie, D.L., Hope, A.G., Belelli, D. *et al.* (1994) *Neuropharmacology*, 33, 473-482.
Belelli, D., Balcarek, J.M., Hope, A.G., *et al* (1995) *Mol. Pharmacol.*, 48, 1054-1062.
Sugita, S., Shen, K.Z., North, R.A. (1992) *Neuron*, 8, 199-203.
Isenberg, K.E., Ukhun, I.A., Holstad, S.G. *et al.* (1993) *Neuroreport.*, 5, 199-203.

294 P THE INHIBITION OF K_{ATP} CHANNELS IN THE RAT INSULINOMA CELL LINE CRI-G1 BY THE INTRACELLULAR APPLICATION OF PURINERGIC ANTAGONISTS

I.C.M. Rowe & M.L.J. Ashford, Dept of Biomedical Sciences, Marischal College, University of Aberdeen, Aberdeen. AB9 1AS, UK.

ATP at micromolar concentrations is able to regulate a number of cation selective channels by binding to specific sites associated with the channel proteins rather than upon ATP hydrolysis and protein phosphorylation. These binding sites may be present on the extracellular aspect (P_{2x} purinergic receptors) or intracellular surface (e.g K_{ATP} channels) of mammalian cells. As these intracellular and extracellular binding sites exhibit similar sensitivities to ATP we have examined whether they display similar pharmacological profiles and have investigated the sensitivity of K_{ATP} channels in the CRI-G1 cell line (Sturgess et al., 1986) to purinergic antagonists (Harden et al., 1995) .

Single channel activity was recorded using the inside-out and outside-out configurations of the patch clamp technique. For outside-out recordings the bath solution (A) consisted of (in mM): NaCl 135.0, KCl 5.0, $CaCl_2$ 1.0, $MgCl_2$ 1.0, HEPES 10.0 (pH 7.2 with NaOH) while the pipette contained (solution B) (in mM) : KCl 140.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, K-EGTA 10.0, HEPES 10.0, pH 7.2 with KOH (resulting in free Ca2+ and Mg2+ concentrations of 25nM and 0.65mM respectively). In experiments using the inside out patch configuration, the pipette contained (in mM): KCl 140.0, CaCl2 1.0, MgCl2 1.0, HEPES 10.0, pH 7.2 with KOH while the bath contained solution B (Mg2+ present), or a Mg2+ "free" solution (in mM): KCl 140.0, K-EDTA 10.0, $CaCl_2$ 4.6, HEPES 10.0, pH 7.2 with KOH (resulting in free Ca2+ and Mg2+ concentrations of ~25 nM and <5 nM respectively).. All data in the text are presented as the mean ± s.e.mean of the indicated number of experiments (n).

The application of the P_{2X} antagonist suramin (100nM-200μM) to the intracellular surface of inside-out excised patches produced a concentration-dependent inhibition of K_{ATP} channel activity which was voltage-independent and reversed upon washout of the drug. The IC_{50} values for suramin in the presence and absence of Mg^{2+} ions were 15±4μM and 8±3μM respectively (n=3-5 for each concentration). The P_{2Y} antagonist reactive blue 2 (1-200μM) also significantly inhibited K_{ATP} channel activity with an IC_{50} of 24±8μM in the absence of Mg^{2+}ions (n=4). In a further series of experiments, the addition of suramin (200μM) or reactive blue 2 (500μM) to the extracellular surface of outside-out patches failed to inhibit K_{ATP} channel activity (n=3 for each).

These data indicate that the P_{2X} antagonists suramin and reactive blue 2 can mimic the action of ATP and inhibit the activity of the K_{ATP} channel. This effect appears to be dependent on interactions with an intracellular ATP binding site as extracellular applications failed to alter channel activity. Further research will be required to determine whether these purinergic agents, at concentrations below those which inhibit channel activity, can antagonise the effect of ATP on the K_{ATP} channel.

This work was supported by the Wellcome Trust.

Sturgess, N.C. et al. (1986). *J. Endocr.* **109**, 201-207.
Harden, T.K., Boyer, J.L. & Nicholas, R.A. (1995) *Ann. Rev. Pharm. Toxicol.* **35**, 541-579.

---

295 P INTRANUCLEAR INJECTION OF SELECTIVE ANTISENSE-GENERATING PLASMIDS SHOWS G-PROTEIN $G_{\alpha 12}$ COUPLES M_1 MUSCARINIC RECEPTORS TO INHIBIT M-TYPE K+ CURRENT IN RAT SYMPATHETIC NEURONES

M. P. Caulfield, F. C. Abogadie, P. Delmas, J. E. Haley, Y.Vallis & N. J. Buckley, Wellcome Laboratory for Molecular Pharmacology, University College London, London WC1E 6BT.

Activation of M_1 muscarinic receptors in rat superior cervical ganglion (SCG) neurones inhibits the M-type K^+ current through a *Pertussis* toxin-insensitive G-protein-linked mechanism. Selective antibodies against $G_{\alpha q/11}$ partially reduced, but did not abolish muscarinic M-current inhibition (Caulfield et al., 1994), indicating that $G_{\alpha q}$ and/or $G_{\alpha 11}$, and possibly other G-proteins may be involved.

G-protein α-subunit expression was investigated in SCG neurones from 15-19 day rats, dissociated and cultured for 2-4 days, acetone-fixed, and immunostained using selective anti-G protein α-subunit antibodies (1:1000). Visualization was *via* alkaline phosphatase-conjugated swine anti-rabbit IgG. SCG neurones stained intensely with antibody raised against $G_{\alpha 12}$. Plasmids were constructed which could generate antisense RNA directed against a region (246bp) of the $G_{\alpha 12}$ gene located 3' to the coding region. Neurones injected intranuclearly with this plasmid (400μg/ml) showed reduced $G_{\alpha 12}$ staining two days after injection, while staining for $G_{\alpha o}$ and $G_{\alpha q}$ was normal. An anti-$G_{\alpha o}$ antisense plasmid (vs 147 bases in the 3' untranslated region: 400μg/ml) reduced $G_{\alpha o}$ staining, but did not affect $G_{\alpha 12}$ staining. The perforated patch technique was used to voltage clamp neurones (32°C) at about -25mV, and M-current deactivation relaxations were measured (Caulfield et al., 1994). Table 1 shows that oxotremorine-M (Oxo-M) inhibition of M-current was substantially reduced at all agonist concentrations in neurones injected with $G_{\alpha 12}$, but not $G_{\alpha o}$ antisense construct. Thus, $G_{\alpha 12}$, like $G_{\alpha q/11}$, is involved in transducing muscarinic inhibition of M-current.

$G_{\alpha 12}$ does not couple to stimulate phospholipase C (PLC; Kozasa & Gilman, 1995), so our demonstration of $G_{\alpha 12}$ involvement in muscarinic M-current inhibition provides further support for the already substantial evidence against PLC products (including Ca^{2+} released from IP_3-sensitive stores) as mediators of this response (reviewed by Brown et al., 1995). Selyanko & Brown (1996) recently described substantial inhibition of M-channels in SCG neurones by depolarization-induced increases in Ca^{2+}, so it may be that an alternative (and as yet undetected) mechanism exists for muscarinic receptor-induced elevation of Ca^{2+}, which then inhibits M-current.

Brown, D.A., Buckley, N.J., Caulfield, M.P. *et al.* (1995) in *Molecular Mechanisms of Muscarinic Acetylcholine Receptor Function* ed. Wess, J. pp165-182. Austin: R.G. Landes.
Caulfield, M.P., Jones, S., Vallis, Y. *et al.* (1994) *J.Physiol.* 477, 415-422.
Kozasa, T. & Gilman, A.G. (1995) *J.Biol.Chem.* 270, 1734-1741.
Selyanko, A.A. & Brown, D.A. (1996) *Neuron* 16, 151-162.

Table 1. Anti-$G_{\alpha 12}$ antisense construct reduces muscarinic inhibition of M-current (data are means ± se mean)

Oxo-M (μM)	Uninjected (n=8)	Anti-$G_{\alpha o}$-injected (n=4)	Anti-$G_{\alpha 12}$-injected (n=8)
0.3	32.8 ± 3.1	26.6 ± 3.7	6.3 ± 1.1
1	57.1 ± 4.6	45.5 ± 3.3	15.6 ± 2.5
3	76.1 ± 3.8	65.1 ± 2.7	34.9 ± 5.0
10	82.5 ± 3.9	81.9 ± 4.2	52.8 ± 5.9

296 P THE SUBUNIT SELECTIVE ACTIONS OF THE ANAESTHETIC ETOMIDATE AT HUMAN RECOMBINANT $GABA_A$ RECEPTORS ARE DETERMINED BY A SINGLE AMINO ACID

D.Belelli, C. Hill-Venning, J.A. Peters, J.J. Lambert, K.A. Wafford[1] & P.J. Whiting[1]. Neurosciences Institute, Dept. of Pharmacology & Clinical Pharmacology, Ninewells Hospital & Medical School, Dundee University, Dundee DD1 9SY & [1]Neuroscience Research Centre, Merck Sharp & Dohme, Terlings Park, Essex CM20 2QR.

Recent studies have highlighted the $GABA_A$ receptor as an important locus of action for a variety of structurally diverse general anaesthetics. Here, the influence of the α and β subunit $GABA_A$ isoforms upon the actions of the general anaesthetic etomidate was investigated in *Xenopus laevis* oocytes (co-injected into the cytoplasm 2-12 days previously with cRNAs encoding human $\alpha_x\beta_y\gamma_{2L}$ $GABA_A$ subunits, where x = 1,2,3,6 and y = 1,2) using the two electrode voltage-clamp technique. At a holding potential of -60mV, inward currents evoked by an EC_{10} concentration of GABA were potentiated by etomidate at all human receptor subunit combinations tested. However, for all α isoforms, the calculated EC_{50} for etomidate was 9-12 fold higher for β_1- than for β_2-containing receptors (Table 1). Furthermore, with the exception of the α_3-containing receptor, the magnitude of the potentiation was greater for β_2- than for β_1-containing receptors, this difference being most evident for the $\alpha_6\beta_1\gamma_{2L}$ *cf* $\alpha_6\beta_2\gamma_{2L}$ receptors (Table 1). For β_2-containing receptors etomidate, in the absence of GABA, induced an inward current which was antagonised by picrotoxin (30μM: see Table 1). Such GABA-mimetic actions of the anaesthetic were minimal, or absent for β_1-containing receptors (Table 1). The β isoform dependence of both the GABA-modulatory and -mimetic actions of etomidate was investigated further by the construction of β_1 and β_2 chimeras. For receptors composed of α_6 and γ_2 subunits and a chimeric β subunit (β_1/β_2Lys237-Gly 334) containing the N-terminal extracellular domain of the β_1 subunit ($\beta_{1\text{-}2}$) the GABA-modulatory and -mimetic actions of etomidate was similar to those determined for the $\alpha_6\beta_2\gamma_{2L}$ receptor (Table 1). By comparison, for α_6 and γ_{2L} subunits coexpressed with a chimeric β subunit (β_2/β_1 Lys238-Gly335) containing the N-terminal extracellular domain of the β_2 subunit, the GABA-modulatory and -mimetic actions of the anaesthetic approached those of the $\alpha_6\beta_1\gamma_{2L}$ receptor (Table 1). The anticonvulsant loreclezole is structurally related to etomidate and exhibits a similar β isoform and domain specificity to the anaesthetic. This characteristic is dictated by a single amino acid asparagine (N) 289 in the M2 region of the β_2 subunit *cf* serine(S) 290 for β_1 (Wingrove *et al.* 1994). Similarly, in this study, the co-expression of the α_6 and γ_{2L} subunits with a mutated $\beta_{1(S289N)}$ subunit yielded a receptor with a GABA-modulatory and -mimetic action of etomidate similar to that of the $\alpha_6\beta_2\gamma_{2L}$ receptor. The demonstration here, that the interaction of etomidate with the $GABA_A$ receptor is greatly modified by a single amino acid, appears counter-intuitive to a mechanism which invokes a non-specific membrane perturbation to explain the behavioural action of a general anaesthetic.

Table 1. Modulation of $GABA_A$ receptor isoforms by etomidate

	Modulation EC_{50}(μM)	Modulation E_{max}(%)	Agonist EC_{50}(μM)	Agonist E_{max}(%)
$\alpha_1\beta_2\gamma_{2L}$	1.2 ± 0.1	127 ± 12	130 ± 2	19 ± 2
$\alpha_1\beta_1\gamma_{2L}$	11 ± 1	79 ± 2	N.D.	4 ± 1
$\alpha_2\beta_2\gamma_{2L}$	0.9 ± 0.1	107 ± 1	50 ± 3	27 ± 8
$\alpha_2\beta_1\gamma_{2L}$	6.3 ± 3	65 ± 3	N.D.	5 ± 1
$\alpha_3\beta_2\gamma_{2L}$	1.0 ± 0.1	88 ± 6	108 ± 4	9 ± 1
$\alpha_3\beta_1\gamma_{2L}$	8.1 ± 0.9	75 ± 8	N.D.	< 2
$\alpha_6\beta_2\gamma_{2L}$	0.6 ± 0.04	169 ± 14	22 ± 1	51 ± 15
$\alpha_6\beta_1\gamma_{2L}$	7.4 ± 0.6	28 ± 2	N.D.	5 ± 2
$\alpha_6\beta_{1\text{-}2}\gamma_{2L}$	0.93 (n=2)	195 (n=2)	34 (n=2)	38 (n=2)
$\alpha_6\beta_{2\text{-}1}\gamma_{2L}$	7.7 ± 1	23 ± 2	N.D.	4 ± 1
$\alpha_6\beta_{1S290N}\gamma_{2L}$	1.6 ± 0.3	150 ± 13	79 ± 6	45 ± 13

N.D. = Not determined due to the small effect.

We acknowledge the financial support of the MRC.

Wingrove, P.B., Wafford, K.A., Bain, C. *et al.*(1994) *Proc.Natl. Acad. Sci., U.S.A.* 91, 4569-4573.

297 P THE EFFECT OF ABT-418 ON MESOLIMBIC DOPAMINE SYSTEMS AND LOCOMOTION IN THE RAT

M.E.M. Benwell & D.J.K. Balfour, Department of Pharmacology and Clinical Pharmacology, University of Dundee Medical School, Ninewells Hospital, Dundee DD1 9SY.

ABT-418, [(S)-3-methyl-5-(1-methyl-2-pyrrolidinyl) isoxazole], binds with high affinity and is a potent and selective agonist at neuronal nicotinic receptors in rat brain (Anderson et al 1995). In the present study, *in vivo* microdialysis has been used to investigate the effect of ABT-418 on the overflow of dopamine (DA) and dihydroxyphenylacetic acid (DOPAC) in the nucleus accumbens (NAcc) in freely moving drug-naive and nicotine-pretreated rats (Benwell & Balfour 1992) tested in a Benwick activity box.

ABT-418 (0.1 or 1.0 mg/kg sc) did not significantly increase extracellular DA in the NAcc of saline or nicotine-pretreated (fig 1a) rats. However, extracellular DOPAC levels in the NAcc were significantly increased ($p<0.01$) in both pretreatment groups following 0.1 mg/kg ABT-418 (fig. 1b). ABT-418 (1mg/kg) also increased ($p<0.05$) DOPAC overflow in the rats pretreated with saline but not nicotine. The activity of both groups of animals was not significantly increased in response to ABT-418 at either of the doses tested (fig. 1c). These results suggest that ABT-418 may increase DA turnover in the NAcc although it does not appear to increase DA release into the extracellular space sampled by the dialysis probe. The data also suggest that nicotine pretreatment does not cause sensitisation of mesolimbic DA or locomotor responses to ABT-418.

The authors acknowledge Foundation VERUM for financial support and Abbott Laboratories for the generous gift of ABT-418.

Anderson, D.J., Williams, M., Pauly, J.R., et al (1995) *J Pharmacol Expt Ther.*, 273, 1434-1441.

Benwell, M.E.M. & Balfour, D.J.K. (1992) *Br J Pharmacol.*, 105, 849-856.

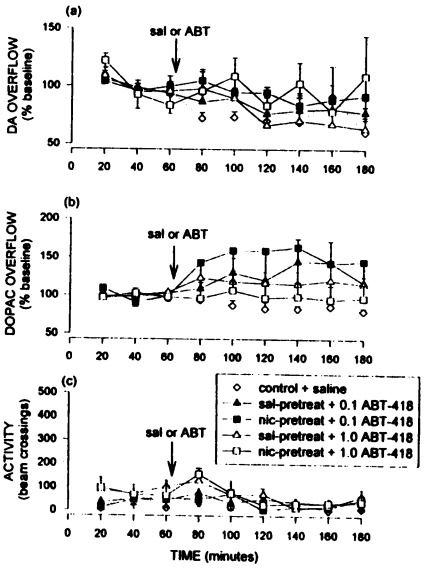


FIGURE 1. Rats were pretreated with saline or nicotine (0.4 mg/kg sc) once per day for 5 days prior to implantation of a dialysis probe in the NAcc. On the following day, the responses were investigated following a challenge with ABT-418 (0.1 or 1.0 mg/kg sc) or saline (controls), administered at the time indicated by the arrow. NAcc DA and DOPAC levels are expressed as percent of baseline. Results are the means±s.e.mean of 4-6 observations.

298 P BLOCKADE OF AMPHETAMINE-INDUCED CONDITIONED TASTE AVERSIONS BY GR205171

K.H.M. McAllister & J.A. Pratt, Dept of Physiology and Pharmacology, University of Strathclyde, Glasgow. G1 1XW.

Neurokinin$_1$ (NK_1) receptor antagonists have been shown to block emesis induced by a variety of emetic stimuli (Gardener et al., 1996). The aim of the present study was to investigate the effect of the neurokinin$_1$ (NK_1) receptor antagonist, GR205171, on amphetamine induced conditioned taste aversion (CTA), a phenomenon which has been hypothesised to recruit similar neural systems to those involved in emesis (Grant, 1987).

CTA responses to amphetamine (0.5 mg kg^{-1}) and GR205171 were investigated in male Lister Hooded rats (weight 250-300g, n=6-8/group) using a 2-trial conditioning procedure (McAllister & Pratt, 1996). Water deprived rats were provided with either a sodium saccharin (0.1%) or a sodium chloride solution (0.9%) and immediately afterwards injected with either drug or vehicle. During the conditioning trials pre-treatment with GR205171 was given 15 minutes prior to flavour presentation. The drug-paired and the vehicle-paired flavours were then presented simultaneously in a two-stimulus choice test to assess CTA. The percentage scores for drug-paired flavour intake were subjected to arc-sine transformations to normalise their distribution and analysed by one-way ANOVA followed by the Newman Keuls test for comparisons between treatment groups.

Results from the 2-stimulus choice test demonstrated that amphetamine produced a clear CTA. The percentage of drug-paired fluid intake was 26.7 ± 2.5%. The NK_1 antagonist, GR205171, blocked amphetamine-induced CTA in a dose dependent manner. Pre-treatment with GR205171 (0.1 mg kg^{-1}) had no significant effect on amphetamine CTA with the mean % of drug-paired flavour intake being 28.0 ± 1.8%. In contrast pre-treatment with GR205171A (0.3 & 0.5 mg kg^{-1}) blocked amphetamine-induced CTA ($p<0.05$). The mean percentages of the amphetamine-paired flavour were 45.5 ± 1.7% and 43.9 ± 1.3% respectively. The NK_1 antagonist GR205171 (0.1 - 1.0 mg kg^{-1}) also blocked apomorphine-induced CTA over a similar dose range. In contrast GR205171A alone (0.1 - 1.0 mg kg^{-1}) did not evoke CTA. Together these results indicate that the NK_1 receptor is important in modifying psychotropic drug-induced CTA. However it remains to be established whether this occurs through an action of GR205171 on NK_1 receptors located in brain stem emetic circuitry or in structures such as the amygdala which have been implicated in aversion learning.

Gardener, C.J. et al. (1996) *Br. J. Pharmacol.* Proc. Suppl. In press.
Grant, L. (1987) *Psychopharmacology* 93, 405-41.
McAllister, K.H.M. & Pratt, J.A. (1996) *Br. J. Pharmacol.* 117, 184P.
K.H.M. McAllister is funded by the University of Strathclyde and Glaxo Wellcome. We thank SHERT for laboratory facilities.

---

299 P METABOLISM OF DOPAMINE AND EXPRESSION OF Fos PROTEIN IN STRIATAL AND LIMBIC RAT BRAIN AREAS FOLLOWING ACUTE AND CHRONIC NICOTINE

L. Ahtee, O. Salminen & T. Seppä, Div of Pharmacology and Toxicology, Dept of Pharmacy, P.O.Box 56, FIN-00140 University of Helsinki, Finland

In mice tolerance seems to develop to the nicotine-induced increase of striatal dopamine (DA) metabolism (Pietilä *et al.*, 1995). However, in the limbic areas of rats no tolerance to and even sensitization of the nicotine-induced increase of DA release was found to occur after repeated nicotine treatment (Damsma *et al.*, 1989; Nisell *et al.*, 1996). In the present experiments we investigated the effects of acute and chronic nicotine treatment on dopamine metabolism in the striatum and limbic forebrain of the same rats. We also studied the expression of Fos protein in these brain areas after acute and chronic nicotine.

Nicotine (4 mg/kg/day) or saline was administered to male Wistar rats (body weight 250-400 g) via subcutaneously implanted osmotic minipumps for 7 days. On the 7th day with the minipumps still in place the rats received saline or 0.5 mg/kg of nicotine s.c. one hour before decapitation (monoamine estimations) or pentobarbital anaesthesia and intracardial perfusion (Fos immunohistochemistry). DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were measured by HPLC with electrochemical detection in the striatum and in the limbic forebrain (containing *inter alia* the olfactory tubercles, medial part of the nucleus accumbens, nucleus amygdaloideus centralis, and part of the paleocortex). Fos-like immunostaining (Fos-IS) was estimated as described previously (Salminen *et al.*, 1996) with minor modifications.

On the 7th day of chronic treatment with nicotine the DOPAC concentration tended to be elevated in the striatum, but was not altered in the limbic area. Acute nicotine elevated DOPAC in the striatum of the control rats, but decreased it after the chronic treatment. In the limbic area acute nicotine elevated DOPAC in the control rats as well as on the 7th day of chronic treatment. Neither limbic nor striatal DA concentrations were altered by any treatment. At 7 days after starting the chronic infusions the number of Fos-positive nuclei in the dorsomedial caudate-putamen (CPU), in the core of nucleus accumbens (NACC), and in the cingulate cortex (Cg) of nicotine-treated rats (n=4) did not differ from those of saline-treated control rats (n=4). Acute nicotine increased the number of Fos-positive nuclei in the CPU from 62±11 (mean±s.e.mean) to 217±40, in the NACC from 143±7 to 395±21, and in the Cg from 208±20 to 395±30 in the control rats (n=4), but no such increase occurred in these brain areas of rats treated chronically with nicotine (n=4).

These findings agree with the earlier findings that chronic nicotine treatment does not produce tolerance in the limbic forebrain areas of the rat to the acute effects of nicotine on dopamine metabolism. In contrast, a tolerance to these effects seems to occur in the striatum. Furthermore, chronic nicotine treatment induced a clear tolerance to the Fos-IS increasing effect of acute nicotine both in the striatal and limbic brain areas studied.

Damsma, G., Day, J., Fibiger, H. (1989) *Eur. J. Pharmacol.* 168, 363-368.
Nisell, M., Nomikos, G., Hertel, P. *et. al.* (1996) *Synapse* 22, 369-381.
Pietilä, K., Laakso, I., Ahtee, L. (1995) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 351, 1-6.
Salminen, O., Lahtinen, S., & Ahtee, L. (1996) *Pharmacol. Biochem. Behav.* 54, 241-248.

300 P INVESTIGATIONS INTO THE EXISTENCE OF MICE OF THE C57 STRAIN WITH A LOW PREFERENCE FOR ETHANOL, AND THE MODIFICATION OF SUCH PREFERENCE

Watson, W.P, Butterworth, A.R.* and Little, H.J., Psychology Department, South Road, Durham, DH1 3LE. *Pharmacology Department, University of Bristol, Bristol, BS8 1TD.

The inbred C57/Bl6 strain of mice has traditionally been widely used as an alcohol preferring strain. When given a free choice of 8% ethanol or water they drink a large percentage of their fluid as ethanol (Unwin and Taberner, 1982). In this study we describe the existence of C57/Bl6 mice with low alcohol preference, the effect of selective breeding from these mice, and the manipulation of their alcohol preference by environmental influences.

The mice (25-30g) were male or female C57/Bl6 mice bred exclusively in house at Bristol University for over 15 years. Screening for ethanol preference consisted of singly housing the mouse in a cage with ad lib access to water, food and 8% ethanol. The volume of each fluid drunk was monitored every 2 days for 6 days. The amount of 8% ethanol drunk as a proportion of total fluid intake was calculated and averaged over the 3 measurements. Mice were then identified as high or low preference mice on the basis of these data (high ratio >0.8, low ratio <0.4 -though most of the latter values are lower than this).

Selective breeding of high with high, and low with low was performed for 5 generations. The ethanol preference of the offspring were screened as above. In another experiment low preference mice were left undisturbed for 3 months. They were then re-screened in the room containing their home cages, before being taken in a lift to a second laboratory and re-screened within 5 days.

Table 1: Effect of selective breeding over 5 generations on the proportion of low preference C57's

Generation	Low preference parents	High preference parents
2	33% (n=238)	30% (n=299)
3	28% (n=113)	27% (n=257)
4	20% (n=44)	24% (n=147)
5	11% (n=28)†	21% (n=115)

In the first set of mice examined (generation 1) 41% of males and 54% of females showed a low preference for ethanol (n=348). There was no correlation with sex or body weight (P>0.1), and the distribution of preference ratios appeared to be two distinct populations.

The results in Table 1 indicate that low preference mice were produced in each generation, but selective breeding did not increase this proportion. Indeed, a significant reduction was noted in the proportion of low preference offspring produced from low preference parents between generations 2 and 5. Table 2 demonstrates that whilst the number of low preference mice declined when tested over time (retested 3 months later), there was a marked and significant reduction in numbers following a change in environment.

It may be that the changes seen in Table 2 occur in transit from suppliers, so that this phenomenon has not been evident earlier. Further work continues to examine the role of stress and pharmacological manipulation on this delayed expression of the genotype as, so far, the change from a low to a high preference state appears to be irreversible.

Unwin, J.W and Taberner, P.V (1982) *Psychopharmacology*, 78, 361-364

Table 2: Effect of time and environmental disruption on the proportion of mice showing a low preference for ethanol

Start of experiment	3 months later	New environment
100% low drinkers (21 male, 13 female)	43% low drinkers (12 male, 7 female)	16% low drinkers* (4 male, 3 female)

*P < 0.02 c.f. 3 months (Fisher's exact probability test)

†P < 0.05 c.f. with generation 2 (Fisher's exact probability test)

301 P SB 216641 AND BRL 15572 PHARMACOLOGICALLY DISCRIMINATE BETWEEN h5-HT1B AND h5HT1D RECEPTORS

G.W. Price, M.J. Burton, C. Roberts, J. Watson, [1]M. Duckworth, [1]L. Gaster, D.N. Middlemiss and B.J. Jones
Departments of Psychiatry Research and [1]Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex, CM19 5AW.

Despite the modest sequence homology between 5-HT_{1B} and 5-HT_{1D} receptors, separation of their pharmacologies has proven difficult. GR127935 has proved most valuable as a selective 5-$HT_{1B/1D}$ receptor antagonist (Skingle *et al.* 1996; Roberts *et al.* 1996) but it fails to discriminate between these receptor subtypes. We now report on two compounds, SB 216641 (N-[3-(2-dimethylamino)ethoxy-4-methoxyphenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-(1,1'-biphenyl)-4-carboxamide) and BRL 15572 (1-phenyl-3-[4-(3-chlorophenyl) piperizin-1-yl] phenylpropan-2-ol) which discriminate between these two subtypes of 5-HT receptors *in vitro*.

Recombinant h5-HT_{1B} and h5-HT_{1D} receptors were stably expressed in CHO cells, yielding receptor levels of 0.65 and 7.90 pmoles/mg protein, respectively. SB 216641 and BRL 15572 were characterised in these cell lines using [^{3}H]5-HT radioligand binding, [^{35}S]GTPγS binding and cAMP accumulation assays (for methods, see Watson *et al.* 1996), to measure their affinities and functional efficacies at h5-HT_{1B} and h5-HT_{1D} receptors. Results from these assays are summarised in Table 1.

SB 216641 showed 30 fold higher affinity for 5-HT_{1B} than for 5-HT_{1D} receptors, whereas BRL 15572 had over 100 fold greater affinity for 5-HT_{1D} receptors. Both compounds have partial agonist activity similar to that published for GR127935 (Watson *et al.* 1996). However, as with GR127935, little intrinsic activity has been observed in native tissues (Skingle *et al* 1996). Using these compounds in an *in vitro* [^{3}H]5-HT release preparation in electrically stimulated guinea-pig cerebral cortex slices, we have clearly demonstrated that SB 216641 inhibited 5-HT (30nM) autoreceptor activation (100% inhibition at 1μM). BRL 15572 (1μM) however, was without effect. Therefore, using these compounds we have shown that the 5-HT terminal autoreceptor in guinea-pig cortex is of the 5-HT_{1B} subtype.

Table 1. SB 216641 and BRL 15572; potencies at 5-$HT_{1B/1D}$ receptors

	Binding (pKi)		GTPγS (pEC_{50})		cAMP (pA_2)	
	5-HT_{1B}	5-HT_{1D}	5-HT_{1B}	5-HT_{1D}	5-HT_{1B}	5-HT_{1D}
SB 216641	9.0	7.5	8.2	7.9	9.3	7.3
BRL 15572	5.6	7.8	6.2	7.9	<6	7.1

All values are at least n=3, all s.e.m. < 0.3

Roberts C., Watson J., Burton M. *et al.* (1996) Br. J. Pharmacol. **117**, 384-388.
Skingle M., Beattie D.T., Scopes D.I.C. *et al.* (1996) Behav. Brain Res. **73**, 157-161.
Watson J., Burton M.J., Price G.W. *et al.* (1996) Eur. J. Pharmacol. *in press*

302 P COMBINED ADMINISTRATION OF N$^{\omega}$-NITRO-L-ARGININE AND INDOMETHACIN INCREASES INFARCT SIZE IN RABBIT LANGENDORFF PERFUSED HEARTS

Keri A. Aitchison & Susan J. Coker, Department of Pharmacology & Therapeutics, University of Liverpool, Ashton Street, Liverpool L69 3BX.

Nitric oxide (NO) and prostacyclin (PGI_2) are putative cardioprotective agents in animal models of myocardial ischaemia and reperfusion. However, treatment with inhibitors of NO or PGI_2 synthesis has yielded conflicting results. For example, inhibitors of NO synthase (NOS) either increased (Williams *et al.*, 1995) or decreased (Patel *et al.*, 1993) infarct size in *in vivo* rabbit models. There is evidence that inhibition of either NO or PGI_2 synthesis can lead to an increased release of the other mediator (Barker *et al.*, 1996), indicating that there may be a reciprocal relationship involved in their production. Our aim was to establish whether concomitant administration of N$^{\omega}$-nitro-L-arginine (L-NOARG; a NOS inhibitor) and indomethacin (Indo; a cyclooxygenase inhibitor) would increase infarct size in a rabbit Langendorff perfused heart model of ischaemia and reperfusion.

Male NZW rabbits (2.0-3.0kg) were anaesthetized with Hypnorm (0.5 ml kg^{-1} i.m.) and sodium pentobarbitone (12 mg kg^{-1} i.v. as required) and artificially ventilated on room air via a tracheal cannula. Hearts were exposed and a ligature placed around the left coronary artery. After administration of heparin (250 IU kg^{-1}), hearts were rapidly excised and perfused with modified Krebs solution (37°C; 95% O_2/ 5% CO_2) at constant pressure (80cm H_2O). A bipolar ECG was recorded and coronary flow determined by timed collection of coronary effluent. After a 20 min stabilisation period, hearts were assigned randomly to one of four treatment groups of n=6: control (Krebs only), L-NOARG 100μM, Indo 3μM or L-NOARG 100μM + Indo 3μM. Hearts were perfused with test solution for 15 min prior to ischaemia and for the duration of the experiment. After 30 min of ischaemia, the ligature around the coronary artery was released and the myocardium reperfused for 2h. The ligature was then retied and 5ml of MTT (Thiazoyl blue, 5% w/v) injected via the aorta to delineate the area at risk (AAR). Hearts were then sliced and incubated in triphenyl tetrazolium chloride (1% w/v; 37°C; 15 min) to stain viable tissue. Slices were then fixed, photographed and AAR and infarct size (IS) measured by image analysis.

AAR was similar in all groups, however, IS was reduced by L-NOARG alone, but increased in hearts that received both L-NOARG and Indo (Table 1). Coronary flow was significantly reduced in both groups that received L-NOARG when compared to the controls. There were no differences in heart rate among groups, either prior to ischaemia or after reperfusion.

Table 1. Values (mean ± s.e. mean) for AAR and IS (%), for heart rate (HR, beats min^{-1}) and coronary flow (CF, ml min^{-1} g^{-1}) 1 min prior to ischaemia. *P<0.01 **P<0.001 compared with control, one way analysis of variance with Bonferroni corrected t-test.

	Control	*L-NOARG*	*Indo*	*Both*
AAR	44.0±1.1	43.3±0.8	44.2±1.2	46.2±1.3
IS	37.4±0.4	20.8±1.3**	33.4±2.3	44.0±1.9*
HR	200±8	197±10	186±11	192±7
CF	6.0±0.5	4.5±0.4*	5.6±0.2	4.5±0.4*

The reduction in IS by L-NOARG was not related to changes in HR or CF. The mechanism by which Indo reversed this effect of L-NOARG, such that IS was increased compared to control, warrants further investigation.

KAA is a recipient of a Wellcome Prize Studentship.

Barker, J.E. *et al.* (1996) *Br. J. Pharmacol.*, 118, 643-648.
Patel, V.C., *et al.* (1993) *Biochem. Biophys. Res. Comm.*, 194, 234-238.
Williams, M.W. *et al.* (1995) *Cardiovasc. Res.*, 30, 79-86.

---

303 P PEROXYNITRITE-INDUCED RELAXATION OF RAT AORTIC RINGS: THE ROLE OF GLUCOSE

Fiona J. Dowell & William Martin. CRI, West Medical Building, University of Glasgow, G12 8QQ.

Nitric oxide reacts with superoxide to form peroxynitrite ($ONOO^-$; PN) which exerts weaker but more prolonged relaxant actions (Liu *et al.*, 1994). PN is unstable at physiological pH and thus it must be assumed that its long acting vasorelaxant action is due to the formation of a secondary, more stable species. Some studies have suggested that PN may react with glucose to form this relaxant (Moro *et al.*, 1995) and we wished to test this in rat aorta.

PN was synthesised according to the method of Beckman *et al.* (1990). To investigate the formation of stable vasodilator substances PN (1 mM) was mixed with a solution of glucose (11 mM) and then neutralised to remove any unreacted PN. The vasorelaxant actions of PN and its derivatives were investigated on endothelium-denuded rings of aorta from male Wistar rats (300g), killed by stunning and exsanguination. The rings were mounted under 1 g of tension, bathed in Krebs' solution, gassed with 95% O_2 / 5% CO_2 and maintained at 37°C. After equilibration rings were precontracted with phenylephrine to study the relaxant properties of PN and its derivatives.

PN induced a concentration-dependent relaxation of rat aortic rings suspended in normal glucose-containing Krebs' (Table 1). When the solution of PN was neutralised (NEU) it exhibited weaker relaxant activity, consistent with the presence of unreacted nitrite. The relaxant potency of PN was completely unaffected when added to aortic rings suspended in glucose-free Krebs (PN-GFK). In contrast, the reaction of glucose with PN (G-PN) led to the formation of a more potent relaxant. The addition of L-cysteine (L-cys; 1 mM) to the bath had no relaxant action itself and had no effect on the relaxation induced by either PN or NEU. In contrast, the presence of L-cys significantly potentiated the relaxation produced by G-PN (Table 1).

Table 1. Log EC50 values for relaxation by PN and its derivatives (mean ± s.e.mean, n≥5).

	Control	+ L-cys (1 mM)
PN	-5.22 ± 0.03	-5.32 ± 0.05
NEU	-4.80 ± 0.04***	-4.88 ± 0.08
PN-GFK	-5.34 ± 0.06	-
G-PN	-5.78 ± 0.02***	-6.18 ± 0.03$^{\$\$\$}$

* p<0.001 cf. PN, $^{\$}$ p<0.001 in the absence of L-cys (ANOVA).

These results confirm that PN can react with glucose to form a novel vasorelaxant whose ability to release NO is augmented by L-cys. The relaxation produced when PN is added to aortic rings is not, however, dependent upon this reaction since it occurs in glucose free Krebs' and is not potentiated by L-cys. The nature of the relaxant produced when PN is added to aortic rings therefore remains elusive.

This work was supported by the British Heart Foundation.

Beckman, J.S. *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, 86,1620-1624.
Liu, S. *et al.* (1994) *J. Pharmacol. Expt. Ther.*, 268,1114-1121.
Moro, M.A. *et al.* (1995) *Br. J. Pharmacol.*, 116,1999-2004.

304 P POTENTIATION OF HYDROGEN PEROXIDE-INDUCED IMPAIRMENT OF VASODILATOR ACTIVITY OF RAT AORTA BY 3-AMINO-1,2,4-TRIAZOLE

W. Martin & K.B. Mian, CRI, West Medical Building, University of Glasgow, Glasgow, G12 8QQ

In addition to its ability to remove the damaging oxidant, hydrogen peroxide, endogenous catalase is responsible for the liberation of nitric oxide from certain nitrovasodilators, including hydroxylamine and azide. Consequently, 3-amino-1,2,4-triazole (AT), which inhibits catalase (Margoliash & Novogrodsky, 1958), selectively blocks the vasorelaxant actions of hydroxylamine and azide but not glyceryl trinitrate, which is metabolised by a separate pathway (Mian & Martin, 1995). The aim of this study was to determine if inhibition of catalase by AT potentiated the damaging actions of hydrogen peroxide in rat aorta.

Male Wistar rats were killed by stunning and exsanguination. The thoracic aorta was removed, cleared of fat and connective tissue and cut into rings 2.5 mm wide. Endothelium was removed from some rings by gentle abrasion. Rings were then suspended in Krebs' solution at 37°C for tension recording and relaxation was studied following the generation of phenylephrine (PE)-induced tone. Relaxation to acetylcholine (ACh, 10 nM-3 μM) was assessed on endothelium-containing rings whereas that to glyceryl trinitrate (GTN, 1-100 nM) and isoprenaline (ISO, 10 nM-3 μM) was assessed on endothelium-denuded rings. Endogenous catalase was inhibited in aortic rings by incubation with AT (50 mM) for 90 min. Statistical analysis was performed by ANOVA followed by Fisher's test.

Following induction of submaximal PE-induced tone, ACh, GTN and ISO each induced concentration-dependent relaxation (maximal relaxation of 95.2 ± 3.2 %, 99.1 ± 0.6 % and 93.4 ± 4.6 %, respectively, n=6 for each. Addition of hydrogen peroxide (1 mM) for 30 min following precontraction resulted in powerful relaxation of endothelium-containing (96.2 ± 1.2 %, n=6) and endothelium-denuded (95 ± 1.6 %, n=6) rings. Following washout of hydrogen peroxide, subsequent maximal relaxation induced by ACh, GTN and ISO was inhibited (by 21.0 ± 0.7 %, 32.3 ± 1.9 % and 32.1 ± 1.3 %, respectively, $P<0.05$ for each vs. control, n=6 for each). Treatment with catalase (1000 units ml^{-1}) completely prevented the ability of hydrogen peroxide (1 mM, 30 min) to inhibit relaxation to ACh, GTN or ISO. Pretreatment with AT (50 mM, 90 min) to inhibit endogenous catalase had no effect on the relaxant actions of hydrogen peroxide, ACh, GTN or ISO. In contrast, pretreatment with AT powerfully potentiated the ability of hydrogen peroxide (1 mM, 30 min) to inhibit relaxation to ACh, GTN and ISO (maximal relaxation inhibited by 68.0 ± 6.2 %, 73.1 ± 7.1 %, and 64.0 ± 5.5 %, respectively, $P<0.05$ for each vs. hydrogen peroxide alone, n=6 for each).

The ability of the oxidant, hydrogen peroxide, to inhibit the endothelium-dependent relaxation to ACh and the endothelium-independent relaxation to GTN and ISO suggests it acts by a non-selective damaging action on vascular smooth muscle. Potentiation of these damaging actions by AT is consistent with a role for endogenous catalase in protecting vascular tissue from oxidant damage by hydrogen peroxide.

This work was supported by the British Heart Foundation and the Medical Research Funds of the University of Glasgow.

Mian, K.B. & Martin, W. (1995). *Br. J. Pharmacol.*, 116, 3302-3308.
Margoliash, E. & Novogrodsky, A. (1958). *Biochem. J.*, 68, 468-475.

---

305 P IODINATED RADIOGRAPHIC CONTRAST MEDIA (IRCM) CAUSE BOTH ENDOTHELIUM-INDEPENDENT RELAXATION AND CONTRACTION OF RABBIT AORTA

M.R.Pitman, J.O.G.Karlsson*,& T.M.Griffith (introduced by M.J.Lewis).
[1] Cardiovascular Sciences Research Group, Dept. of Diagnostic Radiology, UWCM, Heath Park, Cardiff, CF4 4XN and * Nycomed Imaging AS, Oslo, Norway.

Iodinated radiographic contrast media (IRCM) are well recognised to cause peripheral vasodilatation following intravascular administration in both the clinical situation (Pugh *et al* 1993) and animal models (Almen 1987; Pugh *et al* 1995). This phenomenon has been theorised to be a consequence of the high osmolality of the contrast agents. However, previous studies in this laboratory have shown that even iso- osmolar formulations of IRCM may cause significant vasorelaxation in vivo and in isolated arterial preparations (Pugh *et al* 1993, 1995). In the present study we have shown that iohexol (a triiodinated monomer) and iodixanol (a newer, non-ionic hexaiodinated dimer) induce endothelium-independent relaxation of isolated rings of rabbit thoracic aorta constricted by 0.3 μM phenylephrine (PhE). When considered in terms of iodine concentration, relaxation was significantly greater with iohexol (58.1 ± 6.2 %; n=12) than iodixanol (46.1 ± 2.9 %; n=10; $P < 0.05$) at 100 mg Iodine/ml. However, when expressed in terms of molarity (maximum 240 and 245 mM for iodixanol and iohexol, respectively) the concentration-relaxation curves to these agents were found to superimpose. When the "inert" sugar mannitol (1-180 mM) was used to increase the osmolality of the tissue buffer to levels comparable with those produced by iohexol and iodixanol there was only a small relaxant effect (< 15 % of induced tone).

Paradoxically, following a small initial relaxation, in preparations not actively preconstricted by phenylephrine iohexol and iodixanol both evoked an increase in tone, at the highest concentrations employed (100 and 200 mg Iodine/ml for iohexol and iodixanol respectively). This phenomenon was more pronounced with iohexol (1.36 ± 0.13 g ; n=13) than iodixanol (0.45 ± 0.24 g ; n=8) and first became apparent at similar molarities rather than similar iodine concentrations. Contraction to iohexol was unaffected by incubation of the aortae with verapamil (10 μM; n=6) or Ca^{2+}- free buffer containing 5 mM EGTA (n=6), and therefore independent of extracellular Ca^{2+} influx. In the absence of exogenously-induced tone (by PhE) mannitol caused a small concentration-dependent contraction (0.3 ± 0.1 g at 180 mM) but no intial relaxation was observed as in the case of iohexol and iodixanol.
In summary :- (i) neither the presence of the iodine atom nor hyperosmolality appear to be principal determinants of the vasoactive properties of IRCM, (ii) contraction to IRCM may depend on Ca^{2+} release from internal stores, and (iii) at any given concentration the net clinical effects of IRCM upon the vasculature are likely to depend on the balance between competing dilator and constrictor mechanisms.

Almen T (1987) *Acta. Radiologica.* (suppl 370) 69-72.
Pugh ND, Sissons GRJ, Ruttley M *et al* (1993) *Clin. Radiol.* 47, 96-99.
Pugh ND, Hutcheson IR, Edwards DH *et al* (1995) *Br. J. Radiol* 68, 23-26

306 P HETEROGENEITY OF K+-INDUCED RELAXATIONS IN RAT SMALL MESENTERIC ARTERIES AND CORONARY SEPTAL ARTERIES

Harrie C.M. Boonen, Birgitte Roed and Richard D. Carr

Diabetes Pharmacology, Diabetes Discovery, Novo Nordisk A/S, DK 2880 Bagsvaerd, Denmark

Insulin has been shown to either exert vasoconstrictive or vasodilatory effects depending on the vascular bed studied (1,2). The vasodilatory effect of insulin may involve activation of smooth muscle Na^+/K^+-ATPase (3). We hypothesise that vascular heterogeneneity of insulin responses can be explained by differences in vascular smooth muscle Na^+/K^+-ATPase. We have therefore assessed Na^+/K^+-ATPase activity as a function of K^+-induced relaxing responses in small mesenteric arteries (MA) and coronary septal arteries (CA) isolated from adult male Sprague Dawley rats.

Vessels (6 MA and 4 CA) were mounted at optimal diameter for isometric force development in a wire myograph and bathed at 37°C in bicarbonate buffered Krebs solution (KRB). K^+-induced relaxations were obtained 1) by adding 12 mM K^+ to vessels precontracted with 10 μM noradrenaline (NA) and incubated in normal KRB or 2) by adding K^+ cumulatively (2 - 12 mM in MA and 0.5 - 3 mM in CA) to vessels precontracted with 10 μM NA and preincubated for 15 minutes in K^+-free KRB (KRB_{K0}). K^+ relaxations were repeated in the continuous presence of 50 μM Ba^{2+} (inhibitor of inward rectifying K^+ -channels) or 100 μM ouabain (inhibitor of Na^+/K^+-ATPase). All experiments were performed in the presence of 1 μM propranolol.

Maximal K^+-induced relaxations were expressed as a residual fraction of the precontraction to NA (E_{max}): 0.97 ± 0.01 in MA; 0.11 ± 0.12 in CA and 0.14 ± 0.02 in MA; -0.09 ± 0.04 in CA, in vessels incubated in KRB and KRB_{K0}, respectively. Incubation with Ba^{2+} in vessels bathed in KRB significantly reduced K^+ relaxations in CA (E_{max}: 0.89 ± 0.02) but had no effect in MA (E_{max}: 0.96 ± 0.01), whereas Ba^{2+} had no significant effect on maximal K^+-induced relaxations or on K^+ sensitivity in vessels preincubated in KRB_{K0} (E_{max}: 0.06 ± 0.04 in MA; -0.01 ± 0.02 in CA, EC_{50}: 5.6 ± 0.5 in MA; 1.3 ± 0.2 in CA). On the other hand, in vessels preincubated in KRB_{K0}, ouabain significantly reduced maximal K^+ relaxations in MA (0.63 ± 0.09) but not CA (0.18 ± 0.12). Ouabain, furthermore, reduced sensitivities for K^+ in CA (EC_{50} (in mM) 0.9 ± 0.2 vs. 2.6 ± 0.5) and to a much larger extent in MA (EC_{50} (in mM) 4.0 ± 0.7 vs. 7.8 ± 1.1).

Judging from the differences in K^+- and ouabain-sensitivity, these data suggest that different isoforms of Na^+/K^+-ATPase may be present in CA and MA. Vascular heterogeneity in K^+-induced relaxations may be explained by a difference in the number of functional Ba^{2+}-sensitive inward rectifying K^+ -channels in CA and MA.

1) Henrion,D et al. (1994), *Can. J. Physiol. Pharmacol.* 72:849-54
2) McNally, PG et al. (1995), *Diabetologia.* 38:467-73
3) Kahn,AM et al. (1993), *Hypertension* 22:735-42

---

307 P NITRIC OXIDE SYNTHASE INHIBITION AFFECTS NONADRENERGIC-NONCHOLINERGIC RELAXATION IN HUMAN COLON

A. Hassanali, I.F. Stamford, I.A. Tavares & J.A. Rennie. Department of Surgery, King's College School of Medicine & Dentistry, The Rayne Instutute, 123 Coldharbour Lane, London SE5 9NU.

There is evidence of an inhibitory role for nitric oxide (NO) in nonadrenergic-noncholinergic (NANC) relaxations to electrical field stimulation (EFS) in human colon (Burleigh, 1992, Boeckxstaens *et al.*, 1993); however the results are variable and suggest a possible contribution by other transmitters to the NANC response. In an attempt to further clarify the role for NO, the NO synthase (NOS) inhibitors N^Gnitro-L-arginine (L-NOARG) and N^Gnitro-L-arginine methyl ester (L-NAME) have been studied to compare the contribution of NO to EFS induced NANC relaxations in two different colonic regions.

Macroscopically normal sigmoid and ascending colonic circular muscle strips were obtained from specimens resected for malignant disease. Muscle strips (approximately 5x20mm) were suspended in Krebs' solution (O_2 /CO_2, 95:5%, 37°C) and equilibrated (60-90min). Submaximal isotonic responses to acetylcholine (ACh) and noradrenaline (NA) were established. The tissues were then electrically stimulated (EFS) via two parallel electrodes at parameters of 5Hz, 1ms pulse width and 30s pulse duration. Voltage response curves were constructed and a submaximally effective voltage selected. A complete inhibition of adrenergic and cholinergic responses was achieved throughout the experiment using phentolamine, propranolol and hyoscine (all 30μM). Reproducible submaximal relaxations to EFS (10min intervals) maintained throughout the experiment, were followed by incubation (30min) with increasing concentrations (non-cumulative) of L-NAME, L-NOARG, or equivalent saline control volumes. Prior to the increasing concentration of NOS inhibitor, relaxation to sodium nitroprusside (SNP) was established. An attempt was made to counteract any effect of L-NAME or L-NOARG with L-arginine (L-ARG) at the end of each experiment.

Results are expressed as the percent change of relaxation in test tissues compared to control tissues. Statistical analysis used Student's t-test (unpaired) with data expressed as mean ± s.e.mean. A large degree of concentration-dependent inhibition was achieved with L-NAME and L-NOARG in both colonic regions. IC_{50} values for L-NAME and L-NOARG differed in sigmoid colon (23±8.2μM, n=5 and 3.7±0.7μM, n=4 respectively) as did the maximum percentage inhibition (82±7% and 92±8%). In the ascending colon the mean IC_{50} values were 9.6±2.1μM, n=5 and 2.8±0.9μM, n=5 for L-NAME and L-NOARG, maximally inhibiting relaxation by 91±4% and 85±7% respectively. L-ARG (100μM) counteracted the NO synthase inhibition after 30min by 10% for L-NAME and 16% for L-NOARG treated strips. A rise in muscle tone of strips occurred on incubation with L-NAME (10μM) and L-NOARG (1μM) compared to the vehicle control tissues (both $p<0.05$).

Assuming selectivity of L-NAME and L-NOARG for NO synthase, these results suggest that in human ascending and sigmoid colonic circular muscle, NO has an inhibitory role and is likely to be a principal mediator of NANC relaxation.

A.Hassanali is funded by the King's Medical Research Trust.

Boeckxstaens, G., Pelckmans, P.A., De Man, J.G. *et al.* (1993) Gastroenterology. 104, 690-683.
Burleigh, D.E. (1992) Gastroenterology. 102, 697-683.

308 P THE PHARMACOLOGY OF FR173657, A NEW, POTENT AND SELECTIVE NON-PEPTIDE BRADYKININ ANTAGONIST: *IN VITRO* STUDIES IN GUINEA-PIG, RAT AND RABBIT TISSUES

F.J. Legat, W. Sametz*, S. Diethart*, S. Hammer* & T. Griesbacher. Dept. of Experimental & Clinical Pharmacology, and *Dept. of Animal Biology, University of Graz, Austria.

Bradykinin (BK) is recognized to be an important mediator in inflammation. We describe here the pharmacological characteristics *in vitro* of (E)-3-(6-acetamido-3-pyridyl)-N-[N-[2,4-dichloro-3-[(2-methyl-8-quinolinyl) oxymethyl] phenyl]-N-methylaminocarbonyl-methyl]acrylamide (FR173657), a recently disclosed nonpeptide BK antagonist (Inamura *et al.*, 1996).

Contractions of the guinea-pig ileum induced by BK (30 nM) were significantly ($P<0.05$) inhibited in a concentration-dependent manner by FR173657 (3–300 nM) with a pIC_{50} value of 7.4±0.1 ($n=6$). Up to 90 min were required for a full recovery of the BK effect following 300 nM FR173657. Cumulative concentration-response curves for BK (1 nM – 9 μM) were shifted to the right by FR173657 (30–300 nM) with a concomitant decrease in the maximum effect. The contractile effects of acetylcholine, histamine, 5-hydroxytryptamine, angiotensin II, substance P or caerulein were not affected.

The concentration-response curve for contractions of the circular smooth muscles of the isolated guina-pig trachea in response to BK (10 nM – 10 μM) was shifted to the right by FR173657 (10–100 nM, $n=4$–10). At 100 nM, FR173657 almost completely abolished ($P<0.001$) the effects of the highest BK concentration (10 μM) tested. Contractions by acetylcholine and relaxations in response to isoproterenol were the same as in control tissues.

Relaxations of the isolated rat duodenum in response to BK (0.1–1000 nM) were significantly ($P<0.05$) and concentration-dependently inhibited by FR173657 (10–300 nM, $n=4$–10). While the concentration-response curve to BK was shifted to the right, a decrease in the BK maximum could not be clearly seen since no concentrations of BK higher than 1 nM were tested. The duration of the inhibition was similar to that in the guinea-pig ileum. B_1 receptor-mediated relaxations by des-Arg^9-BK (10–1000 nM) were unchanged by FR173657 (300 nM).

In the isolated perfused ear of the rabbit, BK produced a short-lasting venoconstriction as measured by the reduction in the venous effluent. The reduction of flow was dose-dependent between 21±3% at 30 pmol and 84±3% at 10 nmol BK ($n=6$). When FR173657 was added to the perfusion medium at 30 nM, the effects of all doses of BK were reduced by at least 60% ($P<0.05$, $n=5$), while 300 nM FR173657 completely abolished ($P<0.05$, $n=4$) the effects of BK. Venoconstriction induced by angiotensin II or by noradrenaline was completely unaffected.

BK-induced prostaglandin (PG) release was measured by scintillation counting of ^{14}C-prostanoids in thin-layer chromatography fractions of the 20 min effluent from isolated perfused ears prelabelled with ^{14}C-arachidonic acid (Juan, 1981). BK (10 nmol) caused a significant increase in PGE_2 (3.3±0.3 Bq vs 0.8±0.1 Bq before BK, $P<0.05$) and PGI_2 (2.8±0.2 Bq vs 0.9±0.1 Bq, $P<0.05$) ($n=4$). In the presence of FR173657 (300 nM) the release of prostaglandins after BK (0.9±0.2 Bq PGE_2 and 0.9±0.4 Bq PGI_2, $n=4$) was not different from the basal values.

In summary, FR173657 is a highly selective non-peptide BK B_2 receptor antagonist. Its potency and mode of action *in vitro* is similar to that of current peptide BK antagonists. Since the compound exhibits similar properties *in vivo* (Griesbacher & Legat, 1996), it can be expected that FR173657 will be a major advance for potential clinical uses of BK antagonists.

Griesbacher T. & Legat F.J. (1996). *Br. J. Pharmacol.*, this meeting.
Inamura N. *et al.* (1996). *Peptide Receptors*, Montréal, 28 July–1 August
Juan H. (1981). *Naunyn-Schmiedeb. Arch. Pharmacol.*, 317, 345–350

---

309 P THE PHARMACOLOGY OF FR173657, A NEW, POTENT AND SELECTIVE NON-PEPTIDE BRADYKININ ANTAGONIST: *IN VIVO* STUDIES IN RATS AND GUINEA-PIGS

T. Griesbacher & F.J. Legat. Department of Experimental and Clinical Pharmacology, University of Graz, Universitätsplatz 4, A-8010 Graz, Austria.

The novel, non-peptide bradykinin (BK) B_2 receptor antagonist, (E)-3-(6-acetamido-3-pyridyl)-N-[N-[2,4-dichloro-3-[(2-methyl-8-quinolinyl)oxymethyl] phenyl]-N-methylaminocarbonyl-methyl] acrylamide (FR173657; Inamura *et al.*, 1996), is highly potent, selective and long-acting *in vitro* (Legat *et al.*, 1996). Hence, the compound was tested against the actions of BK in anaesthetized rats and guinea-pigs.

In anaesthetized (40 mg kg^{-1} pentobarbital, i.p.) rats pretreated with captopril (50 μmol kg^{-1}, i.p.), the i.v. injection of BK (50 pmol) caused shortlasting (less than 2 min) falls of carotid blood pressure by 20–35 mmHg. Following s.c. injection of FR173657 (100 nmol kg^{-1}) the effects of the subsequent BK injections decreased gradually. At 60 min the effect was reduced to 36±9% ($P<0.05$, $n=5$) of the effect before FR173657. At 300 nmol kg^{-1}, FR173657 abolished the hypotensive effecs of BK from 45 min onwards. No recovery of the inhibition was seen for a further period of at least 3 h.

In order to ascertain whether the inhibition was reversible, FR173657 (300 nmol kg^{-1}), or its solvent (DMSO, 0.5 ml kg^{-1}), was given s.c. 24 h prior to the experiment. While BK caused a fall in blood pressure by 16±2 mmHg ($n=5$) in controls, it was 17±2 mmHg ($n=5$, ns) in rats which had received FR173657.

Visceral plasma protein extravasation was determined as extravasation of Evans blue (E.b.), given i.v. (20 mg kg^{-1}) in anaesthetized and captopril-treated rats. BK (11 nmol kg^{-1} in 20 min, i.v.) caused a prominent accumulation of E.b. in the pancreas (582±34 μg g^{-1} dry wt, $n=6$). FR173657 (30 nmol kg^{-1}, s.c. 60 min before BK) reduced the pancreas content of E.b. to values (83±14 μg g^{-1}, $n=6$, $P<0.01$) which were the same as in rats infused with saline (3.8 ml kg^{-1} in 20 min) instead of BK (71±10 μg g^{-1}, $n=5$). A partial inhibition was caused by 10 nmol kg^{-1} FR173657 while 3 nmol kg^{-1} were inactive.

The subplantar injection of 30 nmol BK into one hindpaw of anaesthetized rats caused an increase in paw volume of 59±8% ($n=6$) at 60 min. FR173657 at 300 nmol kg^{-1} (s.c., 60 min before the experiment) reduced this value slightly (50±8%, $n=7$) but not significantly, while 1 μmol kg^{-1} reduced it to 36±5% ($P<0.05$, $n=6$). Higher doses of FR173657 caused no further inhibition since this part of the oedema is due to 5-hydroxytryptamine (5HT) release from mast cells (Griesbacher *et al.*, 1996). Paw oedema induced by 5HT or histamine was not affected.

In anaesthetized (2 g kg^{-1} urethane, i.p.) and respirated guinea-pigs, BK (20 nmol kg^{-1}, i.v.) caused increases in pulmonary inflation pressure by 200–600 Pa. The effect was reduced to 56±12% of the basal value ($P<0.05$, $n=5$) 60 min after FR173657 (1 μmol kg^{-1}, s.c.) whereas only 9±7% ($P<0.05$, $n=5$) remained after 10 μmol kg^{-1}. Histamine-induced bronchoconstriction was unaffected by FR173657.

In summary, vascular and bronchial effects of BK which are related to its pathophysiological role are inhibited by FR173657 potently and selectively *in vivo*. The inhibition caused by FR173657 lasts for several hours, but is fully reversible.

Griesbacher T., Rainer I. & Griengl S. (1996). *Br. J. Pharmacol.* 117: 89P
Inamura N. *et al.* (1996). *Peptide Receptors*, Montréal, 28 July–1 August
Legat F.J., Sametz W., *et al.* (1996). *Br. J. Pharmacol.*, this meeting.

310 P A GENERAL OPERATIONAL MODEL OF PHARMACOLOGICAL SYNERGISM

G. R. Martin[1], S. J. MacLennan[1], M. Maxwell & R. R. Smith, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS. [1]Present address : Institute of Pharmacology, Neurobiology Unit, Roche Bioscience, Palo Alto, CA94304, USA

A greater-than-additive effect is often produced by two pharmacological agents acting at distinct receptors on the same cell. Such response super-additivity may be evident at threshold, but not higher, concentrations of the interactants as described by Leff (1987). In other cases, super-addition of agonist effects is evident over the entire range of an agonist concentration-response curve (Fig. 1B; MacLennan *et al.*, 1993) and may even result in the unmasking of receptors that are otherwise 'silent' (McGrath *et al.*, 1990). Here, we propose a general theoretical model to account for both forms of pharmacological synergism.

It is assumed that two agonists activate distinct receptors to form AR_1 and AR_2 which in turn promote the saturable formation of transducer elements ART_1 and ART_2 respectively. $[ART_1]$ and $[ART_2]$ act additively to generate response, but $[ART_1]$ also facilitates $[ART_2]$-mediated transduction by increasing $[ART_2]$ formation. In both cases, generation of [ART] is described by :

$$[ART] = ART_{MAX}.\rho.a/((a+1)+\rho.a) \quad ...(1)$$

where ART_{MAX} is the maximum amount of [ART] formed, a is the normalised agonist concentration ($[A]/K_A$) and ρ, the ratio of receptor concentration ($[R_0]$) over the value of [AR] at $0.5ART_{MAX}$ (K_{ART}), describes the efficiency of [ART] production. However, when $[ART_1]$ modifies $[ART_2]$ formation :

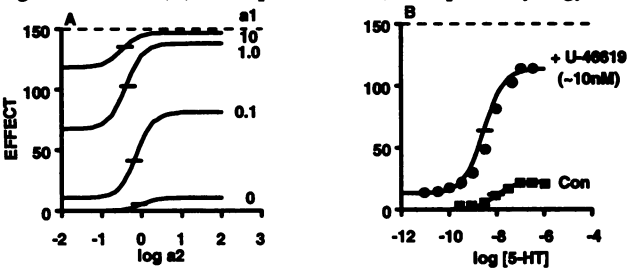
$$[ART_2] = ART_{2MAX}/(((a_2+1)/((\sigma.[ART_1]+1))\rho_2.a_2)+1) \quad ..(2)$$

where σ, the slope of the $[ART_1]$: $[ART_2]$ relation, is a factor that determines the stoichiometry of the cross-talk. Final effect, E_x is described by :

$$E = E_{max}.[ART_1+ART_2]^n/([ART_1+ART_2]^n+K^n) \quad ...(3)$$

where n is the slope of the $E/[ART_1+ART_2]$ relation and K is a constant. Fig.1A shows curves simulated using equation (3) with increasing a_1 and $\sigma=100$. Synergy manifests as a modest, progressive potentiation accompanied by a profound increase in curve amplitude. Fig.1B shows the effect of the TP agonist U46619 on $5\text{-HT}_{1B/1D}$ receptor-mediated contractions in rabbit femoral artery. The lines superimposed on the data, produced using equation (3) with previously determined estimates of E_{max}, K_A and n for 5-HT, illustrate the ability of the model to describe experimental synergy.

Fig. 1. Simulated (A) and experimental (B) examples of synergy.



Conclusions : Synergism of this type is frequently reported between $G_{i/o}$- (e.g. α_2, $5\text{-HT}_{1B/1D}$, NPY_1, A_1) and $G_{q/11}$-coupled (e.g. H_1, AT_1, TP) receptors. The operational analysis described here might assist in better understanding the molecular mechanisms involved.

Leff, P. (1987) *J. Pharmacol. Exp. Ther.*, **243**, 1035-1042.
MacLennan, S.J., Bolofo, M.L. & Martin G.R. (1993) *Biochem. Soc. Trans.*, **21**, 1145-1150.
McGrath, J.C. *et al.*, (1990) *Blood Vessels*, **27**, 146-152.

---

311 P THE ROLE OF ENDOTHELIN IN THE EVOLUTION OF ISCHAEMIA FOLLOWING HAEMORRHAGIC STROKE IN SPONTANEOUSLY DIABETIC (BB) AND NON-DIABETIC RATS

I.P. Fouyas, P.A.T. Kelly, I.M. Ritchie & I.R. Whittle, Department of Clinical Neurosciences, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU.

Factors released from the blood increase the bioavailability of endothelin (ET) (Ohlstein *et al.*, 1991), and ET has been implicated in the pathophysiological response to intracerebral haemorrhage. ET antagonists reduce infarct volume following occlusive stroke in rat (Barone *et al.*, 1995). In this study we have examined the possible involvement of ET in the development of delayed perilesional ischaemia following intracerebral haemorrhage, and have addressed the contribution to final outcome of the chronic cerebrovascular dysfunction associated with diabetes mellitus (Fouyas *et al.*, 1996).

During a brief period of anaesthesia, 50µl of arterial blood was injected unilaterally into the striatum of spontaneously diabetic, insulin-treated BB rats (8-12 weeks after onset of diabetes, with glycosylated haemoglobin levels between 7 and 12%) and non-diabetic controls. After 24h, local cerebral blood flow (LCBF) was measured using [^{14}C]-iodoantipyrine autoradiography as we have previously described (Fouyas *et al.*, 1996). A group of diabetic (n=4) and non-diabetic (n=6) rats received the ET antagonist SB209670 (Ohlstein *et al.*, 1991) (10mg.kg^{-1} i.p.) every 6h, starting 30min prior to the induction of the haematoma. The remaining diabetic (n=5) and non-diabetic (n=5) rats received saline injection. Volumetric analysis of striatal ischaemia was by densitometric analysis of autoradiographic images, with thresholds set at LCBF <35ml.100g^{-1}. min^{-1} (Hossmann 1994). Adjacent sections were stained with cresyl violet, for correlation of ischaemic thresholds with neuropathological evidence of stroke. Data (mean ± s.e.mean) were analyzed by Bonferroni t-test, with significance set at P<0.05.

Haematoma volume measured by planimetry *post mortem* revealed no differences in the burden of primary insult in the various groups.

At the time of LCBF measurement, there were no significant differences in physiological variables in non-diabetic rats treated with either saline or SB209670. However, MABP in SB209670-treated diabetic rats (104 ± 3mmHg) was lower than in those treated with saline (115 ± 4mmHg), although this failed to reach significance. Saline-treated diabetics had a significantly larger volume of striatal ischaemia compared to the non-diabetics (4.89 ± 1.57 vs 0.68 ± 0.21mm^3) 24h after the haematoma. In non-diabetic rats, SB209670 treatment significantly reduced the volume of ischaemia by 85% (0.1 ± 0.05 vs 0.68 ± 0.21 mm^3), but SB209670 failed to reduce the volume of ischaemia in diabetic animals (5.64 ± 1.93 vs 4.89 ± 1.57 mm^3). In areas distant from the haematoma, e.g. contralateral striatum, SB209670 treatment produced no effect upon LCBF in the non-diabetics (123 ± 3 vs 126 ± 6 ml.100g^{-1}.min^{-1} in saline-treated rats) whereas in diabetic rats LCBF increased (from 92 ± 14 to 116 ± 11 ml.100g^{-1}.min^{-1}), although this was not significant.

These results indicate that in normal rats ET may contribute (in part at least) to the development of delayed perilesional ischaemia following intracerebral haemorrhage, and that ET antagonists may have therapeutic potential in this setting. However, ET does not appear to be involved the increased vulnerability of diabetic rats to cerebral ischaemia, and there is no evidence that SB209670 alters the ischaemic burden in these rats. These findings are of particular importance in that a background of cerebrovascular dysfunction and associated pathology, is very common in human sufferers of occlusive as well as haemorrhagic stroke.

Barone, F.C. *et al.* (1995) *J. Cardiovasc. Pharmacol.* **26**, S404-S407.
Fouyas, I.P. *et al.*, (1996) *Br. J. Pharmacol.* **118**, 243-248.
Hossmann K-A. (1994) *Ann. Neurol.* **36**, 557-565.
Ohlstein, E.H. *et al.* (1991) *Circ. Res.* **69**, 832-841.

312 P EFFECT OF CANNABINOID AGONISTS AND SR141716A ON ACETYLCHOLINE RELEASE FROM THE MYENTERIC PLEXUS OF THE GUINEA-PIG SMALL INTESTINE

A.A. Coutts & R.G. Pertwee, Department of Biomedical Sciences, University of Aberdeen, Aberdeen AB9 1AS.

Cannabinoid receptor agonists inhibit electrically-evoked contractile responses of the myenteric plexus-longitudinal muscle preparation of the guinea-pig small intestine and this action can be reversed or prevented by the selective cannabinoid CB1 receptor antagonist, SR141716A, (Coutts *et al.*, 1995). It is assumed that the mechanism of action of cannabinoids is prejunctional since these drugs have no significant effect on the sensitivity of the preparation to exogenously applied acetylcholine (ACh). In the present experiments, we sought to demonstrate the presynaptic localisation of cannabinoid receptors by direct measurement of changes in evoked ACh release following exposure to cannabinoids.

Strips of myenteric plexus-longitudinal muscle were dissected from the small intestines of male albino Dunkin-Hartley guinea-pigs (300-800 g) as described by Pertwee *et al.* (1992). Donor preparations were suspended in 3ml organ baths containing choline chloride (20 μM) in modified Krebs solution at 37° C and bubbled with 95% O_2 and 5% CO_2. Tissues were stimulated continuously at supramaximal voltage (110%) with 0.5 ms pulses at 0.1 Hz and isometric contractions were recorded until they were constant. Subsequently, the preparations were incubated without stimulation for 1 h in the presence of the acetylcholinesterase inhibitor, eserine (7.7 μM). Samples of donor bath fluid were collected at the end of 4-min collection periods during which the preparations were stimulated at 0.1 Hz. ACh was assayed against standard solutions of ACh, on strips of myenteric plexus-longitudinal muscle mounted in 3 ml organ baths to measure ACh-induced isotonic contractions (Waterfield, 1973). Donor preparations were preincubated for 8 min in the presence of test drugs before the start of collection of samples.

The cannabinoid receptor agonists, WIN 55212-2 and CP 55940 (15 to 200 nM), caused a dose-related inhibition of ACh release which was reversed by SR141716A (100 to 400 nM) but not by an equivalent amount of its vehicle, Tween 80 (n=3 to 14). In the presence of a maximal concentration (200 nM) of WIN 55212-2 or CP 55940, ACh release fell from its predrug level ($P < 0.05$; Student's paired t test) by 35.2 ± 4.2% and 34.7 ± 4.4% respectively (mean ± s.e.m.; n=4 or 14). The cumulative dose response curves for WIN 55212-2 on both twitch response (n=8) and ACh output (n=6) were shifted to the right ($P < 0.05$; symmetrical 2+2 dose assay) in the presence of SR141716A (100 to 400 nM). In concentrations of up to 1000 nM WIN 55212-3, the (–)-enantiomer of WIN 55212-2, was devoid of activity as a twitch inhibitor (n=6) and had little effect on ACh release (n=4). It is concluded that WIN 55212-2 and CP 55940 inhibit electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation through prejunctional cannabinoid CB1 receptors. By itself, SR141716A (10 to 160 nM) caused small but significant increases in both ACh release ($P < 0.05$; Student's paired t test; n=9) and the amplitude of electrically-evoked contractions ($P < 0.01$; Student's unpaired t test; n=11 or 12). This suggests that SR141716A may be acting as an inverse agonist at cannabinoid receptors in the myenteric plexus-longitudinal muscle preparation or antagonising an endogenously-released cannabinoid.

We thank the Wellcome Trust for financial support, Pfizer for CP 55940 and Sanofi Winthrop for (+)-WIN 55212 and SR141716A.

Coutts, A.A. *et al.* (1995) *Br. J. Pharmacol. (Proc. Suppl.)* 116, 46P.
Pertwee, R.G. *et al.* (1992) *Br. J. Pharmacol.* 105, 980-984.
Waterfield, A.A. (1973) *Ph.D Thesis, University of Aberdeen.*

---

313 P LIPOPOLYSACCHARIDE-INDUCED NITRITE FORMATION IN RAT ANOCOCCYGEUS CULTURED SMOOTH MUSCLE CELLS

J. Zhou, A.D. Struthers & G.A. Lyles. Department of Pharmacology and Clinical Pharmacology, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY.

Treatment with bacterial lipopolysaccharide (LPS) leads to expression of inducible nitric oxide synthase (iNOS) in rat aortic cultured smooth muscle cells (Szabo *et al.*, 1993). Similar mechanisms for NO formation have been implicated in producing vasodilation leading to severe hypotension in patients with septic shock (Moncada & Higgs, 1995). Here we have investigated if LPS treatment can also induce NO formation in non-vascular cultured smooth muscle cells (SMC) from the rat anococcygeus muscle.

Primary cultures of SMC were obtained by collagenase digestion of rat anococcygeus muscles or aortae, and by growth of cells in Medium 199, 10% foetal bovine serum and antibiotics/amphotericin. Cells were identified as SMC by immunofluorescence after treatment with an anti-smooth muscle actin antibody (from mouse), followed by a fluorescently-labelled anti-mouse IgG (from goat). Confluent cells were trypsinized and passaged into 12 well plates. LPS or drugs were added in culture medium to confluent cells, and the medium was sampled from wells at designated times for determination of nitrite by the Griess reaction, as an index of NO production. None of the drugs affected cell viability under the conditions used.

In initial experiments, anococcygeus SMC were treated with LPS (1, 10 and 100 μg/ml) for 96h, involving media/LPS replacement every 24h with fresh solutions immediately after media samples from the cells had been taken for nitrite analysis. Nitrite concentrations (μM) per 24h sample increased with concentration and duration of LPS treatment, reaching an apparent maximum around 72 - 96h. At 24h these nitrite values were: No LPS, 0.6 ± 0.2; 1 μg/ml, 1.2 ± 0.6; 10 μg/ml, 1.6 ± 0.7; 100 μg/ml, 3.3 ± 0.9. At 96h, corresponding values were: No LPS, 1.0 ± 0.4; 1 μg/ml, 2.7 ± 0.6; 10 μg/ml, 5.6 ± 1.3; 100 μg/ml, 8.5 ± 1.8 (mean ± s.e. of 4 cultures from different rats).

Pyrrolidine dithiocarbamate (PDTC), an antioxidant which acts as an inhibitor of transcription factor NF-κB activation, blocks iNOS expression in rat aortic smooth muscle cells (Hattori *et al.*, 1996). Phenylarsine oxide (PAO), a protein-tyrosine phosphatase (PTP) inhibitor, also prevents NF-κB activation implying a role for PTP in the pathway for this activation (Singh & Aggarwal 1995). After 24h treatment, PDTC significantly ($P < 0.05$, t-test) inhibited LPS-induced nitrite formation in SMC from rat aorta (1 μg/ml LPS, 4.8 ± 0.8; LPS + 25 μM PDTC, 0.4 ± 0.1, n=5) and anococcygeus (100 μg/ml LPS, 4.7 ± 0.9; LPS + 12.5 μM PDTC, 1.4 ± 0.8, n=6). Similarly, PAO (65 nM) treatment also significantly inhibited nitrite formation in aortic (1 μg/ml LPS, 7.8 ± 0.8; LPS + PAO, 3.3 ± 1.1, n=7) and anococcygeus SMC (10 μg/ml LPS, 3.8 ± 0.9; LPS + PAO, 1.0 ± 0.3, n=5).

These studies suggest that iNOS expression in both aortic and anococcygeus SMC may be linked to NF-κB activation, although more direct methods of determining iNOS protein and nuclear NF-κB translocation will be required to confirm this.

Supported by Tenovus-Scotland (Tayside) and the Northwood Charitable Trust

Hattori, Y., Nakanishi, N., Kasai, K., *et al.* (1996) *Eur. J. Pharmacol.*, **296**, 107-112.
Moncada, S. & Higgs, E.A. (1995) *FASEB J.* **9**, 1319-1330.
Singh, S. & Aggarwal, B.B. (1995) *J. Biol. Chem.* **270**, 10631-10639.
Szabo, C., Mitchell, J.A., Gross, S.S. *et al.* (1993) *J. Pharmacol. exp. Therap.* **265**, 674-680.