251P ACUTE AND CHRONIC EFFECTS OF SECOND AND THIRD GENERATION PROGESTOGENS ON RAT PLATELET AGGREGATION

J.L. Fraser & S.J. Coker, Department of Pharmacology and Therapeutics, The University of Liverpool, Ashton Street, Liverpool, L69 3GE

It has been reported that the risk of myocardial infarction was lower in users of oral contraceptives containing third generation progestogens, e.g. desogestrel, compared to that with second generation products, such as norethisterone (Lewis *et al.*, 1996). However, a recent publication reports no difference between second and third generation progestogens (Dunn *et al.*, 1999). As platelet aggregation initiates arterial thrombosis, which can lead to myocardial infarction, we have compared the effects of progestogens on platelet aggregation.

Female Wistar rats (200-310g) were ovariectomized under Hypnorm/diazepam anaesthesia. After 12 to 16 days, rats (290-400g) were used either for acute experiments or treated with 0.03 mg kg^{-1} norethisterone or desogestrel, or vehicle (sesame oil) s.c. daily for 7 days. Rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.) and blood was removed from a cannulated carotid artery and diluted 1:1 with saline containing 10 Units ml^{-1} heparin. Aliquots (1ml) of diluted blood were placed in cuvettes, incubated at 37°C for 5 min and platelet aggregation responses to ADP or collagen were measured by impedance aggregometry. In acute experiments, responses obtained following incubation with 0.03, 0.1 and 0.3 μM norethisterone or 3-keto desogestrel (3KD), the active metabolite of desogestrel, were compared to vehicle (0.5% Tween 80 in DMSO), all added in 1 μl volumes for 1 min (n=6 per group).

In vitro the highest concentration of norethisterone (0.3 μM) increased 1 μM ADP-induced aggregation from 14.9 ± 1.1 to 17.8 ± 0.7 Ω ($P<0.05$) whereas 3KD did not alter the response (19.2 ± 0.9 Ω with vehicle, 21.6 ± 1.3 Ω with 0.3 μM 3KD).

With chronic administration of progestogens for 7 days, norethisterone, but not desogestrel, enhanced responses to ADP (1 μM) and to ADP (0.1 μM) in combination with the thromboxane-mimetic U44619 (1 μM). The response to collagen (1 μg ml^{-1}) was not altered by norethisterone and decreased by desogestrel (Table 1).

Table 1. Aggregation responses (Ω) in blood taken from rats given vehicle, norethisterone (NET) or desogestrel (DES).

	Vehicle	NET	DES
ADP	16.4 ± 1.0	20.7 ± 2.8*	14.2 ± 1.7
ADP + U44619	10.1 ± 2.3	16.8 ± 1.5*	9.2 ± 3.2
Collagen	6.3 ± 1.6	7.0 ± 0.6	3.6 ± 0.5*

Values are mean ± s.e. mean, n=6 per group. *$P<0.05$ compared to other groups, Kruskal-Wallis test.

These data indicate that norethisterone enhances platelet aggregation whereas desogestrel either does not alter, or reduces platelet aggregation. The results suggest that if there is a reduced risk of myocardial infarction with third generation progestogens compared to second generation progestogens this can be explained by their actions on platelet aggregation.

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Dunn N. *et al.*, (1999). *Br. Med. J.* 318, 1579-1584.
Lewis M.A., Spitzer W.O. *et al.* (1996). *Br. Med. J.* 312, 88-90.

252P REGIONAL VARIATIONS IN THE LOCATION OF Na-Ca EXCHANGER PROTEIN IN GUINEA-PIG HEART AT CELLULAR AND TISSUE LEVELS

R.L. McDonald, J. Colyer* & S.M. Harrison (Introduced by C. Peers). School of Biomedical Sciences and *School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9NQ

The expression of the Na^+-Ca^{2+} exchanger (NCX) has been qualitatively examined in human heart using the C2C12 monoclonal antibody (Wang *et al.*, 1996) raised to the peptide sequence 371-525 of NCX (Porzig *et al.*, 1993). Although NCX expression was found to be significantly lower in the atria compared to the septum, no significant differences were found between atrial and ventricular tissue. We have used a quantitative Western blotting technique (McDonald *et al.*, 1999) to determine the pattern of expression in guinea-pig heart. The regional expression of NCX protein was quantified in samples from left and right atrium, left and right ventricle and septum. The tissue samples were homogenised in 2% SDS sample buffer and analysed quantitatively using SDS-PAGE and Western blotting with the C2C12 antibody. NCX expression was found to be significantly lower ($P < 0.05$; ANOVA) in both the left atrium and right atrium compared to the left ventricle, right ventricle and septum with 17.5 ± 3.9, 29.2 ± 6.1, 64.7 ± 15.2, 76.8 ± 19.5, 69.4 ± 14.1 pmol.mg $protein^{-1}$ in the left atrium, right atrium, septum, left ventricle and right ventricle, respectively (n = 7).

These differences in NCX expression may reflect regional variations in cellular location of NCX protein. Therefore, we also used confocal immunofluorescence to examine differences in the proportion of fluorescent staining on the surface membrane compared with the interior of the cell (i.e. with a t-tubular location). We found that the general surface membrane staining was 79.1 ± 1.0% of total staining in cells from the atria which was significantly higher ($P < 0.01$) than that seen in cells from the left ventricle, right ventricle and septum with 51.8 ± 2.5%, 43.3 ± 1.8% and 49.5 ± 1.4%, respectively (n = 10).

These results illustrate a similar pattern of NCX expression in guinea-pig and human with expression in atrial tissue significantly lower than in ventricular tissue. Furthermore, this technique of quantitative Western blotting has determined that smaller differences in expression can be differentiated compared to qualitative techniques. In addition, the cellular location of NCX differs regionally; in atrial tissue, NCX is located predominately in the general sarcolemma whereas in ventricular and septal tissue, a considerable fraction of NCX is located within the cell (presumably at the level of t-tubular invaginations).

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McDonald, R.L., Drago, G.A., Colyer, J. & Harrison, S.M. (1999). *Biophys. J.* **76**, A305
Porzig, H., Li, Z., Nicoll, D.A. & Philipson, K.D. (1993). *Am. J. Physiol*, **265**, C748-C756.
Wang, J., Schwinger, R.H.G., Frank, K., *et al.* (1996). *J. Clin. Invest.* **98**, 1650-1658.

253P EFFECTS OF HOMOCYSTEINE ON POTASSIUM CHANNELS IN HUMAN PLATELETS

N.G. John & J.K. Aronson, Oxford Project to Investigate Memory and Ageing (OPTIMA), University Department of Pharmacology, Oxford OX1 3QT, & University Department of Clinical Pharmacology, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, UK

Potassium (K^+) channels, specifically calcium-dependent K^+ channels, function abnormally in the platelets of patients with Alzheimer's disease (De Silva *et al.*, 1998). Homocysteine serum concentrations are raised in patients with Alzheimer's disease (Clarke *et al.*, 1998). Pursuing these two observations we have studied the effects of homocysteine in vitro on K^+ channels in platelets from young healthy volunteers. The results suggest that homocysteine converts normal calcium-dependent K^+ channels to Alzheimer-like channels.

We obtained platelets from healthy volunteers, preloaded them for 2 hours with $^{86}Rb^+$ (used as an analogue of K^+), immobilized them on an inert filter, and measured $^{86}Rb^+$ efflux using a superfusion technique (De Silva *et al.*, 1996) in the following medium (mM): NaCl (119), KCl (4.6), $CaCl_2$ (1.5), NaH_2PO_4 (1.2), $NaHCO_3$ (15), glucose (11), pH 7.4. The platelets were allowed to stabilize for 20 min before $^{86}Rb^+$ efflux was measured at one- or two-minute intervals for 30 min. In experiments with homocysteine the cells were incubated with homocysteine (10 μM) for 2 hours, while they were being loaded with $^{86}Rb^+$. $^{86}Rb^+$ efflux was stimulated with thrombin (0.3 i.u./ml) and inhibited by the inhibitors of calcium-dependent K^+ channels, apamin (100 nM) and charybdotoxin (300 nM).

The results are shown in Table 1. The non-stimulated (control) efflux of $^{86}Rb^+$ was similar to that previously described (De Silva *et al.*, 1998). Thrombin, added for 5 min at the start of the experiment (time 0), significantly increased the rate of $^{86}Rb^+$ efflux, and the thrombin-stimulated efflux was inhibited by both apamin and charybdotoxin. Pre-incubation with homocysteine caused a significant increase in the rate of $^{86}Rb^+$ efflux, similar to that caused by thrombin. The combination of thrombin and homocysteine produced a further increase in $^{86}Rb^+$ efflux, although this was not significant. Neither apamin nor charybdotoxin inhibited the $^{86}Rb^+$ efflux stimulated by the combination of thrombin and homocysteine.

Human platelets lack large-conductance calcium-dependent K^+ channels (De Silva *et al.*, 1997), and so the effects of apamin and charybdotoxin are consistent with the presence of small- and intermediate-conductance channels in these cells. In platelets taken from patients with Alzheimer's disease thrombin-stimulated efflux was normal but was not inhibited by apamin or charybdotoxin, in contrast to the inhibition that occurred in the platelets of both young subjects (De Silva *et al.*, 1997) and age-matched controls (De Silva *et al.*, 1998). In the experiments reported here, homocysteine, in a concentration found in normal serum (7–22 μM), stimulated $^{86}Rb^+$ efflux on its own and prevented the inhibition of thrombin-stimulated $^{86}Rb^+$ efflux by apamin and charybdotoxin, similar to the effect seen in Alzheimer's disease.

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Clarke, R., Smith, A.D., Jobst, K.A., *et al.* (1998) *Arch. Neurol.* 55, 1449-55.
De Silva, H.A., Carver, J.G., Aronson, J.K. (1996) *Clin. Sci.* 91, 725-31.
De Silva, H.A., Carver, J.G., Aronson, J.K. (1997) *Clin. Sci.* 93, 249-55.
De Silva, H.A., Aronson, J.K., Grahame-Smith, D.G., *et al.* (1998) *Lancet* 352, 1590-3.

Table 1. Cumulative $^{86}Rb^+$ efflux (pmol) at 30 min

	Control	H'cys	Thrombin	Thrombin + APA	Thrombin + CTX	Thrombin + H'cys	Thrombin + H'cys + APA	Thrombin + H'cys + CTX
$^{86}Rb^+$ efflux	196	288*	268*	201†	215†	336*	287*	339*
s.e.mean	29	76	55	23	32	125	59	74
n	6	6	6	6	6	2	4	4

H'cys=homocysteine 10 μM; thrombin 0.3 i.u./l; APA=apamin 100 nM; CTX=charybdotoxin 300 nM.
The complete efflux curves (0–30 min) were tested by ANOVA with repeated measures:
*significantly different from control ($P<0.01$); †significantly different from thrombin alone ($P<0.01$).

254P CO-EXPRESSION OF DOMAINS D1-S4 AND S5-S6 OF THE VOLTAGE-GATED POTASSIUM CHANNEL Kv2.1

J.P.A. Bannister, M.J. Main, A. Sivaprasadarao & D. Wray School of Biomedical Sciences, Worsley Building, University of Leeds, Leeds LS2 9JT

Potassium channels can be divided functionally into inwardly rectifying (Kir) channels and voltage-gated (Kv) channels such as Kv2.1 studied here. Tytgat *et al.* (1994) have reported that removal of the first four transmembrane domains (S1–S4) of the voltage-gated *Shaker* channel (Kv1.1) gives a channel which behaves as a strong inward rectifier. In the present study we have investigated whether the removal of the S1-S4 domains of the voltage-gated Kv2.1 channel would give an inward-rectifier type current, and further whether co-expression of domains S1-S4 with domains S5-S6 would reconstitute functional Kv2.1 channels when expressed in oocytes. Such fuctional reconstitution might be expected by analogy with work on receptors such as bacteriorhodopsin (Khan & Engleman 1992) where it has been shown that separate component domains (each lacking in function alone) can restore receptor function when co-expressed together.

Deletion mutants of rat Kv2.1 were prepared by polymerase chain reaction using a 4:1 Taq:Pfu DNA polymerase enzyme mix with primers designed to amplify the required domains and plasmid, followed by ligation. Mutants were confirmed by dideoxy sequencing. cRNA was transcribed *in vitro* using the T7 promoter and *Not*I linearised templates, and injected into *Xenopus* oocytes. Two-electrode voltage-clamp recordings were made 24–48 hours later as previously described (Wilson *et al.*, 1994).

The deletion mutant for the pore-containing domain (S5-S6), when injected alone into oocytes (10ng RNA), gave currents (482 ± 75 nA, n=4) which were not significantly different from those for uninjected oocytes (404 ± 54 nA, n=7), for normal potassium concentrations (2 mM K^+) and using steps from -80 mV to +80 mV. Currents for the pore-containing mutant S5-S6 were also recorded in high potassium solutions (100 mM K^+), but were again found to be not significantly different from those for uninjected oocytes (pore-domain mutant 206 ± 24 nA, n=7, uninjected oocytes 166 ± 14 nA, n=5, using steps from 0 mV to -100 mV). Increasing the amount of S5-S6 cRNA injected up to 50 ng also failed to give currents which were significantly different from currents in uninjected oocytes, for normal and high potassium solutions. Upon co-injection of RNA for the S1-S4 domain and for the S5-S6 domain (25 ng injected of each), currents (213 ± 44 nA, n=4) were again not significantly different from currents in uninjected oocytes (214 ± 42 nA, n=6), using normal potassium solutions and voltage steps from -80 mV to +80 mV.

The results show that removal of the S1– S4 domains of rat Kv2.1 channel does not lead to expression of inwardly rectifying type currents or indeed of any observable potassium current. This is in contrast to the *Shaker* channel, and the difference may come about because of specific sequence differences in the S5-S6 domains between *Shaker* Kv1.1 channel and the rat Kv2.1 channels studied here. Furthermore, channel function could not be restored by co-expression of the S1-S4 domain with the S5-S6 domain of the Kv2.1 channel. One explanation may be that the intact linker between S4 and S5 is of key importance in the functioning of the channel, so implicating this linker as having an important role in transmitting movement of the S4 region into pore opening in S5-S6.

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KHAN TW & ENGELMAN DM (1992) *Biochemistry* **31**, 6144-6151.
TYTGAT J, VEREECKE, J & CARMELIET, E (1994) *Journal of Physiology* **481**, 7-13.
WILSON GG, O'NEIL CA, SIVAPRASADARAO A, FINDLAY JBC & WRAY D (1994) *Pflugers Archiv* **428**, 186-193.

255P BLOCK OF THE CARDIAC MUSCARINIC K^+ CHANNEL Kir3.1/Kir 3.4 BY FLUFENAMIC ACID

K.M. Dibb, R. Leach, M.K. Lancaster, J.B.C. Findlay & M.R. Boyett (introduced by D. Beech). Schools of Biomedical Sciences and Biochemistry & Molecular Biology, University of Leeds, Leeds LS2 9JT

Flufenamic acid is known to block Ca^{2+}-activated Cl^- channels in oocytes from *Xenopus laevis* (White & Aylwin, 1990). In an attempt to reduce large endogenous currents present in Xenopus oocytes which were contaminating recordings from heterologously expressed channels, we applied 100 μM flufenamic acid. In water-injected oocytes, as expected, some reduction in endogenous current amplitude was observed. However, in oocytes expressing the muscarinic K^+ channel, flufenamic acid was also found to cause a substantial reduction in current through the heterologously expressed channel. We therefore went onto investigate the effects of flufenamic acid on the muscarinic K^+ current. mRNA encoding for Kir3.1, Kir3.4 and the human dopamine receptor was injected into Xenopus oocytes. Currents were recorded 48 to 96 h later using the two-microelectrode voltage clamp technique. Oocytes were held at 0 mV and 750 ms voltage pulses applied from -160 to +40 mV in the presence of 10 μM dopamine. Flufenamic acid was found to be a reversible blocker of the muscarinic K^+ current. Current-voltage relationships of the muscarinic K^+ current in the presence of various concentrations of flufenamic acid are shown in Fig. 1. The block of the muscarinic K^+ current was weakly voltage-dependent. The fraction of the electrical field (δ) through which flufenamic acid moves in order to reach its site of block was 0.12 ± 0.02 (n=5). The K_d at 0 mV for flufenamic acid was estimated to be 101 ± 16.9 μM (n=5). It is concluded that flufenamic acid is not a suitable blocker of the endogenous Ca^{2+}-activated Cl^- channel in Xenopus oocytes when studying the muscarinic K^+ channel.

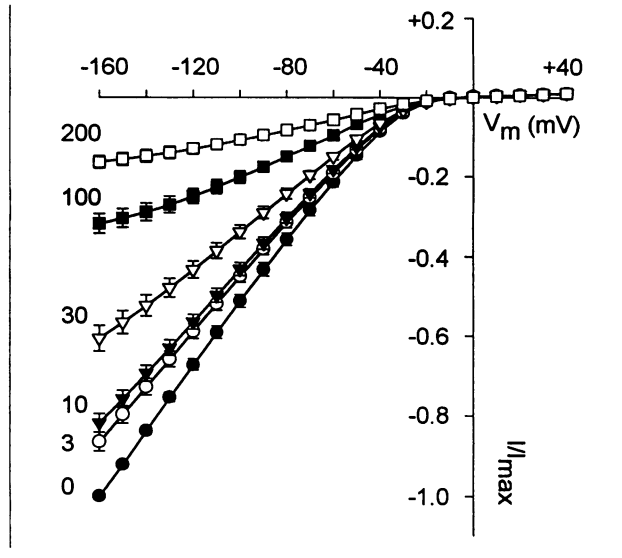


Figure 1. Current-voltage relationships of the muscarinic K^+ current in the presence of various concentrations (values in μM shown) of flufenamic acid. Currents were measured at the end of voltage clamp pulses and are normalised to the current at -160 mV under control conditions. Means ± SEM shown (n=5).

White, M.M. & Aylwin, M. (1990). *Molecular Pharmacology* **37**, 720-724.

256P SUPPRESSIVE EFFECTS OF PROGESTERONE ON RAT PORTAL VEIN CONTRACTILITY MEDIATED BY BK_{Ca} OPENING

M. S. Mukerji, Helen L. Leathard & H. Huddart[1], Department of Nursing Studies, St. Martin's College, Lancaster, LA1 3JD and [1] Department of Biological Sciences, IENS, Lancaster University, Lancaster, LA1 4YQ

Progesterone has been observed to suppress the contractility of a number of different vascular smooth muscles (Leathard & Eccles, 1991). Such vasoactive effects of this hormone have been implicated in changes in blood pressure related to the menstrual cycle (Hall & Leathard, 1998) and in the aetiology of menstrually-related migraine (Leathard & Eccles, 1991). Previous studies had indicated that, on the rat portal vein, progesterone suppresses contractility by opening K^+ channels (Mukerji *et al.*, 1995). The present study was designed to elucidate the specific K^+ channels involved by the use of a variety of K^+ channel blockers (KCBs).

Portal vein preparations were taken from Male Wistar rats (~350 g), killed by cervical dislocation. The tissues were kept in aerated mammalian Tyrode saline (composition (mM): NaCl 137.0, KCl 5.4, $CaCl_2$ 1.79, $MgSO_4$ 1.04, NaH_2PO_4 0.34, $NaHCO_3$ 11.9, Glucose 5.6) maintained at 37 °C and set up in organ baths as 'whole' preparations to give measurable changes in force, recorded via isometric tension transducers.

The effects of 30 min KCB incubations were assessed on responses evoked by 5 - 80 mM cumulative K^+ addition that had been selectively suppressed by progesterone (10^{-6} M, 40 min). These were compared to the effects of respective KCB solvents (ethanol or water) and 'non-progesterone non-KCB' solvent control studies. TEA (1 mM) or 4-AP (1 mM) enhanced the phasic contractions of portal vein at 5 mM K^+ (200 ± 48% increase (mean ± s.e.mean), n=9, and 180 ± 55%, n=8, respectively; both $p<0.05$). These effects were, however, significantly greater than the respective 'non-KCB non-progesterone' solvent controls indicating that TEA and 4-AP were not solely counteracting progesterone. $BaCl_2$ (100 μM) antagonised progesterone-induced suppression of phasic activity at 5 mM K^+ (96 ± 34% increase, n=5, $p<0.05$) indicating an involvement of K_{ATP} channels, but this was not supported by the use of the highly selective K_{ATP} blocker glibenclamide (1 μM). The absence of an effect of apamin (1 μM) indicated a lack of involvement of SK_{Ca} channels. Iberiotoxin (100 nM), however, caused a direct antagonism of progesterone-induced suppression at 5 mM K^+ (107 ± 18% increase, n=6, $p<0.05$) that was not significantly different from 'non-progesterone non-iberiotoxin' solvent control, indicating an explicit role for BK_{Ca}. Statistical analyses utilised Student's t test for unpaired data.

The above data support the observation of Jacob & White (1996) that progesterone opens BK_{Ca} channels in porcine coronary artery cells. Iberiotoxin is a highly specific BK_{Ca} blocker and its antagonism of progesterone's action is consistent with the theory that the hormone suppresses contractility by opening BK_{Ca} channels in rat portal vein.

Hall, V.L. & Leathard, H.L. (1999). *J. Physiol.*, 515P, 78P.
Jacob, M. & White, R. (1996). *F.A.S.E.B.*, 10, 384.
Leathard, H.L. & Eccles, N.K. (1991) in *New Advances in Headache Research 2.* ed. F. Clifford Rose pp 187-192. London: Smith-Gordon.
Mukerji, M., Leathard, H. & Huddart, H. (1995). *Br. J. Pharmacol.* 116, 196P.

257P ARE β_1-ADRENOCEPTORS INVOLVED IN THE ISOPRENALINE-EVOKED DILATATION OF THE HUMAN DORSAL HAND VEIN?

A.H. Abdelmawla, R.W. Langley, E. Szabadi & C.M. Bradshaw, Division of Psychiatry, University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH

The non-selective ß-adrenoceptor agonist isoprenaline (IPNA) dilates the dorsal hand vein (Aellig, 1994). Although there is good evidence for dilator β_2-adrenoceptors in this structure (Eichler et al. 1990), there is relatively little indication of the involvement of β_1-adrenoceptors. In an early study White & Udwadia (1975) reported that IPNA-induced dilatation of the dorsal hand vein could be antagonized by the β_1-adrenoceptor antagonist practolol. However, this observation has not been replicated or repeated with more modern β_1-adrenoceptor antagonists. Bisoprolol is a highly selective β_1-adrenoceptor antagonist recommended as a tool for the identification of β_1-adrenoceptors (Brodde, 1986). We compared the effects of bisoprolol and nadolol, a non-selective β_1/β_2-adrenoceptor antagonist, on IPNA-evoked venodilatation, using the dorsal hand vein compliance technique (Aellig, 1994).

12 males (18-30 years) participated in 4 weekly sessions. In the first (preliminary) session a dose-response curve to locally infused phenylephrine was constructed and the dose yielding 50-75% of the maximum venoconstricting response for each individual subject was determined. The subsequent (experimental) sessions were associated with the ingestion of one of the following treatments: bisoprolol 5 mg, nadolol 40 mg, or placebo. Subjects were allocated to treatments and sessions according to a double-blind balanced design. In each experimental session local infusion started 150 min after drug ingestion. Six doses of IPNA (3.33-1000 ng min^{-1}) were infused and dose-response curves were constructed. A fixed dose of phenylephrine, as determined in the preliminary session, was co-infused with each dose of IPNA in order to pre-constrict the vein. Venodilator responses were transformed to percent maximum response. Systolic and diastolic blood pressure (BP), and heart rate (HR) were also measured. Data were analyzed by ANOVA (repeated measures) with individual comparisons using Dunnett's test. A probability level of $P<0.05$ was regarded as significant for all statistical tests.

Nadolol decreased the venodilatation to IPNA; bisoprolol had no significant effect. The antagonism of the venodilator response is reflected in the value of $logED_{50}$ in the presence of nadolol (Table 1). IPNA increased HR and systolic BP, and reduced diastolic BP; these effects were antagonized by both bisoprolol and nadolol.

The antagonistic effects of nadolol on IPNA-evoked venodilatation could have been mediated by both β_1- and β_2-adrenoceptors. However, the lack of effect of bisoprolol argues against the involvement of β_1-adrenoceptors.

Table 1 Parameters of dose-response curves to IPNA

	ED50 (ng min^{-1})		
	log ED_{50} (mean ± s.e.mean)	*Geometric Mean*	*E_{max} (%) (mean ± s.e.mean)*
Placebo	1.79 ± 0.10	60.98	84.13 ± 8.09
Bisoprolol	1.68 ± 0.11	47.45	68.07 ± 7.91
Nadolol	2.10 ± 0.12*	126.62	68.09 ± 12.81

Differences from placebo condition: * $P<0.01$

Aellig, W.H. (1994). *Br. J. Clin. Pharmacol.*, 38, 289-305
Brodde, O-E. (1986). *J. Cardiovasc. Pharmacol.*, 8 (Suppl. 11), S29-S35
Eichler, H-G. *et al.* (1990). *J. Allergy Clin. Immunol.*, 86, 714-7
White, C. & Udwadia, B.P. (1975). *Br. J. Clin. Pharmacol.*, 2, 99-105

258P DIFFERENTIAL EXPRESSION OF RENAL ADENOSINE A_1 RECEPTORS INDUCED BY ACUTE RENAL FAILURE

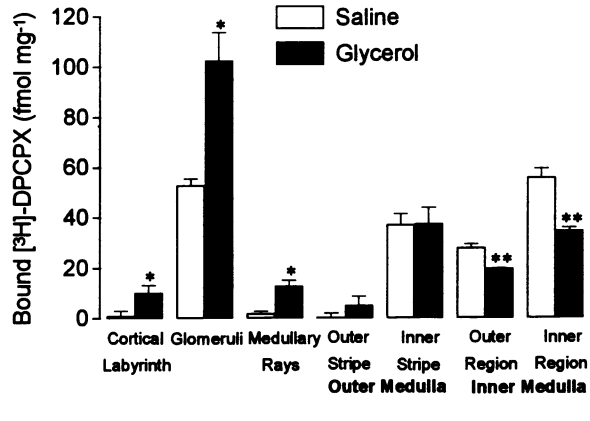
J.A. Smith, C.J. Bowmer & M.S. Yates, School of Biomedical Sciences, Worsley Medical and Dental Building, The University of Leeds, Leeds LS2 9JT

Adenosine is a haemodyamic mediator in acute renal failure (ARF) induced by myohaemoglobinuria (produced by glycerol injection) and renal adenosine A_1 receptor density and mRNA levels are elevated in this form of ARF (Gould *et al.*, 1997). In ARF induced by $HgCl_2$, adenosine does not play a pathophysiological role and A_1 receptor density and mRNA levels are unaltered. The aim of this study was to identify any regional changes in receptor density in the kidneys of rats with ARF induced by either glycerol or $HgCl_2$.

ARF was induced in male Wistar rats (200 - 250g) by either i.m. injection of 50% v/v glycerol in saline (10ml kg^{-1}) or s.c. injection of $HgCl_2$ (2 mg kg^{-1}). Control animals received equivalent saline injections. Following induction of ARF, both kidneys were removed and frozen in isopentane cooled in liquid nitrogen. Kidney sections (20 µm) were incubated for 4h at 4°C in the presence of 0.3 nM [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]-DPCPX). Sections were apposed to coverslips coated in nuclear emulsion and left to expose at 4°C for 12 weeks in light proof boxes.

Sixteen hours following the induction of glycerol-induced ARF, there was a 34% ($P<0.01$) increase in labelling in the glomeruli, compared to saline-injected controls; whilst by 48h, glomerular labelling had increased two-fold. In addition, 48h following glycerol injection, significant labelling was now detected in the cortical labyrinth and medullary rays whilst, in both inner and outer regions of the inner medulla, labelling had decreased by 29-38% (Figure 1). The only statistically significant change noted 48h following induction of $HgCl_2$-induced ARF was a 39% decrease ($P<0.01$) in labelling in the inner and outer regions of the inner medulla. Glycerol-induced ARF results in differential expression of renal adenosine A_1 receptors. The increase in density of A_1 receptors in glomeruli and cortical labyrinth may account for the increased renal vasoconstrictor response to adenosine and depressed glomerular filtration rate noted previously in this type of ARF.

Figure 1 Binding of [^{3}H]-DPCPX to kidney regions of rats 48h following induction of ARF with glycerol. Results are given as mean + s.e.mean (n = 3-5). * $P<0.05$, ** $P<0.01$ (Students *t*-test) relative to saline group.



Gould, J., Morton, M.J., Sivaprasadarao, A. *et al.* (1997). *Br. J. Pharmacol.*, 120, 947-953.

259P ADRENOMEDULLIN IS AN EXTREMELY POTENT VASODILATOR OF HUMAN SMALL MUSCULAR PULMONARY ARTERIES

M.R. MacLean, M. Gallacher, C Hillier, JV McMurray,[1] & MC Petrie.[1] Division of Neuroscience and Biomedical Systems, IBLS, Glasgow University, Scotland and [1]Department of Cardiology, Western Infirmary, Glasgow.

Adrenomedullin (ADM) is a newly identified vasodilator (Richards et al., 1996). There is increased production of ADM in pulmonary hypertension with plasma ADM levels increasing in proportion to the extent of pulmonary hypertension (Kakishita et al., 1999). Hence, its ability to induce pulmonary vasodilation is of interest.

Here we compared the potency of ADM in human small muscular pulmonary arteries (hSMPAs) and compared this with the potencies of acetylcholine (ACh) and sodium nitroprusside (SNP) as well as its potency in human small subcutaneous arteries (hSCAs). hSMPA preparations (~250μi.d.) were obtained from macroscopically normal sections of lung removed with bronchial carcinomas. hSCAs were obtained from buttock biopsies. Vessels were set up at 37°C in myographs in Krebs solution and bubbled such that the PaO_2 equalled 110-120 mmHg (hSMPAs). Tone was raised with a submaximal concentration of endothelin-1 (3-10nM, hSMPAs) or phenylephrine (~0.1μM, hSCAs). Once a stable contraction was maintained cumulative concentration response curves to ADM, SNP or ACh were constructed. Relaxations were expressed as a % of precontraction and individual pIC_{50}s were calculated. The results are shown in Table 1.

The results show that ADM was considerably more potent in the pulmonary than systemic arteries. In addition it was more potent than both SNP and ACh in the pulmonary vessels whilst it was equipotent to SNP and ACh in the systemic arteries. It may, therefore, play a significant role in the control of pulmonary vascular tone.

Table 1 pIC_{50}s for adrenomedullin (ADM), sodium nitroprusside (SNP) and acetylcholine (ACh) in human small muscular pulmonary arteries (hSMPAs) and a comparison with the potency of ADM in human small subcutaneous arteries (hSCAs). n=number of subjects.

	pIC_{50}s	n
ADM (hSMPAs)	10 ± 0.2	4
ADM (hSCAs)	7.7 ± 0.4	9**
SNP (hSMPAs)	8.5 ± 0.3	5
SNP (hSCAs)	6.4 ± 0.9	9
ACh (hSMPAs)	6.8 ± 0.3	7***†
ACh (hSCAs)	6.4 ± 0.8	9

Statistical analysis was by one-way analysis of variance followed by a Tukey-Kramer Multiple comparisons test. Versus ADM (hSMPAs) **P<0.01; ***P<0.001. Versus SNP (hSMPAs) †P<0.05.

In conclusion, ADM is an extremely potent vasodilator of hSMPAs. This work was funded by The British Heart Foundation.

Kakishita, M., Nishikimi, T., Okano, Y., Satoh, T., Kyotani, S. et al. (1999). *Clin. Sci.* (Colch.), **96**, 33-9
Richards, A.M., Nicholls, M.G., Lewis, L. & Lainchbury, J.G. (1996). Adrenomedullin. *Clin. Sci.*, **91**, 3-16.

260P EVIDENCE FOR PHARMACOLOGICAL SYNERGISM IN RAT SMALL MUSCULAR PULMONARY ARTERIES

I Morecroft & M.R. MacLean Div. of Neuroscience & Biomedical Systems, IBLS, University of Glasgow, Glasgow G12 8QQ

Pharmacological synergism has been observed in several blood vessel types (Movahedi & Purdy, 1997; MacLennan & Martin, 1992) where Gi-coupled 5-HT_1-receptor induced vasoconstriction is 'uncovered' by increased vascular tone. 5-hydroxytryptamine (5-HT) has been implicated in pulmonary hypertension (PHT) (Herve *et al*; 1995) and we have recently shown in the chronic hypoxic rat model of PHT that pulmonary arterial responses to 5-HT are enhanced and guanosine 3':5'-cyclic monophosphate ([cGMP]i) levels decreased (MacLean *et al*, 1996). The effect of increased vascular tone and decreased cGMP on 5-HT receptor induced vasoconstriction in pulmonary arteries is of interest. Here we examined the effect of increasing vascular tone and decreasing cGMP on 5-carboxamidotryptamine (5-CT; 5-HT_1 agonist) vasoconstriction in rat small muscular pulmonary arteries (smPAs).Wistar rats were killed with sodium pentobarbitone ($60mgkg^{-1}$). smPAs (150-250μm internal diameter) were dissected out mounted on wire myographs under tension (in Krebs at 37°C) and bubbled with 16% O_2/6%CO_2 balance N_2. The smPAs were preconstricted with a threshold concentration of either endothelin-1 (ET-1,Gq-linked receptor activation) (0.1-1.0nM), KCl (opening of calcium channels) (10mM) and subsequently, once a stable plateau had been reached, cumulative concentration response curves to 5-CT (1.0nM-0.1mM) were obtained in the presence/absence of the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; decreases cGMP) (0.1mM). Results are shown in table 1. 5-CT evoked a concentration dependent vasoconstriction only at concentrations ≥ 1μM, which did not produce a maximum response at the highest concentration. Prior exposure to ET-1 or KCl caused a significant potentiation of the 5-CT response, which was further augmented by L-NAME. These results show that in rat smPAs, increased vascular tone via either Gq-coupled receptor activation or increased intracellular calcium can potentiate Gi-coupled 5-HT_1 receptor-induced vasoconstriction. This synergism may therefore be of pathophysiological importance in pulmonary hypertension characterised by elevated pulmonary vascular tone.

Herve, P., Launay, J.M., Scrobohaci, M.L., *et al.* (1995) Am. J. Med. 99, 249-254.
MacLean, M.R, Sweeney, G., Baird, M., *et al.* (1996) Br. J. Pharmacol. 119, 917-930.
MacLennan, S.J., Martin, G.R. (1992) Br. J. Pharmacol. 107, 418-421.
Movahedi, H., Purdy, R.E. (1997) J. Pharmacol. Exp. Ther. 283, 653-660.

Table 1. Effect of elevated tone on 5-CT-induced vasoconstriction in rat smPAs (n,n ≥5,5).

Group	Response (% 50mM KCl) at control 5-CT threshold concentration (1μM)	
	- L-NAME	+ L-NAME
5-CT	0.5 ± 0.5	3.2 ± 1.3
+ET-1-tone	6.4 ± 0.2**	14.8 ± 2.4**ΨΨ
+KCl-tone	10.5 ± 2.7**	31.0 ± 9.0*Ψ

Mean data ± s.e.mean.n,n = number of vessels/number of animals. Significance of difference from a) 5-CT control response,*P< 0.05 **P<0.01.b) 5-CT response in presence of L-NAME , ΨP <0.05; ΨΨP <0.01. Statistical analysis by one way analysis of variance with Tukey multiple comparison test.

261P EFFECT OF NITRIC OXIDE ON CALCIUM SIGNALLING IN VASCULAR MYOCYTES FROM THE RAT PULMONARY ARTERY

J.P. Savineau, O. Pauvert & R. Marthan.
Laboratoire de Physiologie Cellulaire Respiratoire (INSERM E9937), Université Bordeaux 2, 33076 Bordeaux, France.

Pulmonary vascular tone is controlled by a variety of endogenous factors including the endothelium-derived factor nitric oxide (NO). NO is known to relax vascular smooth muscle cells primarily by stimulating guanylyl cyclase to produce cyclic GMP (cGMP). However, the precise mechanisms by which NO/cGMP induce vasorelaxation are not fully understood. Since the contraction-relaxation cycle of smooth muscle is mainly dependent on a variation in the cytoplasmic calcium concentration ($[Ca^{2+}]_i$), we studied the effect of the NO-donor sodium nitroprusside (SNP) on both ($[Ca^{2+}]_i$) and mechanical activity in the rat isolated extrapulmonary artery (RPA).

The effect of SNP on $[Ca^{2+}]_i$ was studied in myocytes freshly isolated from RPA dissected from pentobarbitone anaesthetised Wistar rats. Myocytes were loaded with 1μM indo-1acetoxymethyl ester for 30 min. $[Ca^{2+}]_i$ was estimated fluorimetrically from the 405:480 nm ratio using a specific calibration (Guibert *et al.*, 1997). Isometric contraction was recorded in arterial rings using a computerised isolated organ bath system (Roux *et al.*, 1997). Owing to the existence of a low basal tone, RPA were prestimulated by ATP or endothelin-1 (ET-1). SNP (0.01-500 μM) concentration-dependently relaxed ATP (10 mM, n = 4) and ET-1 (0.1μM, n = 4)-precontracted RPA. In isolated myocytes, short (30s) application of ATP (100 μM) or ET-1 (0.1 μM) induced 3-6 cyclic rises in $[Ca^{2+}]_i$ (Ca-oscillations) of decreasing amplitude, the first one reaching 409 ± 16 nM and 635 ± 34 nM (mean ± SEM), respectively from the resting value of 70 ± 2 nM (n = 50). After removal of extracellular calcium for 10 min, ATP and ET-1 again induced Ca-oscillations (n = 20). Preincubation of cells with SNP (10-250 μM) for 10 min had no effect on the resting $[Ca^{2+}]_i$ value, but progressively abolished the oscillations. A similar effect was obtained with 8-bromo-cGMP (100-500 μM). 1H-[1,2,4]oxadiazolol [4,3,-a]quinoxalin-1-one (ODQ, 10 μM) and methylene blue (10 μM), two potent inhibitors of the cytosolic guanylyl cyclase fully reversed the effect of SNP on ATP-and ET-1-precontracted RPA and ATP-induced $[Ca^{2+}]_i$ oscillations. In contrast, N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide (H8, 10 μM) and KT5823 (0.1 μM), two potent inhibitors of cGMP-dependent protein kinase (PKG) did not alter the effect of SNP. Caffeine (5 mM) induced a transient contraction in RPA strips (n = 20) and only one transient $[Ca^{2+}]_i$-increase (660.3 ± 50 nM, n = 24) in isolated myocytes. The amplitude of caffeine-induced both contraction and $[Ca^{2+}]_i$ response was altered neither by SNP nor 8-bromo-cGMP. In conclusion, our results show in RPA that the relaxing effect of NO is mainly due to its action on the Ca-signalling pathway. The effect of NO involves an increase in cGMP but appears independent of activation of PKG. Moreover, NO interacts with inositol trisphosphate pathway but not with that involving the ryanodine-sensitive receptor.

Guibert, C., Marthan, R. & Savineau, J.P. (1997) *Cell Calcium* 21, 421-429

Roux, E., Guibert, C., Savineau, J.P. et al., (1997) *Br. J. Pharmacol.* 120, 1294-1301

262P ROLE OF 5-HT_{1B} AND 5-HT_2 RECEPTORS IN THE DECREASES IN INTERNAL CAROTID BLOOD FLOW INDUCED BY 5-HT IN THE DOG

D. Centurión, A. Sánchez-López, M. Ortiz, P. De Vries, P. Saxena & C.M. Villalón. Pharmacology, CINVESTAV - IPN, Apdo. Postal 22026, 16020 México D.F.

Intracarotid (i.c.) infusion of 5-hydroxytryptamine (5-HT) decreases internal carotid blood flow (ICBF) in anaesthetized dogs (Vidrio & Hong, 1976). Since little is known on the mechanisms mediating this response, we have analysed the pharmacological profile of the receptors involved in 5-HT-induced decrease in ICBF (by ultrasonic flowmetry) in 24 male mongrel dogs (18-22 kg) anaesthetized with pentobarbital (30 mg kg^{-1}). As shown in Table 1, 5-HT (0.1-10 μg min^{-1}) elicited dose-dependent decreases in ICBF (mean±s.e.mean) without changes in blood pressure. These responses were not significantly affected after i.v. administration of saline or the antagonists ritanserin (5-HT_2) and tropisetron (5-HT_3 and 5-HT_4), SB224289 (5-HT_{1B}) or BRL15572 (5-HT_{1D}). In contrast, these effects were antagonised by GR127935 (5-$HT_{1B/1D}$). Interestingly, in the animals receiving GR127935 or SB224289, the subsequent administration of ritanserin unmasked a dose-dependent vasodilator response to 5-HT which was not elicited in the animals treated with BRL15572 (see Table 1). In addition, the vasoconstrictor responses to 5-HT were mimicked by the agonists sumatriptan (5-$HT_{1B/1D}$) or DOI (5-HT_2), but not by *m*-chlorophenyl-biguanide (5-HT_3) or cisapride (5-HT_4) (data not shown). It is therefore suggested that 5-HT-induced canine internal carotid vasoconstriction is predominantly mediated by both 5-HT_{1B} and 5-HT_2 receptors.

Table 1. Effect of various compounds (i.v.) on the decreases in ICBF (% basal) induced by 5-HT (μg min^{-1}, i.c.) in dogs

Compound	n	Dose	0.1		0.3		1.0		3.0		10	
		mg kg^{-1}	Before	After	Before	After	Before	After	Before	After	Before	After
Saline[a]	5	-	-3±1	-9±5	-8±2	-11±3	-20±4	-32±5	-38±6	-36±4	-54±12	-52±2
Rit. + Trop.	4	0.1+3	-2±1	-5±3	-7±1	-5±4	-19±5	-17±9	-34±8	-30±9	-46±9	-33±8
GR127935	7	0.03	-4±2	-1±1	-11±3	-2±1	-23±4	-4±3*	-32±4	-7±4*	-42±4	-5±4*
GR + Rit.	7	0.03+0.1	-4±2	+11±3*	-11±3	+17±4*	-23±4	+28±6*	-32±4	+46±11*	-42±4	+68±13*
SB224289	5	0.3	-5±3	-4±4	-11±2	-7±7	-25±2	-10±7	-41±2	-21±14	-59±4	-31±18
SB + Rit.	5	0.3+0.1	-5±3	+17±5*	-11±2	+20±8*	-25±2	+28±12*	-41±2	+35±14*	-59±4	+48±17*
BRL15572	3	0.3	-4±2	-6±3	-7±2	-10±4	-21±5	-21±3	-26±4	-28±2	-35±8	-35±1
BRL + Rit.	3	0.3+0.1	-4±2	-4±2	-7±2	-6±1	-21±5	-13±3	-26±4	-25±3	-35±8	-23±5

[a], 0.1 ml kg^{-1}, *, P<0.05 vs corresponding value before antagonists. Rit.: ritanserin; Trop.: tropisetron; GR: GR127935; SB: SB224289; BRL: BRL15572

Vidrio, H. & Hong, E. (1976) *J. Pharmacol. Exp. Ther.*, **197**, 49-56

263P ALTERED BALANCE OF ENDOTHELIUM-DERIVED RELAXING FACTORS IN THE ACETYLCHOLINE-MEDIATED RELAXATION OF SUBCUTANEOUS RESISTANCE ARTERIES ISOLATED FROM PATIENTS WITH NORMAL PRESSURE GLAUCOMA

C. Buckley[1], P.W.F. Hadoke[2], E. Henry[3] & C. O'Brien[3]. (Introduced by G.A. Buckley) Departments of [1]Medicine & [3]Ophthalmology, Royal Infirmary of Edinburgh and [2]Department of Medical Sciences, Western General Hospital, Edinburgh.

Vascular disease is widespread in patients with normal pressure glaucoma (NPG) leading to the suggestion that this condition has a vascular aetiology (Geijssen & Greve, 1995). Acetylcholine (ACh)-mediated vasodilatation is reduced in the forearm of patients with NPG (Henry *et al.*, 1999). In contrast, however, this response was not impaired in isolated subcutaneous resistance arteries in a similar group of patients (Buckley *et al.*, 1999). This study aimed to determine the relative contributions made by different endothelium-derived factors in mediating ACh-induced relaxation in patients with NPG.

Gluteal fat biopsies were taken under local anaesthesia from 7 patients with NPG (4 male; age 63±2 years) and 6 healthy control subjects (3 male; age 53±3 years). Arteries were isolated and mounted in a myograph containing physiological salt solution at 37°C, gassed with 95%O_2; 5%CO_2 and equilibrated at their optimum resting force (Mulvany & Halpern, 1977). Cumulative concentration-response curves to ACh were obtained following sub-maximal contraction with noradrenaline (NA, 10^{-7}-10^{-6}M). These were repeated following incubation with either: N^G-nitro-L-arginine (L-NOARG; 10^{-4}M) and indomethacin (Indo; 10^{-5}M; 45mins) to inhibit nitric oxide (NO) synthase and cyclo-oxygenase; 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10^{-5}M; 10mins) to inhibit soluble guanylyl cyclase; charybdotoxin (ChTx; $5x10^{-8}$M) and apamin ($3x10^{-8}$M; Apa; 10mins) to inhibit the effects of endothelium-derived hyperpolarising factor (EDHF) or a combination of L-NOARG, Indo, ChTx and Apa.

Results are mean ± s.e.mean and were compared using Student's unpaired *t*-test. Ethical approval was obtained for the study protocol and all subjects gave written informed consent.

ACh induced similar endothelium-dependent relaxation in arteries from both patients and controls, as demonstrated previously (Buckley *et al.*, 1999). Incubation with L-NOARG/Indo or ODQ significantly reduced ACh-mediated relaxation in arteries from both controls and patients (Table 1). In contrast, ChTx/Apa produced a small, but significant, reduction in ACh-induced relaxation in arteries from controls, but had a far more pronounced effect in arteries from patients (Table 1). A combination of L-NOARG/Indo with ChTx/Apa virtually abolished relaxation to ACh in arteries from both groups (Table 1).

This study has demonstrated that ACh-induced relaxation in arteries from both controls and patients is mediated by both NO-dependent and independent pathways. In patients with NPG impaired NO-mediated relaxation appears to be balanced by an enhanced contribution from EDHF. This may explain why ACh-mediated relaxation is not impaired in subcutaneous resistance arteries from patients with NPG.

Buckley, C, Hadoke, P.W.F., Henry, E. & O'Brien, C. (1999). Nottingham BPS Meeting.
Geijssen, H.C. & Greve, E.L. (1995) *Curr. Opin. Ophthalmol.* **6**, 71-77.
Henry, E., Newby, D.E., Webb, D.J. & O'Brien, C. (1999) *Invest. Ophthalmol. Vis. Sci.* In Press
Mulvany, M.J. & Halpern, W. (1977) *Circ. Res.* **411**, 19-26.

Table 1. Maximum Relaxation (E_{max}; %) and sensitivity (-log IC_{50}) values for ACh in the absence and presence of inhibitors.

	Control		Glaucoma	
	E_{max}	-log IC_{50}	E_{max}	-log IC_{50}
Control	95.41±2.19	7.43±0.09	93.47±1.99	7.70±0.11
L-NOARG/Indo	74.43±3.52†	6.91±0.11†	72.04±5.06†	6.84±0.15†
ODQ	71.27±7.58†	6.98±0.27†	81.26±2.59†	7.15±0.22†
ChTx/Apa	81.84±6.04†	6.85±0.21†	55.39±8.06*†	6.92±0.06†
L-NNA/Indo/ChTx/Apa	15.93±5.02†	-----‡	0.73±0.44†	-----‡

Results are mean ± s.e.mean, (n=4-7). *P<0.05 compared with arteries from control subjects. †P<0.05 compared with relevant arteries in the absence of inhibitors. ‡ -log IC_{50} not measurable due to small relaxation.

264P HETEROGENEITY OF ENDOTHELIAL CELL FUNCTION IN VESSELS ISOLATED FROM HUMAN SUBCUTANEOUS FAT

C.H.Buckley[1], C-A.McIntyre[2] and P.W.F.Hadoke[2] (Introduced by B.C.Williams). [1]Department of Medicine, Royal Infirmary and [2]Department of Medical Sciences, Western General Hospital, Edinburgh University, Edinburgh, U.K.

Gluteal fat biopsies provide a convenient source of resistance arteries for investigating the vascular consequences of a variety of conditions, including hypertension (Deng *et al.*, 1995) and diabetes (McNally *et al.*, 1994). Arteries and veins are both present in gluteal biopsies but can be distinguished under a light microscope by differences in physical characteristics. In addition, however, we have identified a third type of vessel which appears larger than resistance-sized arteries but is not readily identifiable as either an artery or vein. The aim of the present study was to characterise the functional properties of this larger vessel (LV).

Gluteal fat biopsies were taken under local anaesthesia. Vessels were dissected from the biopsies and mounted in a myograph containing physiological salt solution at 37°C, gassed with 95% O_2; 5% CO_2 for measurement of isometric force. A normalisation procedure (Mulvany & Halpern, 1977) was performed in order to determine the optimum resting force and internal diameter of each vessel. Cumulative concentration-response curves were obtained to noradrenaline (NA; 10^{-9}-$3x10^{-5}$M). Following contraction with a submaximal concentration of NA (10^{-7}-10^{-6}M), cumulative concentration-response curves were obtained for the endothelium-dependent vasodilators acetylcholine (ACh; 10^{-9}-$3x10^{-5}$M) and bradykinin (BK; 10^{-10}-$3x10^{-6}$M), and the endothelium-independent nitric oxide donor 3-morpholinosydnonimine (SIN-1; 10^{-8}-10^{-4}M). Results are mean ± s.e.mean and were compared using Student's unpaired *t*-test. Ethical approval was obtained for the study and all subjects gave written informed consent.

As expected, the LV had a larger internal diameter (485±56μm; P=0.0002) than resistance arteries (186±16μm). Veins were difficult to normalise and had an internal diameter of approximately 532±82μm. Resistance arteries relaxed in response to both ACh and BK whereas these agonists had no effect on the veins (Table 1). In contrast, LV relaxed in response to BK but was unaffected by ACh (Table 1). Resistance arteries (Emax; 100±0%) and LV (Emax; 102.4±2.4%) relaxed in a similar (P=0.99) manner in response to SIN-1. NA produced concentration-dependent contractions in all three types of vessel. This appeared to be greater in LV (Table 1) but any differences were abolished when the contraction in resistance arteries (102.84±5.42%), veins (92.48±3.13%) and LV (104.61±5.11%) were expressed as a percentage of the maximum response to NAK (125mM K^+ + $5x10^{-6}$M NA).

Table 1. Maximum (Emax) contraction (mN/mm) or relaxation (%) and sensitivity (pD_2) values for the three types of vessel identified

	Normal (n=11)		Vein (n=3)		LV (n=11)	
	E_{max}	pD_2	E_{max}	pD_2	E_{max}	pD_2
NA	2.0±0.4	7.0±0.2	1.9±0.3	7.6±0.3	3.1±0.4	7.4±0.1
ACh	95.7±1.9	7.3±0.1	25.0±9.0*	7.8±0.7	17.1±3.8*	7.3±0.2
BK	89.0±4.3	7.5±0.2	26.1±2.2*	-----‡	78.7±3.9	7.9±0.2

Results are mean ± s.e.mean. *P<0.05 compared with resistance artery. ‡pD_2 not measurable due to small relaxation.

This study has demonstrated the existance of three functionally distinct vessel types in human gluteal fat biopsies. Microscopic distinction between resistance arteries and veins was reinforced by the demonstration that the latter were difficult to normalise and, unlike arteries, did not respond to endothelium-dependent vasodilators. In contrast, the LV was larger than the resistance arteries and appeared to combine physical characteristics of arteries and veins. Furthermore, these vessels were also functionally distinct as they relaxed in response to BK but not ACh. Therefore, care must be taken when isolating arteries from human gluteal fat biopsies to ensure that these vessels are responsive to both ACh and BK.

Deng, L-Y., Li, J-S, Sciffrin, E.L. (1995) *Clin Sci*, **88**, 611-622.
McNally, P.G. Watt, P.A., Rimmer, T. *et al.*, (1994), *Clin. Sci.*, **87**, 31-36
Mulvany, M.J. & Halpern, W. (1977) *Circ. Res.* **411**, 19-26.

265P ENDOTHELIUM-DEPENDENT POTASSIUM CHLORIDE-MEDIATED RELAXATION OF HUMAN RESISTANCE ARTERIES

P.W.F.Hadoke[1], C-A.McIntyre[1], C.H.Buckley[2], G.C.Jones[1], A.I.Elliott[3], J.McKnight[3], B.C.Williams[1] & B.R.Walker[1]. [1]Department of Medical Sciences & [3]Metabolic Unit, Western General Hospital, and [2]Department of Medicine, Royal Infirmary, University of Edinburgh, Edinburgh, U.K.

Nitric oxide (NO) and prostaglandin (PG)-independent relaxation of small resistance arteries is mediated by release of a hyperpolarising factor (EDHF) from the vascular endothelium (Feletou & Vanhoutte, 1988). This can be inhibited by blocking calcium activated potassium channels on the vascular endothelium (Doughty *et al.*, 1999). It has recently been demonstrated that the interaction of endothelium-derived K^+ with the barium-sensitive inwardly rectifying potassium channel (K_{IR}) and the ouabain-sensitive-Na^+/ K^+ pump accounts for the activity of EDHF in rat resistance arteries (Edwards *et al.*, 1998). This study aimed to determine whether K^+ could also be responsible for NO/ PG-independent relaxation in human sub-cutaneous resistance arteries.

Sub-cutaneous resistance arteries (internal diameter 236±18μm) were obtained from gluteal biopsies taken under local anaesthesia (2% lignocaine hydrochloride) from normal healthy volunteers (2M; 6F; age 31±4). Arterial sections from each biopsy were suspended on intraluminal wires in a small vessel myograph, containing physiological salt solution at 37°C, continuously perfused with 95% O_2; 5% CO_2, for measurement of isometric force. The endothelium was removed from some sections by rubbing the lumen with a single hair. Arteries were sub-maximally contracted with noradrenaline (NA; 10^{-8}-10^{-6}M) and cumulative concentration-response curves obtained for KCl (2.5-25mM) and acetylcholine (ACh; 10^{-9}-$3x10^{-5}$M). Responses to KCl were repeated following incubation with either: N^G-nitro-L-arginine (L-NOARG; 10^{-4}M) plus indomethacin (Indo; 10^{-5}M; 45min), to inhibit NO synthase and cyclo-oxygenase activity; L-NOARG+Indo with charybdotoxin (ChTx; 50nM) and apamin (Ap; 30nM; 10min), to further inhibit large and small conductance calcium activated potassium channels; or L-NOARG+Indo with $BaCl_2$ (30μM) and ouabain (1mM; 10min), to further inhibit K_{IR} channels and the Na^+/ K^+ pump. Results are mean ± s.e. mean, expressed as a percentage of the contraction induced by NA, and were compared using Student's unpaired t-test. Use of human arteries was approved by the Lothian Research Ethics Committee.

ACh-induced relaxation (82.2±6.6%; n=5) was abolished by removal of the endothelium (13.3±6.2; n=5; $P<0.0001$). Low concentrations (2.5-10mM) of KCl also produced a relaxation (76.0±5.2%; n=6) but this was followed by contraction at higher (15-20mM) concentrations. This response was unaffected by incubation with L-NOARG+Indo (68.1±5.58%; n=5; $P=0.33$) or by the combination of L-NOARG+Indo+ChTx+Ap (79.7±10.0%; n=3; $P=0.72$). KCl-mediated relaxation was abolished, however, by removal of the endothelium (15.5±9.2%; n=5; $P<0.001$) or incubation with L-NOARG+Indo+$BaCl_2$+Ouabain (1.7±1.2%; n=4; $P<0.0001$).

These results indicate that potassium produces an endothelium-dependent, NO-independent relaxation of human sub-cutaneous resistance arteries. This response is insensitive to inhibition of calcium activated potassium channels but is abolished by inhibition of the K_{IR} channel and Na^+/ K^+ pump. This supports the suggestion that K^+ acts as an endothelium-derived hyperpolarising factor in human resistance arteries but, in contrast to rat mesenteric arteries (Edwards *et al.*, 1998), this response is dependent upon an intact endothelium.

Doughty, J.M., Plane, F. & Langton, P.D. (1999) *Am J Physiol*, 276, H1107-H1112.
Edwards, G., Dora, K.A., Gardener, M.J. *et al.* (1998) *Nature*, 396, 269-272
Feletou, M. & Vanhoutte, P. (1988) *Br J Pharmacol*, 93, 515-24

266P GRAPE SKIN EXTRACT INDUCES BOTH ENDOTHELIUM-DEPENDENT AND ENDOTHELIUM-INDEPENDENT VASORELAXATION IN RABBIT AORTA

J.S McGinn & M.R. MacLean Div. of Neuroscience & Biomedical Systems, IBLS, University of Glasgow, Glasgow G12 8QQ

Previous studies have shown that certain red wine and grape products cause endothelium-dependent vasorelaxation (Andriambeloson *et al.*, 1997, 1998; Fitzpatrick *et al.*, 1993). Current interest in the proposed beneficial effects of moderate red wine consumption in protecting against coronary heart disease (CHD) remains controversial.
The present study was undertaken to determine the possible effects and mechanisms of a red grape skin extract on vascular function *in vitro*. The role of the endothelium, and the effects of the nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine-methyl-ester (L-NAME, 10^{-4}M) and the prostaglandin cyclo-oxygenase inhibitor Indomethacin (Indo, 10^{-5}M) on vasorelaxation were also investigated.
Aortic rings from male New Zealand White rabbits (3.5 kg) were set-up at 37°C in organ baths (20ml) filled with Krebs buffer solution and continuously bubbled with 16% O_2, 5% CO_2 and 79% N_2. Vessels were precontracted with phenylephrine (PE, 10^{-7}M). Once a stable plateau had been reached, cumulative concentration response curves for the diluted grape skin extracts (2 x 10^{-4} - 1.4 x 10 $mgml^{-1}$) were then obtained.
Grape skin extract evoked a biphasic, concentration-dependent relaxation in the aortic rings. The initial phase being abolished by Indo. (16.5±5 % relaxation of PE-contraction) but not by L-NAME or the removal of the endothelium, suggesting endothelium-NO-independent relaxation. The second phase of the relaxation was abolished by both L-NAME and endothelium removal (Table 1).
These results show that grape skin extract induced vasorelaxation in rabbit aorta in both endothelium-independent and also endothelium-dependent NO dependent mechanisms.
If such responses occur *in vivo*, they may contribute to a reduced incidence of CHD.

Andriambeloson, E., Kleschyov, A.L., Muller, B., *et al.*, (1997) British Journal of Pharmacology 120: 1053-1058
Andriambeloson, E., Magnier, C., Haan-Archipoff. G., *et al.*, (1998) Journal of Nutrition 128: 2324-2333
Fitzpatrick, D.F., Hirschfield. S.L., Coffey, R.G. (1993) American Journal of Physiology 265: H774-H778

Table 1. Maximum % relaxation values (± sem) obtained with grape skin extracts in rabbit aorta. n = number of animals.

	Max. % Relaxation	n	p
GS (control)	49 ± 10	8	
GS (-endo)	13 ± 3	7	**
GS & Indo.(10^{-5}M)	53 ± 9	7	ns
GS & Indo.(10^{-5}M) (-endo)	9 ± 2	7	**
GS & L-NAME (10^{-4}M)	17 ± 5	7	**
GS & L-NAME (10^{-4}M) & Indo.(10^{-5}M)	23 ± 4	7	*

Statistical analysis was carried out using a one-way analysis of variance, followed by a Dunnetts Multiple comparisons test.
ns = not significant, *P< 0.05, **P<0.01, compared to control.

267P VASORELAXANT EFFECT OF NITROFLURBIPROFEN IN RAT AORTA AND KIDNEY

J. Keeble, R. Clifford, M. Deraet, G. Munro, P. del Soldato[1] & P.K. Moore, Messengers and Signalling Group, Hodgkin Building, G.K.T. School of Biomedical Sciences, Guy's Campus, St.Thomas' Street, London SE1 and [1]NicOx SA, Sophia-Antipolis, France.

Nitro-nonsteroidal antiinflammatory drugs (NO-NSAID) such as nitroflurbiprofen (NOF) exhibit potent antiinflammatory activity in a range of animal models (Wallace et al.,1994). However, NOF causes less marked gastric damage (c.f. flurbiprofen) most probably due to release of nitric oxide (NO) to cause gastric mucosal vasodilatation. Interestingly, the effect of NO-NSAID on vascular smooth muscle calibre has received little attention. We have now compared the vasodilator effect of NOF with that of sodium nitroprusside (SNP) in the isolated rat aorta and perfused rat kidney.

Rats (male, Wistar, 250-300g) were killed and aortic rings (2mm) mounted (2g tension) for isometric tension recording in 20ml organ baths. Kidneys were also removed and perfused (5ml/min) with warmed (37°C) and oxygenated (95% O_2/5%CO_2) Krebs solution via a cannula inserted into the renal artery (Bhardwaj & Moore, 1988). After 60 min preincubation, preparations were precontracted with an approximate EC_{70} of noradrenaline (NA; aorta: 1 μM; kidney; 31.3 μM) and thereafter exposed to cumulative addition of NOF, SNP or flurbiprofen.

Both NOF and SNP caused dose related relaxation of the rat aorta. However, SNP was considerably more potent (EC_{50}, 83 ± 7.91 nM, E_{max}, 1.4 ± 0.1 g, n=4) than NOF, which even at the highest concentration used (1 mM) relaxed the aorta by only 0.5 ± 0.1 g (n=3). In contrast, NOF caused a dose related decrease in NA-evoked perfusion pressure in the rat kidney (EC_{50}, 35.5 ± 3.6 μM, E_{max}, 59.8 ± 3.4 mm Hg, n=5). SNP was less potent than NOF in this preparation (e.g. 100 μM lowered perfusion pressure by 19.0 ± 5.7 mm Hg, n=5). Flurbiprofen (1-100 μM) did not exhibit significant vasodilator activity in either preparation used. Furthermore, the inclusion of ascorbic acid (50 and 400 μM) or DMSO (vehicle, 0.2 ml/l) into the Krebs solution did not affect the vasodilator response to SNP or NOF in any tissue.

These results suggest that the ability of NOF to cause vasorelaxation is dependent on the blood vessel/vascular bed studied. Further experiments to investigate the effect of NOF on cardiovascular function in vivo and to elucidate the mechanism(s) of NO release from NOF are required.

Bhardwaj, R. & Moore, P.K. (1988) *Br. J. Pharmacol.*, **95,** 835-843.
Wallace, JL, Reuter, B, Cicala C et al., (1994) *Gastroenterology,* **107,** 173-179.

268P HAEME OXYGENASE INDUCTION AND THE REGULATION OF THE RAT RENAL VASCULATURE

M.R. Mustafa & E.J. Johns, Department of Physiology, The Medical School , Birmingham B15 2TT.

Carbon monoxide (CO) has been implicated in the regulation of vascular tone, analogous to nitric oxide (NO). It activates soluble guanylate cyclase and has been shown to relax vascular smooth muscle (Wang *et al.*, 1997). CO is produced endogenously as a product of haeme catabolism involving 2 isoforms of haeme oxygenase (HO): HO-1: an inducible type and HO-2: a constuitive type. A number of stimuli such as ischaemia-reperfusion, hypoxia and agents including hemin have been reported to induce HO-1 enzyme *invivo* or *invitro* (Maines, 1997). The present study aimed to investigate the contribution of the HO system in the regulation of the rat renal vasculature normally and in hypertension.

Male Wistar or Stroke-Prone Spontaneous Hypertensive (SPSHR) rats (300-350 g) were given either hemin (30 mg/kg, iv) or vehicle (saline). 24 h later the animals were anesthetised with pentobarbitone (65 mg/kg, i.p) and the right kidney was isolated and perfused with Krebs solution (plus indomethacin, 5 μM) and maintained at 37 °C, filtered through a 4.5 μm filter and gassed with oxygen in 5% CO_2. Renal perfusion pressure (RPP) was monitored via the renal arterial cannula. In some animals, tin protoporphyrin (SnPP, 33mg/kg, i.v.) was administered 60 min before removing the kidney. Following a 30 min equilibration period at a baseline pressure of 65-85 mmHg, phenylephrine (PE,1 μM) was infused for 10 min. After a further 20 min, response curves to PE (1-300 nM) were constructed. All data, mean ± s.e, were compared by ANOVA with Bonferroni's post hoc test in groups of 3-5 animals with significant level at $P < 0.05$.

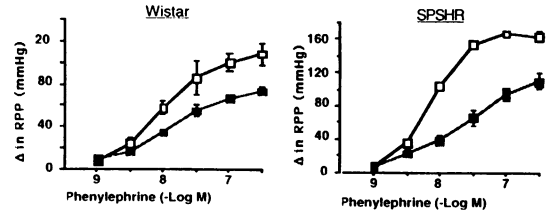


Figure 1. Effect of hemin (■) and vehicle (□) on PE induced rise in in RPP in Wistar and SPSHR animals.

Figure 1 shows in both Wistar and SPSHR animals, PE evoked a concentration dependent increase in the RPP (maximum pressure: 108 ± 11 mmHg (Wistar) and 167 ± 4 mmHg (SPSHR). Pretreatment of the animals with hemin, significantly reduced the PE (10 nM)-induced increase in RPP, in Wistar by 36.4 % and SPSHR by 59.6 % of vehicle treated animals. Preliminary data suggest that SnPP pretreatment of Wistar animals restored the PE-induced increased in RPP as in vehicle treated animals.

In conclusion, hemin treatment significantly reduced the reactivity of rat renal vasculature to PE which may be due to HO induction and increased CO synthesis. The proportionately greater suppression of the vasoconstrictor responses following hemin in the SPSHR suggests a greater HO activity in the hypertensive animals.

MRM is the recipient of a research fellowship from the Royal Society.

Maines, M.D. (1997). Annu. Rev. Pharmacol., 37, 517-554.
Wang, R., Wang, Z. & Wu, L. (1997). Br. J. Pharmacol., 121, 927-934.

269P ENDOGENOUS GM-CSF MODULATES COX-2 ACTIVITY IN HUMAN VENOUS SMOOTH MUSCLE CELLS

S. J. Stanford, J.R. Pepper and J.A. Mitchell
Imperial College of Science, Technology and Medicine, Unit of Critical Care Medicine, Royal Brompton Campus, Sydney Street, London SW3 6NP

Neutrophil recruitment and activation are primary events in the development of a number of vascular diseases. Once present in the vessel wall neutrophils do not differentiate and rapidly die. Neutrophil survival can be promoted by the cytokine granulocyte macrophage-colony stimulating factor (GM-CSF). GM-CSF is released from a variety of cell types. We have recently shown that human venous smooth muscle cells can be induced to release GM-CSF and to express cyclo-oxygenase-2 (COX-2) when stimulated with inflammatory cytokines such as IL-1β (Mitchell *et al.*, 1998). Furthermore, we have shown that GM-CSF release from these cells is further increased when COX-2 activity is blocked with a range of NSAIDs including the selective COX-2 inhibitor, L-745,337, an effect that is reversed by PGE_2 (Stanford *et al.*, 1998: 1999). In this study we have used binding antibodies to investigate the possibility that GM-CSF may reciprocally regulate COX-2 activity in human venous smooth muscle cells.

Samples of saphenous vein (SV) were dissected clean, cut into small pieces and placed in supplemented culture medium as described previously (Bishop-Bailey *et al.*, 1997). Following explantation, cultured human venous smooth muscle cells were plated onto 96 well plates for use in experiments. When cells reached confluence culture medium was replaced with new medium. Cells were then stimulated with increasing concentrations of rhIL-1β (0.01-10ng/ml). In some experiments cells were pre-treated with increasing concentrations of anti-GM-CSF antibody (1.0-10μg/ml; raised to rhGM-CSF in goat; purchased from R & D Systems). After 24-hours the medium was removed and PGE_2 release was measured by RIA (Mitchell *et al.*, 1993).

As observed previously IL-1β induced a concentration dependent increase in PGE_2 release from venous cells (Bishop-Bailey *et al.*, 1998). The presence of the anti-GM-CSF antibody significantly inhibited, in a concentration-dependent manner, PGE_2 release induced by the lowest concentration of IL-1β tested (0.01ng/ml) (*Figure 1a)*. At all other concentrations of IL-1β tested *(e.g. 0.1ng/ml; Figure 1b)* the antibody had no effect on PGE_2 release.

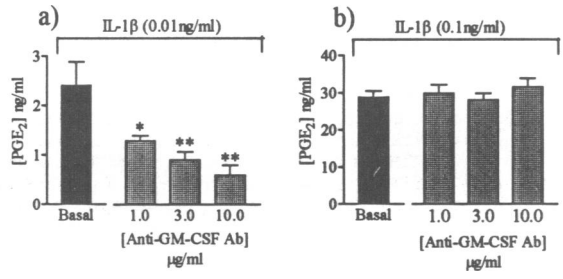


*Figure 1. Effect of increasing concentrations of anti-GM-CSF antibody (1.0-10μg/ml) on PGE_2 release from human cultured venous smooth muscle cells stimulated with IL-1β at a concentration of a) 0.01ng/ml and b) 0.1ng/ml. Data is shown as mean±s.e.m: n=6: *P<0.05, **P<0.01 (One way ANOVA: Post test, Dunnet).*

Here we show that an anti-GM-CSF antibody reduces COX-2 activity in cells stimulated with a low concentration of IL-1β (0.01ng/ml). We suggest that in the presence of higher concentrations of IL-1β the increased levels of GM-CSF are able to overcome the quenching effect of the antibody. This study, together with our previous observations, indicates the existence of reciprocal regulatory mechanisms between GM-CSF and COX-2 in human venous smooth muscle cells.

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Bishop-Bailey, D., *et al.*, (1997). *Br. J. Pharmacol.* 120, 73P.
Bishop-Bailey, D., *et al.*, (1998). *Arterioscler. Thromb. Vasc. Biol.* 18, 1655-1661.
Mitchell, J.A., *et al.*, (1998). *Br. J. Pharmacol.* 123, 74P.
Mitchell, J.A., *et al.*, (1993). *Proc. Natl. Acad. Sci., USA.* 90, 11693-11697.
Stanford, S.J., Pepper, J.R. & Mitchell, J.A. (1998). *Br. J. Pharmacol.* 125, 20P.
Stanford, S.J., Pepper J.R. & Mitchell, J.A. (1999). *Br. J. Pharmacol.* 126, 42P.

270P TREATMENT OF EQUINE ENDOTHELIAL CELLS WITH SUBSTANCE P INDUCES EOSINOPHIL ADHERENCE

S.R. Bailey, F.M. Cunningham, Dept of Veterinary Basic Sciences, Royal Veterinary College, Hawkshead Campus, Herts. AL9 7TA

Eosinophils accumulate in large numbers in the skin of horses with allergic skin disease. Substance P induces endothelial adhesion molecule expression leading to increased neutrophil and eosinophil accumulation in human skin (Smith *et al*, 1993). The present study has examined the effects of substance P on the adherence of equine eosinophils to equine endothelial cells.

Equine digital vein endothelial cells (EDVEC) were obtained by enzymatic digestion, using a modification of the technique used by MacEachern *et al* (1997). EDVEC were cultured in Dulbecco's modified Eagle's medium containing 10% each of foetal and newborn calf serum and penicillin/ streptomycin, passaged once, transferred to 48 well plates and allowed to form a confluent monolayer. Equine blood eosinophils were obtained from a mixed population of native breed adult ponies and adherence to EDVEC quantified after 25min using a colorimetric assay specific for eosinophil peroxidase, as previously described (Foster *et al*, 1997). In preliminary experiments, EDVEC were treated with substance P (0.2 mM) for 1-24h, washed three times in PBS and the adherence of unstimulated eosinophils from 4 animals assayed. The effects of human recombinant IL1 (1 ng/ml) were examined for comparison. A preincubation time of 18h was chosen for further experiments in which EDVEC were treated with substance P (0.1 μM - 1 mM) or IL1 (1 pg/ml - 100 ng/ml). The effects of the NK_1 receptor antagonist CP96345 (10 μM) were also examined.

Maximum eosinophil adherence was observed at approximately 20h after treatment of EDVEC with substance P (0.2 mM) or IL1 (1 ng/ml) (11.6±3.4 % for substance P (background adherence 3.5±1.1 %) and 16.7±3.2 % for IL1 (background adherence 1.7±0.3 %)). Substance P and IL1 caused a concentration dependent increase in eosinophil adherence following treatment of EDVEC for 18h, the EC_{50} value for IL1 being 0.3±0.04 ng/ml; maximum adherence 18.7±4.4 %. The response to substance P did not reach a maximum at the highest concentration tested (1 mM; 10.6±2.6% adherence). CP96345 (10 μM) caused significant inhibition of the response to substance P (0.3 mM)(5.0±0.2 % vs. 8.9±0.2 % for control; paired *t*-test, $P<0.05$) whilst the inactive enantiomer CP96344 (10 μM) was without effect. Neither compound affected the response to IL1.

These data show that substance P and IL1 can activate equine endothelial cells, increasing adherence of equine eosinophils in a time- and concentration-dependent manner. The effects of substance P appear to be mediated via the NK_1 receptor. Substance P, if released in the skin of horses with allergic skin disease, may contribute to the eosinophil recruitment observed.

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Foster, A.P., Lees, P., Cunningham, F.M. (1997) *Vet Immunol Immunopathol* 56, 205-220
MacEachern, K.E., Smith G.L., Nolan, A.M. (1997) *Res Vet Sci* 62, 147-152
Smith, C.H., Barker, J.N., Morris, R.W. *et al*, (1993) *J Immunol* 151, 6, 3274-82

271P COMPARISON OF INHALED SPASMOGEN AS DETERMINANTS OF AIRWAY HYPERRESPONSIVENESS IN A MURINE MODEL OF ASTHMA

C.H. Battram, M. Birrell, M. Foster, S.E. Webber and M.G. Belvisi.
Rhône-Poulenc Rorer Ltd, Pharmacology Department, Dagenham Research Centre, Rainham Road South, Dagenham, Essex, RM10 7XS.

Non-specific airway hyperresponsiveness (AHR) is a cardinal feature of asthma which is characterised by an increase in responsiveness to inhaled spasmogens. Whilst there is a wealth of literature describing animal models of AHR to spasmogens acting directly on the airway smooth muscle (ASM) such as methacholine (MCh), the extent of the AHR produced is not representative of the 7 to 75 fold increase seen in asthmatics (Crowther *et al.*, 1997). However it has previously been demonstrated that both bradykinin (BK) (Fuller *et al.*, 1987; Polosa and Holgate, 1990) and adenosine (or 5' adenosine monophosphate (AMP) which is dephosphorylated to yield adenosine) (Cushley & Holgate, 1985, Crimi *et al.*, 1988) cause bronchoconstriction in asthmatics but not in normal volunteers. BK is thought to provoke bronchoconstriction, in part by acting directly on airway smooth muscle. Interestingly, both BK and AMP may also evoke constrictor responses indirectly via activation of sensory nerves and in addition, for AMP, through mast cell activation. We have used whole body plethysmography to evaluate ovalbumin (OA) induced AHR to inhaled AMP and BK, compared this with MCh and determined the modulatory effect of dexamethasone (Dex).

Male balb\c mice (20g) were immunised with 10μg of OA in 0.2ml of saline (sal) with 20mg of $AL(OH)_3$ i.p. on days 0 and 14. From day 21 the animals were challenged (chall) with aerosolised OA (5%) in saline (or saline alone) for 20 minutes on 6 consecutive days. Vehicle (veh) (1% carboxymethylcellulose in distilled H_2O) or Dex was administered twice daily p.o. in a dose volume of 10ml kg^{-1}, the day before the first OA challenge, 2 hrs prior to and 6 hrs after subsequent challenges and on the morning of AHR determination.

Twenty-four hours after the last OA challenge mice were anaesthetised with urethane (2g kg^{-1}, i.p.) and connected, via a tracheal cannula, to a ventilator set at 200 breaths min^{-1}. Measurement of air flow was by whole body plethysmography. A water filled, oesophageal cannula was placed such that transpulmonary pressure could be recorded. Administration of aerosolised spasmogen was by ultrasonic nebuliser connected in line with the ventilator. Resistance (R_L) (cmH_2O/ml/s) was continuously computed on a Buxco XA-analyser. AHR was assessed with either a single concentration of BK (as BK is tachyphylaxic) or increasing concentrations of MCh or AMP. Maximal changes in airways resistance were determined by the analyser over 2 mins for MCh, 15 mins for BK or 5mins for AMP. Reactivity to all three spasmogens, was significantly increased by ovalbumin compared to saline challenge and for both BK and AMP was significantly reversed in a dose related manner by Dex. (see table). Data is presented as the mean ± s.e.m. (n=8-12).

	Change in airways resistance (cmH_2O/ml/s)				
MCh mg ml^{-1}	Saline challenge	OA challenge			
2	0.3±0.07	0.9±0.22*			
4	0.4±0.11	2.0±0.49*			
8	0.8±0.18	2.4±0.44**			
16	1.2±0.18	3.6±0.69**			
32	1.8±0.28	5.4±1.10*			
AMP mg ml^{-1}	Sal chall Veh p.o.	OA chall Veh p.o.	OA chall / Dex mg kg^{-1} p.o. 0.3	1.0	3.0
0.3	0.3±0.04	0.8±0.26$^{\blacklozenge\blacklozenge}$	0.3±0.02††	0.2±0.05††	0.2±0.11††
1	0.3±0.04	1.2±0.23$^{\blacklozenge\blacklozenge}$	0.5±0.11††	0.3±0.06††	0.3±0.09††
3	0.4±0.08	1.7±0.30$^{\blacklozenge\blacklozenge}$	0.7±0.12††	0.3±0.07††	0.3±0.06††
10	0.3±0.05	1.7±0.29$^{\blacklozenge\blacklozenge}$	0.7±0.19††	0.4±0.06††	0.4±0.07††
30	0.3±0.04	1.8±0.30$^{\blacklozenge\blacklozenge}$	0.8±0.15††	0.5±0.09††	0.5±0.06††
100	0.4±0.07	1.7±0.30$^{\blacklozenge\blacklozenge}$	1.2±0.23	0.6±0.11††	0.6±0.05††
BK 1mM	0.1±0.02	0.6±0.14$^{\blacklozenge\blacklozenge}$	0.2±0.09††	0.3±0.06†	0.1±0.03††

*p<0.05, **p<0.01 Student's t test.
$^{\blacklozenge}$p<0.05, $^{\blacklozenge\blacklozenge}$p<0.01 ANOVA one way, Dunnett's v saline/vehicle control.
†p<0.05, ††p<0.01 ANOVA one way, Dunnett's v OA/vehicle control.

The profile of these spasmogens presents a similar picture to that seen in the clinic i.e. there was AHR to all three spasmogens following OA challenge but with BK and AMP little or no response in the saline challenged animals. Furthermore, AHR demonstrated by spasmogens which can activate inflammatory mechanisms in addition to producing direct contraction of ASM and be reversed by steroid treatment maybe a more appropriate model in which to examine the action of anti-asthma drugs.

Crimi et al. (1988). *J. Allergy Clin. Immunol.* 83: 921-925.
Crowther et al. (1997). *Clin. Exp. Allergy.* 27:606-616.
Cushley & Holgate. (1985). *Allergy Clin. Immunol.* 75: 272-278.
Fuller et al. (1987). *Am. Rev. Resp. Dis.* 135:176-180.
Polosa & Holgate. (1990). *Am. Rev. Resp. Dis.* 142:1367-1371.

272P DETECTION OF NITRATED PROTEIN IN A RAT MODEL OF LOCAL CUTANEOUS THERMAL INJURY

A. Rawlingson, S. Greenacre and S.D. Brain, Division of Pharmacology & Therapeutics and Centre for Cardiovascular Biology & Medicine, King's College London, Manresa Road, London SW3 6LX, UK.

Peroxynitrite ($ONOO^-$) is formed *in vivo* on reaction of nitric oxide ($NO^\bullet$) and superoxide ($O_2^{\bullet-}$). $ONOO^-$ injected intradermally has pro-inflammatory microvascular effects (Ridger *et al.*, 1997) and protein bound 3-nitrotyrosine (3NT), a commonly measured marker of peroxynitrite formation has been measured *in vivo* in response to a number of inflammatory stimuli (Ischiropoulos, 1998). We have investigated the possibility that $ONOO^-$ and/or other reactive nitrogen species (RNS) are formed in rat cutaneous tissues following thermal injury.

Male Wistar rats (230-280 g) were anaesthetised with thiopentone sodium (100 mg kg^{-1}, i.p.). The abdominal skin was closely shaven and depilated. Animals were intubated to aid respiration and body temperature was maintained at 37°C. For oedema experiments animals received [^{125}I]-albumin (100 kBq) i.v. Local cutaneous thermal injury was induced with a temperature-controlled skin heater (Pinter *et al.*, 1999). Animals were killed at either 60 or 180 min following burn. Heated and unheated skin was removed and homogenised for the measurement of 3NT by an ELISA assay (Khan *et al.*, 1998) or the accumulation of [^{125}I]-albumin which was used to calculate plasma extravasation (Ridger *et al.*, 1997). All results are expressed as mean ± s.e.mean and were analysed by a paired Student's t-test.

Thermal injury to rat abdominal skin caused a significant increase in both 3NT and oedema formation when compared to unheated control sites at the 180 min time point post-burn (Table 1), but did not cause an increase in 3NT immunoreactivity at the 60 min time point.

	3-nitrotyrosine (n=7-8)		Oedema (n=6-8)	
	60 min	180 min	60 min	180 min
Unheated	10.16 ±1.17	11.32 ±0.98	8.09 ±0.64	19.60 ±4.02
Heated	13.62 ±2.25	19.38 ±1.11*	34.63 ±6.66*	175.04 ±22.42***

Table 1. Effect of thermal injury (50°C, 10 min) on 3NT formation (nitrated BSA equivalents nmol cm^2 $skin^{-1}$) and plasma extravasation (μl cm^2 $skin^{-1}$) in rat abdominal skin. *P<0.05, ***P<0.001 compared to unheated sites in the same animal.

This data provides evidence of protein nitration in thermally injured, oedematous skin and strongly suggests that $ONOO^-$ is generated in thermally damaged cutaneous tissue. This may explain why superoxide dismutase, a $O_2^{\bullet-}$ scavenger has been reported to attenuate thermally induced oedema formation (Bjork & Artuson, 1983).

AR and SG are in receipt of MRC and ARC Ph.D. studentships respectively.

Bjork J. & Artuson G. (1983) *Burns* 9, 249-256.
Ischiropoulos H. (1998) *Arch. Biochem. Biophys.* 356, 1-11.
Khan J., *et al.* (1998) *Biochem. J.* 330, 795-801.
Pinter E., *et al.* (1999) *Eur. J. Pharmacol.* 369, 91-98.
Ridger V.C., *et al.* (1997) *Br. J. Pharmacol.* 122, 1083-1088.

273P FcεRI IMMUNOCONJUGATE INHIBITS IgE-MEDIATED PASSIVE CUTANEOUS ANAPHYLAXIS (PCA) IN THE RAT

K. Holland, N. Gozzard, R. Owens & R. Foulkes. Celltech Therapeutics Ltd., Slough, SL1 4EN.

IgE has been implicated in the pathology of a number of allergic diseases, including asthma, rhinitis and anaphylaxis (Sutton and Gould, 1993). IgE can bind, via its Fc region, to inflammatory cells (e.g. mast cells and basophils) expressing FcεRI and/or FcεRII. Crosslinking of receptor bound IgE with antigen elicits cellular degranulation and release of inflammatory mediators (e.g. histamine and leukotrienes). These mediators can cause plasma protein leakage (PPL) and oedema in tissues (Wedmore & Williams, 1981). pRO129 is an immunoconjugate comprising two FcεRI α-chains linked by a human IgG_4 Fc region (Shi *et al.*, 1997), which can bind free IgE. The binding of free IgE may prevent its association to effector cells and their subsequent activation in response to antigen (Ag).

Using the rat passive cutaneous anaphylaxis (PCA) model, the ability of pRO129 to block IgE-mediated plasma protein leakage was studied.

CD rats (male, 250-320g, Charles River, UK) were anaesthetised with isoflurane. Anti-DNP (di-nitrophenyl) IgE (diluted 1:10000) or saline was injected intradermally (100µl, i.d.) into previously shaved dorsal skin sites. 24h later the rats were anaesthetised prior to intravenous administration of 0.5ml of DNP-HSA antigen (Ag; 2mg.ml^{-1}) in Evan's blue (0.1%) containing [^{125}I]-BSA (1µCi.ml^{-1}). After 1h rats were terminally anaesthetised, a blood sample taken and the dorsal skin removed. Radioactivity levels of individual skin sites were determined and expressed relative to plasma radioactivity levels. The degree of radioactivity in skin sites being proportional to PPL. Values are mean±s.e.mean and data analysed by Kruskall-Wallis & Dunn's post test. Degree of significance indicated as * ($p<0.05$), ** ($p<0.01$) & *** ($p<0.001$).

Intravenous administration of Ag (DNP-HSA) caused a significantly greater PPL in anti-DNP IgE injected skin sites (348±27µl) compared to saline injected skin sites (108±4µl), ($p<0.001$). pRO129 was co-incubated with anti-DNP IgE *in vitro* (24h @ 4°C) prior to intradermal injection into skin sites. Increasing mole ratios of pRO129 caused a concentration-dependent decrease in PPL compared to an isotype matched control antibody (CDP850). Percent inhibition of PPL with respect to pRO129:IgE mole ratios were 4:1 (82±6% ;$p<0.001$), 2:1 (67±8%; $p<0.001$), 1:1 (62±2%; $p<0.001$), 0.5:1 (47±6%; $p<0.001$) and 0.25:1 (14±7%; $p>0.05$) (n=9-19).

In a seperate study, the effect of systemic administration of pRO129 on IgE-mediated PPL was investigated. Intravenous administration of Ag (DNP-HSA) caused a significantly greater PPL in anti-DNP IgE injected skin sites (269±11µl; n=24) compared to saline injected skin sites (112±6µl, n=24), ($p<0.001$), producing an IgE-mediated PPL of 157±10µl. Intravenous administration of pRO129, given 24 and 1 hour prior to anti-DNP IgE injection, caused a dose-dependent inhibition of PPL (figure 1).

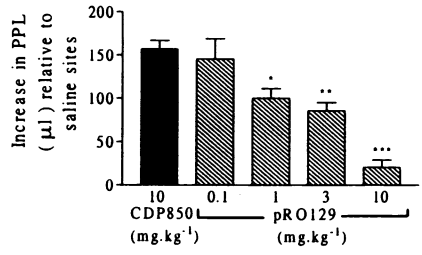

Figure 1:Dose-dependent inhibition of PPL in the rat (n=7-24).

These data demonstrate that the systemic administration of the anti-IgE immunoconjugate pRO129 inhibits IgE-mediated PPL in the rat skin. pRO129 may be useful in suppressing IgE-mediated diseases.

Shi, J., *et al* (1997) Biochemistry 36, 2112-2122.
Sutton, B. & Gould, H. (1993) Nature 366, 421-428.
Wedmore, C. & Williams, T. (1981) Nature 289, 646-650.

274P INHIBITORY ACTIONS ON RAT MYELOPEROXIDEASE OF MOLECULES ISOLATED FROM ANTI-INFLAMMATORY EXTRACTS OF *COMMIPHORA KUA*

G.R. Battu[1], I.J. Zeitlin[1], A.I. Gray[2] & P.G. Waterman, Depts. of [1]Physiology & Pharmacology and [2]Pharmaceutical Sciences, University of Strathclyde, Glasgow G4 0NR, Scotland U.K.

The resin exudate from *C. kua*, family Burseraceae, is a traditional Kenyan anti-inflammatory medicine. We have previously reported that the octanordammaranes, mansumbinone and mansumbinoic acid are the main anti-inflammatory molecules in *C. kua* resin (Duwiejua *et al.*, 1993). Myeloperoxidase (MPO) is a major constituent of neutrophils, catalysing the formation of hypochlorite and free radical hydroxyl from hydrogen peroxide and Cl^-. The products kill phagocytised bacteria and viruses and cause tissue damage in chronic inflammatory disease. We now report the purification of molecules from *C. kua* extracts, which inhibit the formation of myeloperoxidase products.

Rat paw inflammation was induced by subplantar injection of carrageenan in hind limbs of male Sprague Dawley rats. Animals were killed and inflamed paws were used as a source of MPO activity. Skinned paws were homogenised with hexadecyl trimethyl ammonium bromide in 50 mM phosphate buffer (pH 6.0), centrifuged and the supernatants were collected. For MPO assay (Bradley et al, 1982), supernatant, with or without test molecules, was incubated with buffer containing o-dianisidine and H_2O_2. The reaction was stopped at 60 min. with 1.2M HCl. Absorbance was measured at 440 nm. Statistical analysis was carried out using Student's t-test and, where appropriate, analysis of variance. Inhibitory molecules were purified by vacuum liquid chromatography from extracts of the resin and the structures determined using NMR spectral data.

Four active molecules have been purified; mansumbinone (M^{one}), mansumbinoic acid (M^{oic}), picropolygamain (P) and lignan-1 (L1; methoxy-1,2,3,4-tetrahydropolygamain). Figure 1 shows the conc.-effect relationships. The limited solubility of the compounds prevented determination of maximum inhibition. The equipotent molar ratios for M^{oic} : L1 : Indomethacin : M^{one} : P at 40% inhibition were 1 : 1.8 : 4.7 : 44.4 : 209.7 respectively.

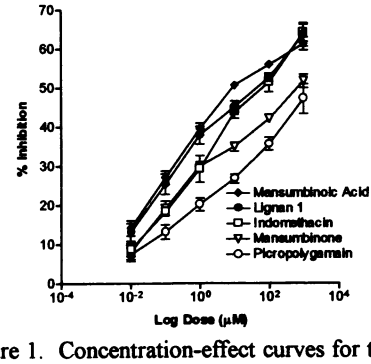

Figure 1. Concentration-effect curves for the inhibition of rat myeloperoxidase by molecules purified from *C. kua* resin (Mean±SEM, n=6).

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Bradley, P.P., Priebat, D.A., Christensen, R.D. *et al.* (1982) *J. Invest. Dermatol.* 78, 206-209.
Duwiejua, M., Zeitlin, I.J., Waterman, P.G., *et al.* (1993) *Planta Med.* 59, 12-16.

275P CHARACTERISTICS OF AIRWAY HYPERREACTIVITY, CELL INFLUX AND STEROID SENSITIVITY AFTER INHALED LPS IN CONSCIOUS GUINEA-PIGS

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

Aerosolised lipopolysaccharide (LPS) inhalation induces airway hyperreactivity (AHR) and infiltration of predominantly neutrophils and macrophages into the airways (Folkerts *et al.*, 1988), which are features of chronic obstructive pulmonary disease (COPD; Vrugt & Aalberes, 1993). The degree of AHR correlates with the severity of COPD and asthma (Rijcken *et al*, 1997). Corticosteroid treatment attenuates the AHR and pulmonary cell influx (Postma & Kerstjens, 1998). Unlike COPD, asthmatics (Cushley *et al.*, 1983) and atopic guinea-pigs (Thorne & Broadley, 1994), exhibit a bronchoconstriction to inhaled 5' adenosine monophosphate (5'AMP). We therefore characterised AHR, cell influx and steroid sensitivity in LPS-challenged guinea-pigs as a potential model of COPD.

Specific airway conductance (sGaw) was measured in groups (n=6) of conscious Dunkin-Hartley guinea-pigs (male 300-350g) by whole-body plethysmography (Griffiths-Johnson *et al.*, 1988). Baseline sGaw values were obtained and 30 min later they received a nose-only exposure to threshold doses of nebulised (0.2 $ml.min^{-1}$) histamine (Hist, 1mM, 20s) or methacholine (MCh, 0.1mM, 20s) and sGaw recorded at 0, 5 and 10min. 24h later the guinea-pigs were exposed to either nebulised LPS (30$\mu g.ml^{-1}$) or vehicle (0.9% LPS-free saline), for 60 min and sGaw measured at 0, 15, 30, 60 min, and hourly thereafter. Airway reactivity to Hist was re-assessed at 0.5, 1, 2, 4 or 24 h post LPS and to MCh at 1 h. Due to tachyphalaxis, airway reactivity to nebulised 5'AMP (box exposure, 3mM, 60s) was assessed in naive animals and at 1 h after saline or LPS. After assessing airway reactivity, animals were overdosed with pentobarbitone sodium (0.6 $mg.100g^{-1}$, i.p.), the lungs lavaged (1% EDTA, 1 $ml.100g^{-1}$, twice) and the cell content of the BALF determined.

There was an initial transient bronchoconstriction after exposure to saline or LPS (-16.2±4 and -13.1±2.6 peak % changes from baseline sGaw, respectively). LPS exposure produced AHR to Hist at 30 ($p<0.05$), 60 ($p<0.02$) and 120 min, bronchoconstriction occurring after LPS (-13.5±7.8, -26.3±7.9 and -24.3±14.4%) compared with before LPS (+18.0±8.7, +16.1±5.9 and +6.7±11.4%, respectively). Reactivity was restored by 4h. LPS also induced AHR to MCh at 60 min (% change from baseline sGaw −7.51±1.0 and -21.2±4.5 before and after LPS, $p<0.02$), but not saline. The cells in the BALF, showed a peak neutrophilia at 24h (36.3±2.3x10^6 cells per sample) and a time-dependent increase in macrophages and eosinophils (30 mins:4.6±0.4 and 0.1±0.05; 48h:31.0±6.0 and 1.8±0.3 x10^6 cells per sample, respectively), compared to 60 min after saline exposure (neutrophils, eosinophils and macrophages: 0.1±0.0, 0.1±0.0 and 2.2±0.3x10^6 cells per sample, respectively). Dexamethasone (20$mg.kg^{-1}$, i.p. daily) administered 2 days prior to LPS challenge inhibited the LPS-induced AHR to Hist at 60 and 120 min. It also reduced the pulmonary influx of neutrophils and eosinophils at 24h by 38.5 & 31%, respectively, but caused a 42% increase in macrophages (22.3±2.8, 0.9±0.2 and 39.1±5.4x 10^6 cells per sample, respectively). Naive animals exposed to 5'AMP produced a small bronchodilation (+8.4±8.5% increase sGaw). At 1h after saline or LPS exposure, 5'AMP caused small bronchoconstrictions (-15.2±4.9 & -10.3±4.9% decrease in sGaw).

This study shows that in common with non-atopic COPD, there was AHR to Hist and Mch but not to 5'AMP after LPS inhalation. The AHR and infiltration of granulocytes were sensitive to inhibition by a steroid, although disease progression is not (Postma *et al.*, 1998).

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Cushley MJ *et al.* (1983) *Br J Clin Pharmacol* **15**, 161-5
Folkerts G *et al.* (1988) *Am Rev Respir Dis* **137**, 1441-7
Griffiths-Johnson DA *et al.* (1988) *J Pharmacol Meth* **19**, 233-42
Postma DS, Kerstjens HA (1998) *Am J Respir Crit Care Med* **158**, s187-92
Rijcken JP *et al.* (1997) *Lancet* **350**, 1431–34
Thorne JR, Broadley KJ (1994) *Am J Respir Crit Care Med* **149**,392-9
Vrugt B, Aalberes R (1993) *Respir Med* **87** (supp. B), 3-7

276P CHANGES IN CYTOCHROME P450 ISOENZYMES BY NEW FLUOROQUINOLONES

M.R. Martínez-Larrañaga, M.J. Díaz, M.A. Martínez, M.T. Frejo, M. Martínez, M.L. Fernandez-Cruz, M. Tafur, & A. Anadón. Department of Toxicology, Faculty of Veterinary Medicine, Universidad Complutense, 28040 Madrid, Spain.

Fluoroquinolones represent a growing family of bacterial antimicrobials. The existence of significant drug interactions between fluoroquinolones and the metabolism of other pharmaceutical agents became evident shortly after the initiation of clinical investigations. Evidence suggests that these drug interactions are caused by an inhibition of specific cytochrome P450 (CYP) isoenzymes (Fuhr *et al.*, 1992). This is of considerable interest in light of the abundance of new fluoroquinolones. The present work assesses the effect of an *in vivo* multi-dose administration of ciprofloxacin, a known inhibitor of CYP1A2 (Fuhr *et al.*, 1992) and danofloxacin, difloxacin and marbofloxacin, new fluoroquinolones used in veterinary medicine, on the CYP1A1, CYP1A2 and CYP2B1 isoenzymes by measuring the ethoxy- (EROD), methoxy- (MROD) and pentoxy- (PROD) resorufin *O*-dealkylation activities, respectively, and on the CYP3A1/2 isoenzymes by measuring the erythromycin *N*-demethylation and the testosterone 6ß-hydroxylation.

Groups of 6 male Wistar rats weighing initially 200 g were deprived of food for 6 h before the single daily oral administrations of each antibiotic at the dose of 50 mg/kg for 6 days. The antibiotics were administered by gavage. Control rats received 0.5 ml of saline solution. Antibiotic-treated and control animals were killed 24 h after the last administration and the livers were removed. The livers were individually homogenized and microsomal pellets were prepared for drug metabolising enzyme determinations (Martínez-Larrañaga *et al.*, 1996). The testosterone 6ß-hydroxylation was determined as described (Wood *et al.*, 1983).

Statistical analysis was done by using an unpaired Student's t-test $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared to control group.

Ciprofloxacin and difloxacin significantly inhibited EROD (21% and 24%, $P<0.05$), MROD (17% and 13%, $P<0.05$), and PROD (45%, $P<0.001$ and 37%, $P<0.01$) activities, respectively. Marbofloxacin caused significant inhibitions of EROD (20%, $P<0.05$) and testosterone 6ß-hydroxylase (21%, $P<0.05$) activities. Danofloxacin caused a significant decrease in testosterone 6ß-hydroxylase (21%, $P<0.05$) activity but an increase in EROD (63%, $P<0.001$) and MROD (18%, $P<0.01$) activities.

We conclude that fluoroquinolones tested can decrease CYP1A-, CYP2B- or CYP3A-mediated biotransformation by competitive inhibition and they have the potential to cause drug interactions with agents metabolized by these enzymes. On the other hand, to our knowledge, danofloxacin is the only fluoroquinolone for which CYP1A1 and CYP1A2 induction have been demonstrated.

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Fuhr, U., Anders, E.-M., Mahr, G. *et al.* (1992). *Antimicrob. Agents Chemother.* **36**(5), 942-948.
Martínez-Larrañaga, M.R., Anadón, A., Diaz, M.J. *et al.*(1996). *Toxicol. Appl. Pharmacol.* **141**, 185-194.
Wood, A.W. Ryan, D.E., Thomas, P.E. *et al.* (1983) *J. Biol. Chem.* **258**(14), 8839-8847.

277P TESTOSTERONE HYDROXYLASES IN EVALUATING INDUCTION AND INHIBITION OF CYTOCHROME P450 ISOENZYMES BY TILMICOSIN: COMPARISON WITH ERYTHROMYCIN

M.A. Martínez, M.R. Martínez-Larrañaga, M.J. Díaz, & A. Anadón. Department of Toxicology, Faculty of Veterinary Medicine, Universidad Complutense, 28040 Madrid, Spain.

Tilmicosin is a new macrolide antibiotic used in farm animals for treatment of respiratory diseases. Some macrolide antibiotics cause clinical drug interactions usually by altering metabolism due to complex formation and inactivation of cytochrome P4503A (CYP3A) subfamily in the liver (Wrighton *et al.*, 1983). The effects of macrolide antibiotics as inducers or inhibitors seem to be greatly dependent on their structures. This study has been undertaken to discover if tilmicosin [20-deoxo-20-(3,5-dimethyl piperidinyl)-1-yl-desmycosin] has effects on CYP isoenzymes in rat liver. The testosterone hydroxylations at the 2α-, 16α-, 6ß-, 7α-, and 16ß- positions were chosen as markers for CYP2C11, CYP3A1/2, CYP2A1/2 and CYP2B1/2 activity, respectively (Waxman *et al.*, 1987; Halvorson *et al.*, 1990).

Groups of 6 male Wistar rats weighing initially 200 g were deprived of food for 6 h before the single daily oral administration of tilmicosin at the doses of 20, 40 and 80 mg/kg for 15 days. For comparison, another group received 63 mg erythromycin/kg and a control group received 0.5 ml of saline solution. Tilmicosin-, erythromycin-, and saline-treated animals were killed 24 h after the last dose. The livers were removed, individually homogenized and microsomal pellets prepared as described (Martínez-Larrañaga *et al.*, 1996) and stored at -90°C prior to use. The microsomal hydroxylation of testosterone was determined as described (Wood *et al.*, 1983).

Exposure of rats to tilmicosin at dose of 80 mg/kg (eight times higher than the recommended dose for treatment of bacterial pneumonia) only resulted in a significant decrease in the formation of 16ß-hydroxytestosterone (36.8%, $P<0.01$ compared to the saline-treated control group). In contrast, in comparison with saline-treated control group, erythromycin produced a significant increase in the formation of 6ß-hydroxytestosterone (99.2%, $P<0.01$)), and a significant decrease in the formation of 2α-hydroxy-testosterone (38.9%, $P<0.05$), 16α-hydroxytestosterone (47.4%, $P<0.001$), 7α-hydroxytestosterone (41.7%, $P<0.01$), and 16ß-hydroxytestosterone (38.2%, $P>0.001$).

The results obtained with testosterone indicated that tilmicosin did not result in either induction or inhibition of hepatic CYP2C11, CYP3A1/2 or CYP2A1/2.

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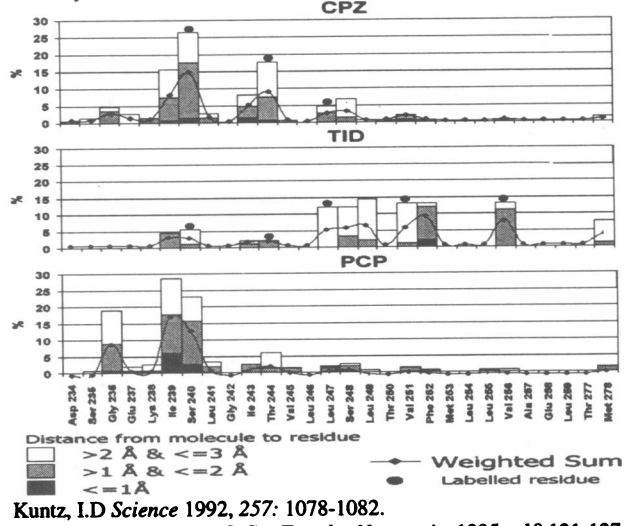
Halvorson, M., Greenway, D., Eberhart, D. *et al.* (1990). *Arch. Biochem. Biophys.* **277**, 166-180.
Martínez-Larrañaga, M.R., Anadón, A., Díaz, M.J. *et al.* (1996). *Toxicol. Appl. Pharmacol.* **141**, 185-194.
Wrighton, S.A., Maurel, P., Schuetz, E.G. *et al.* (1985). *Biochemistry* **24**, 2171-2178.
Waxman, D.J., Lapenson, D.P., Park, S.S. *et al.* (1987). *Mol. Pharmacol.* **32**, 615-624.
Wood, A.W. Ryan, D.E., Thomas, P.E. *et al.* (1983) *J. Biol. Chem.* **258**(14), 8839-8847.

278P ION CHANNEL—CHANNEL BLOCKER INTERACTIONS IN THE NICOTINIC ACETYLCHOLINE RECEPTOR

The nicotinic acetylcholine receptor (nAChR) is the best understood of the Ligand-Gated-Ion Channel superfamily, which also comprises the $5HT_3$, $GABA_A$, and glycine receptors (Ortells & Lunt, 1995). Since no high resolution structure is available, we used an open channel model to predict docking sites of several compounds known to block the nAChR ion channel as noncompetitive antagonists (NCA) and to deduce their interactions patterns with the residues of the channel. The nAChR is an important target for local anaesthetics, toxins, hallucinogens and other important drugs, and several of the blockers analysed behave as such, hence the relevance of a detailed structural understanding of the way they function.

A molecular model of the transmembrane región of the nAChR ion-channel (Ortells et al. 1997) was used as a target for docking the following NCA molecules: Chlorisondamine, Chlorpromazine (CPZ), Ethidium, Hexamethonium, Histrionicotoxin, Phencyclidine (PCP), Quinacrine, QX-222, Tetracaine, 3-(trifluoromethyl)-3-m-(iodophenyl) diazirine (TID), and others. To dock these molecules, the DOCK 3.5 software (Kuntz, 1992) was used. We calculated, for each compound, the percentage of atoms that were at three ranges of distances to each amino acid in the model: 1) lower than 1Å, 2) between 1 and 2Å, and 3) between 2 and 3Å. A weighted sum was also calculated: % of atoms in range 1 + (% of atoms in range 2)/2 + (% of atoms in range 3)/3. This weighting scheme awards higher importance to those atoms in the docked ligand lying at lower distances to a given amino acid in the nAChR..

The interaction patterns with the ion channel residues predicted for those molecules tested for which experimental information is known (on the basis of site directed, photoaffinity labelling, mutagenesis, etc), were reasonably accurate, given that the three components on which the predictions are based are independent of each other. Analysis revealed that all compounds have predicted docking sites well correlated with experimental data. Figure 1 shows the interaction patterns between three of the tested molecules and the nAChR. The good correlation obtained represents a strong support for the models and theoretical approaches, and consequently the predictions made, since otherwise it would have been quite unlikely to obtain sound results.



Kuntz, I.D *Science* 1992, *257:* 1078-1082.
Ortells, M.O.; Lunt, G.G. *Trends Neurosci.* 1995, *18*:121-127.
Ortells, M.O., Barrantes, G.E. et al. *Prot.Engng.*1997, *10:*511-517.
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279P APICAL UPTAKE OF L-DOPA BY L-TYPE AMINO ACID TRANSPORTER IN Caco-2 CELLS

M.B. Sampaio Maia, & P. Soares-da-Silva. Inst. Pharmacol. & Therap., Fac. Medicine, 4200 Porto, Portugal.

There is evidence suggesting that dopamine at the intestinal level may play the role of a local hormone, reducing sodium absorption through inhibition of Na+,K+ ATPase activity and reduce intestinal sodium absorption (Vieira-Coelho et al., 1998). Caco-2 cells, a human epithelial intestinal cell line, were demonstrated to take up L-DOPA and convert it to dopamine, filling some of the criteria to be used as an *in vitro* model of the intestinal dopaminergic system (Vieira-Coelho & Soares-da-Silva, 1998). The aim of the present study was to examine the result of manoeuvres that affect cellular sodium gradients on the apical inward transfer of L-DOPA, the immediate precursor of dopamine, in Caco-2 cells (Vieira-Coelho & Soares-da-Silva, 1998). In an additional set of experiments, 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid (BHC) and N-(methylamino)-isobutyric acid (MeAIB) were used to define the type of amino acid transporter involved in the apical inward transfer of L-DOPA. Caco-2 cells (ATCC CRL 37-HTB; passages 39-49) were grown at 37° C in a humidified atmosphere (5% CO_2) on 2 cm^2 plastic culture clusters or polycarbonate filters in Minimum Essential Medium supplemented with 20% fetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 μg ml^{-1} amphotericin B and 100 μg ml^{-1} streptomycin. After 6 days, the cells formed a monolayer and each 2 cm^2 culture well contained about 100 μg of cell protein; 24 h before the experiments the cell culture medium was changed to a serum free medium. In uptake studies, cells were preincubated (30 min) with Hanks' medium with added tolcapone (1 μM) and benserazide (1 μM). L-DOPA was assayed by h.p.l.c. with electrochemical detection. L-DOPA was applied from the apical cell side at non-saturating (2.5 μM) and saturating (up to 1000 μM) concentrations for 6 min. Results are arithmetic means with s.e.mean, n=4-5. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons. A P value less than 0.05 was assumed to denote a significant difference. L-DOPA uptake was drastically decreased by 2,4-dinitrophenol (91% reduction) and incubation of cells at 4°C (95% reduction). Non-linear analysis of the saturation curve for L-DOPA revealed a K_m value (in μM) of 26±3 and a V_{max} value (in pmol mg $protein^{-1}$ 6 min^{-1}) of 2772±76. Reducing extracellular sodium (from 140 mM to 120, 90, 60, 30 and 0 mM) did not affect the accumulation of L-DOPA (2.5 μM). N-(methylamino)-isobutyric acid (MeAIB; 1 mM) failed to affect the uptake of L-DOPA, whereas 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid (BHC) produced a concentration-dependent inhibition of L-DOPA uptake (IC_{50}=83±1 μM). The inhibitory effect of 1 mM BHC on the accumulation of L-DOPA was of the competitive type, as evidenced by the increase in K_m (78±8) but not V_{max} (2932±94) values for L-DOPA uptake. It is concluded that Caco-2 cells are endowed with the L-type amino acid transporter through which L-DOPA can be taken up.

Vieira-Coelho, M.A. et al. (1998). *Am J Physiol*, **275**, G1317-G1323.
Vieira-Coelho, M.A. & Soares-da-Silva, P. (1998). *Am J Physiol*, **275**, C104-C112

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280P CONTROL OF LIMINAL UPTAKE OF L-DOPA IN HUMAN INTESTINAL EPITHELIAL Caco-2 CELLS BY Ca^{2+}/CALMODULIN-MEDIATED PATHWAYS

M.B. Sampaio Maia, & P. Soares-da-Silva. Inst. Pharmacol. & Therap., Fac. Medicine, 4200 Porto, Portugal.

Dopamine at the intestinal level has been demonstrated to inhibit Na+,K+ ATPase activity and reduce intestinal sodium absorption (Vieira-Coelho et al., 1998). Caco-2 cells, a human epithelial intestinal cell, were demonstrated to take up L-DOPA and convert it to dopamine, filling some of the criteria to be used as an *in vitro* model of the intestinal dopaminergic system (Vieira-Coelho & Soares-da-Silva, 1998). The present study examined the result of manoeuvres that affect molecular mechanisms, namely those concerning protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG), protein tyrosine kinase (PTK) and Ca^{2+}/calmodulin mediated pathways, on the uptake of L-DOPA in Caco-2 (ATCC 37-HTB). Cells (passages 39-49) were grown at 37° C in a humidified atmosphere (5% CO_2) on 2 cm^2 plastic culture clusters or polycarbonate filters in Minimum Essential Medium supplemented with 20% fetal bovine serum and 100 U ml^{-1} penicillin G, 0.25 μg ml^{-1} amphotericin B and 100 μg ml^{-1} streptomycin. After 6 days, the cells formed a monolayer and each 2 cm^2 culture well contained about 100 μg of cell protein; 24 h before the experiments the cell culture medium was changed to a serum free medium. In uptake studies, Caco-2 cells were preincubated (30 min) with Hanks medium with added pargyline (100 μM) and tolcapone (1 μM). L-DOPA and dopamine were assayed by h.p.l.c. with electrochemical detection. Results are arithmetic means with s.e.mean, n=4-5. Statistical differences between experimental groups were determined by ANOVA followed by the Student's t test. Non-linear analysis of the saturation curves for L-DOPA revealed K_m values (in μM) of 26±3 and a V_{max} value (in pmol mg $protein^{-1}$ 6 min^{-1}) of 2772±76; uptake of saturating concentrations of L-DOPA at 4°C was ~ 40% of that occurring at 37°C. Cyclic AMP (0.5 mM), forskolin (50 μM) and cholera toxin (5 μg/ml) failed to affect the accumulation of a non-saturating (2.5 μM) concentration of L-DOPA, whereas isobutylmethylxanthine (0.1 to 1.0 mM) produced a concentration-dependent increase in L-DOPA uptake. Similarly, cyclic GMP (1 mM), zaprinast (30 μM), LY 83583 (30 μM) and sodium nitroprusside (100 μM) failed to affect the accumulation of L-DOPA (2.5 μM). The PKC activator phorbol 12,13-dibutirate (PDBu, 0.1 to 1.0 μM), the inactive phorbol ester 4α-phorbol 12,13-didecanoate (PDDC, 0.1 to 1.0 μM) and the PKC inhibitors chelerythrine (1 μM) bisindolylmaleimide (100μM) also failed to affect the accumulation of L-DOPA (2.5 μM). However, another PKC inhibitor, staurosporine (0.3 to 3 μM) reduced L-DOPA uptake by 86±3%. The PTK inhibitors genistein and tyrphostin 25 failed to change the accumulation of L-DOPA (2.5 μM). The Ca^{2+}/calmodulin inhibitors calmidazolium and trifluoperazine produced concentration-dependent inhibition of L-DOPA (2.5 μM) uptake with IC_{50} 's of 57±2 and 245±2 μM, respectively. The inhibitory effect of calmidazolium (50 μM) and trifluoperazine (300 μM) on the accumulation of L-DOPA was of the non-competitive type, as evidenced by the decrease in V_{max} (1987±52 and 1432±66 pmol mg $protein^{-1}$ 6 min^{-1}), but not K_m (35±3 and 21±4 μM) values for L-DOPA uptake. It is concluded that L-DOPA uptake in Caco-2 cells is a carrier-mediated system that is temperature dependent and appears to be under the control of Ca^{2+}/calmodulin mediated pathways.

Vieira-Coelho, M.A. et al. (1998). *Am J Physiol*, **275**, G1317-G1323.
Vieira-Coelho, M.A. & Soares-da-Silva, P. (1998). *Am J Physiol*, **275**, C104-C112
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281P EFFECT OF STABLE TRANSFECTION OF PRESENILINS ON K⁺ CHANNEL ACTIVITY IN HEK-293 CELL LINE

N Hipkins, J Leafe, M Ramsden, N Hooper* and HA Pearson,
Schools of Biomedical Sciences and *Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT

Mutations in the presenilins (PS) family of proteins are believed to give rise to some forms of familial Alzheimer's disease (AD). Transient transfection of HEK 293 cells with presenilins have been suggested to increase endogenous K^+ channel activity (Malin et al, 1998). The aim of experiments reported here was to determine the effect of stable transfection of presenilins on voltage gated K^+ channel activity in the same cell line.

HEK 293 cells, stably transfected with wild type and mutant presenilins were grown according to the method of Fearon et al (1997). K^+ channel currents were recorded using the whole-cell patch clamp technique as previously described (Gibb et al, 1998). All data are given as means ± SEM. Statistical analyses were carried out using Student's unpaired t-test.

Untransfected HEK 293 cells depolarised from a prepulse potential of −140mV to a test potential of +50mV exibited a small endogenous K^+ channel current with characteristics similar to a delayed rectifier. Mean current density in these cells was 13±2pA/pF (n=32). In contrast, cells transfected with PS-1 had a significantly larger mean current density of 20±2pA/pF (n=29, $p<0.05$). A similarly large current was seen in cells transfected with PS-1 containing an exon 9 deletion mutation common to a Finnish AD lineage (Finn, 24±3pA/pF, $p<0.001$, n=26). No differences were seen between currents in PS-1 or Finn transfected cells. There were no significant differences in resting cell conductance between the transfected and untransfected cell lines, indicating that changes were not due to alterations in non-specific leak channels. Currents in untransfected, PS-1 transfected and Finn transfected cells all showed similar activation and inactivation properties and a similar sensitivity to block by tetraethylammonium, suggesting that endogenous current activity was affected.

In a separate set of experiments K^+ channel currents in cell lines transfected with either PS-2 or PS-2 with a missense mutation associated with familial AD (VG) were measured. In contrast to results with the PS-1 transfected cells no differences in the voltage gated K^+ channel current between the transfected cells and the wild type, untransfected cells were observed (n=19, 33 and 35 for untransfected, PS-2 and VG transfected cells respectively). Similarly, no effect was observed on resting cell conductance.

These data indicate that PS-1 may be involved in K^+ channel expression or trafficking in human cells and broadly agrees with earlier studies in transiently transfected cells. The ten-fold change in current density seen in transiently transfected cells compared to the two-fold change observed in stably transfected cells may represent differences in either PS expression or proteolytic processing of PS.

Fearon IM et al (1997). J. Physiol. 500, :551-6
Gibb, SG et al (1998). Br. J. Pharmacol. 123, 222P
Malin, SA et al (1998). Neurobiol. Diseases 4, 398-409

282P DIFFERENTIAL EFFECTS OF ROPINIROLE, BROMOCRIPTINE AND L-DOPA ON STRIATAL PREPRODYNORPHIN MRNA EXPRESSION IN THE MPTP-TREATED MARMOSET

B.C. Tel, C. Cannizzaro, R.K.B. Pearce, S. Rose & P. Jenner
Neurodegenerative Disease Research Centre, Pharmacology Group, Biomedical Sciences Division, King's College, London SW3 6LX

L-DOPA treatment of Parkinson's disease induces dyskinesia which is associated with an imbalance between striatal output pathways (Bedard *et al.*, 1992). In MPTP-treated common marmosets, L-DOPA, bromocriptine and ropinirole all improve motor function, but L-DOPA rapidly induces marked dyskinesia, whereas bromocriptine induces mild dyskinesia and ropinirole produces only intermittent involuntary movements (Pearce *et al.*, 1998). In the present study, we report the effect of these drugs on the striatal direct and indirect output pathways by measuring the expression of striatal preprodynorphin (PPE-B) (for direct) and adenosine A2a receptor (for indirect) mRNA by *in situ* hybridisation immunohistochemistry.

Adult common marmosets (n=16, 280-360 g, Callithrix jacchus, either sex) were treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropridine hydrochloride) 2 mg/kg (s.c.) once daily for 5 days. Animals were divided into 4 groups and received 10% sucrose solution, bromocriptine (0.5 mg/kg, p.o.), L-DOPA plus carbidopa (12.5 mg/kg plus 12.5 mg/kg, p.o.) or ropinirole (0.3-0.5 mg/kg, p.o.) once daily for 4 weeks. A further 4 animals were used as naive controls. At the end of the study, brains were removed under terminal anaesthesia and flash-frozen. Coronal sections (20 µm) were incubated with ^{35}S-labelled oligonucleotide probes for human PPE-B (Telkov *et al.*, 1998) and A2a receptor (Furlong *et al.*, 1992). Non-specific hybridisation was carried out in excess (100x) unlabelled oligonucleotides. Quantitative evaluation of autoradiograms was undertaken by computerised densitometry (MCID, Imaging Research Inc.) and results were analysed by one way ANOVA followed by *post hoc* Dunnett's test.

PPE-B mRNA expression was decreased in the nucleus accumbens, caudate and putamen in MPTP-treated animals. Bromocriptine and ropinirole did not normalise levels of PPE-B mRNA in the nucleus accumbens, caudate or putamen. In contrast, L-DOPA reversed the decrease in PPE-B mRNA in the caudate nucleus and putamen compared with MPTP-treated marmosets. MPTP-treatment had no effect on A2a mRNA levels in the caudate and putamen and none of the drug treatments have any effect on A2a mRNA expression.

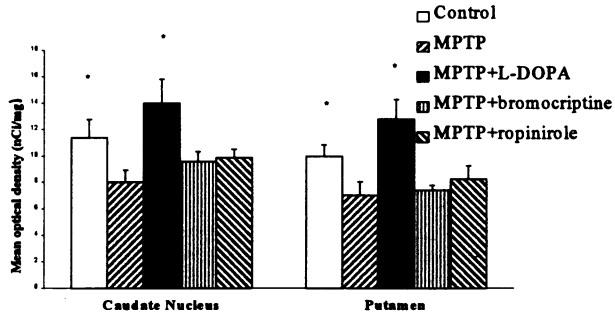


Figure 1. PPE-B mRNA expression, +P<0.05 vs MPTP-treatment (Mean±SEM).

Previously we have shown that chronic L-DOPA but not bromocriptine or ropinirole treatment normalises the decrease in PPT mRNA (direct pathway) but fails to attenuate the elevated PPE-A mRNA (indirect pathway) following MPTP-treatment (Tel *et al.*, 1997). In this study, L-DOPA reversed the decrease in PPE-B mRNA whereas bromocriptine and ropinirole did not. The patterns of alteration in striatal neuropeptide expressions produced by L-DOPA, bromocriptine and ropinirole reflect differential improvement of locomotor activity and appearance of dyskinesia. This suggests that the agonists acting on the indirect pathway may be associated with a lower incidence of dyskinesia.

Bedard, P.J. *et al.*, (1992) *Can. J. Neurol. Sci.* 19:134-137.
Furlong, T.J *et al.*, (1992) *Mol. Brain. Res.* 15:62-65.
Pearce, R.K.B., Banerji, T., Jenner, P. & Marsden, C.D. (1998) *Mov. Disord.* 13:234-241.
Tel, B.C., Zeng, B.-Y., Pearce, R.K.B, *et al.*, (1997). *Br. J. Pharmacol.* 122(SS):161.
Telkov, M., Geijer, T. & Terenius, L. (1998) *Brain Res.* 804:284-295.

283P ANTIPARKINSONIAN POTENTIAL OF THE GROUP II mGluR AGONIST, DCG-IV, FOLLOWING INTRAVENTRICULAR ADMINISTRATION IN THE RESERPINE-TREATED RAT MODEL OF PARKINSON'S DISEASE

L. Dawson, M. Megalou & S. Duty. Neurodegenerative Disease Research Centre, Kings College London, London SW3 6LX.

Overactivity of the subthalamic nucleus (STN) resulting in excess glutamate release in the substantia nigra pars reticulata (SNr) contributes to the symptomatology of Parkinson's disease (PD). Stimulation of Group II metabotropic glutamate receptors (Gp II mGluRs) on STN terminals, which reportedly act as autoreceptors (Shigemoto *et al.*, 1997), may help to reduce this excess glutamate release and hence relieve symptoms. In support of this, we have shown that direct injection of the Gp II mGluR selective agonist, (2S,1R,2R,3R)-2-(2,3-dicarboxycyclopropyl) glycine (DCG-IV), into the SNr reverses the akinesia seen in the reserpine-treated rodent model of PD (Dawson *et al.*, 1999). In the present study, we examined whether DCG-IV displayed similar antiparkinsonian activity when administered via intraventricular injection.

Under general anaesthesia (60 mg / kg i.p. Sagatal), male Sprague Dawley rats (250-270g) were stereotaxically implanted with 23-gauge guide cannulae above the third ventricle. Five days later, rats were treated with reserpine (5 mg / kg s.c.). After 18 hours, when a stable akinesia was attained, animals were placed in rectangular cages, with 5 cm square grids covering the base. Baseline locomotor activity was recorded for 30 min prior to intraventricular injection of DCG-IV (0.125 to 1.5 nmoles in 2 µl PBS, pH 7.0). Locomotor activity, measured over 60 min in 5 min time bins, was scored in arbitrary locomotor units (ALUs), where 1 ALU represented the animals front paws crossing a grid line. Examination of the time-course of action of DCG-IV led to subsequent evaluation of the total locomotor score over the first 30 min. The effects of different doses were compared using a 1-way ANOVA with a Student Newman Keuls post-hoc analysis (significance level, $p<0.05$). To confirm receptor specificity, rats were treated with the Gp II selective antagonist, (2S)-α-ethylglutamic acid (EGLU; 400 nmole / 2 µl) or vehicle (0.3 mM NaOH in PBS) 6 hours after receiving an initial dose of DCG-IV (0.5 nmoles). After 30 min equilibration, DCG-IV (0.5 nmoles) was re-administered and locomotor scores / 30 min compared pre- and post- EGLU/vehicle using a 2-tailed t-test ($p<0.05$).

DCG-IV (≥0.25 nmoles) significantly increased locomotor activity within 5 min of administration. The response returned to baseline levels within 30 min. Over 30 min duration, DCG-IV produced a dose-dependent increase in locomotor activity (Figure 1.). EGLU (400 nmoles) significantly inhibited the response to DCG-IV (0.5 nmoles) by 71.4 ± 10.6 % (mean ± S.E.M.) confirming Gp II mGluR specificity.

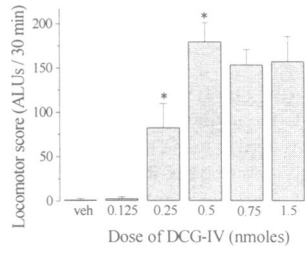


Figure1. Locomotor activity produced by intraventricular administration of DCG-IV or vehicle (veh) in the reserpine-treated rat. Values are mean ± S.E.M. (n=6-8 animals per dose). *Significantly different to previous dose.

In conclusion, DCG-IV retains its antiparkinsonian potential when given via intraventricular injection. Since site-directed targeting of Gp II mGluRs is not necessary, future systemically-active Gp II mGluR agonists may prove useful in the treatment of PD.

The authors wish to thank the Nuffield Foundation for support.

Dawson L., Chadha A. & Duty S. (1999). *Br. J. Pharmacol.*, 126, 60P.
Shigemoto R., Kinoshita A., Wada E. *et al.*, (1997). *J. Neurosci.*, 17, 7503-7522

284P PRIMARY HUMAN ASTROCYTES POSSESS FUNCTIONAL BOMBESIN RECEPTORS

S.McNulty, K.Lee, R.D.Pinnock and S.Mason Parke Davis Neuroscience Research Centre, Cambridge University Forvie Site, Cambridge, CB2 2QB.

Bombesin has been shown to bind to primary cultures of rat astrocytes causing membrane hyperpolarization and increased levels of intracellular free calcium ($[Ca^{2+}]_i$, Hosli *et al.* 1993, Enkvist *et al.* 1989). In the present study, we used imaging and electrophysiological techniques to characterise the effects of peptides from the bombesin family on human astrocytes.

Human astrocytes were obtained and maintained in culture as directed (BioWhittaker Inc., Maryland, USA). For population studies of intracellular free calcium, cells were grown on 96 well plates ($3x10^4$cells/well) and loaded with Fluo 3 for 1 hour prior to analysis using FLIPR (Molecular Devices, California, USA). For single cell imaging experiments, cells were cultured on 22mm coverslips and loaded with 2µM Fura-2AM in physiological saline containing (mM) 135.0 NaCl, 5 KCl, 1.0 MgCl 1.0 $CaCl_2$, 10.0 HEPES, 3.0 glucose, pH 7.3 for 1 hour. Image acquisition and analysis were undertaken using the Merlin analysis system (LSR Inc., Cambridge, UK). For electrophysiological studies, the whole cell configuration of the patch clamp technique was employed. Cells were visually identified and bathed in physiological saline whilst the intracellular (pipette) solution comprised (mM) 120.0 Kgluconate, 10.0 NaCl, 2.0 $MgCl_2$, 0.5 K_2EGTA, 10.0 HEPES, 4.0 Na_2ATP, 0.1 Na_2GTP, pH 7.2.

Population studies performed on astrocytes showed the BB_2-selective agonist neuromedin C (NMC), to increase basal $[Ca^{2+}]_i$ with a 4-fold higher potency (pEC_{50} 7.60±0.11; n=3) than that observed with the BB_1-selective agonist neuromedin B (NMB, pEC_{50} 6.97±0.14; n=3). Addition of the BB_2 receptor antagonist/BB_1 partial agonist, [D-Phe^6] bombesin (6-13) methylester alone was without effect suggesting the absence of BB_1 receptors on these cells. Pre-incubation with either a single (100nM) or full range (0.1nM-30µM) of concentrations of this compound caused a rightward shift of the NMC dose-response curve (pK_b=7.46±0.04; n=3) and full inhibition of a sub-maximal (100nM) dose of NMC (pK_i=7.74±0.06; n=3). Hill coefficient values (1.06±0.04) were close to unity indicating the presence of a homogeneous receptor pool.

Analysis of the $[Ca^{2+}]_i$ of individual astrocytes revealed that treatment with NMC (BB_2 selective, 10nM) or with NMB (10nM) increased $[Ca^{2+}]_i$ in 96±2% (n=8 experiments) and in 78±4% (n=5 experiments) of cells respectively. The mean increases of 340:380 ratio units (RU) observed in responding cells were 0.37±0.01 (n=161) for NMC and 0.26±0.01 (n=45) for NMB. Removal of extracellular calcium did not prevent the response to NMC 0.24±0.01 RU, (n=50) or to NMB 0.29±0.01 RU, (n=53). Co-treatment of cells with NMC (10nM) and the phospholipase C inhibitor U73122 (1µM) significantly inhibited the increase in $[Ca^{2+}]_i$ 0.05±0.01 RU, (n=79) compared to cells treated with NMC alone ($p<0.001$). Simultaneous treatment of cells with NMC (10nM) and the antagonist [D-Phe^6] bombesin (6-13) methylester (100nM) inhibited the increase in $[Ca^{2+}]_i$ 0.05±0.02 RU, (n=49) compared to cells treated with NMC alone, ($p<0.001$). A recovery period of 15 min, prior to treatment with NMC alone enabled a significant recovery in the calcium response to occur 0.23±0.04 RU, (n=49) ($p<0.001$).

In whole-cell current clamp recordings, bath application of the BB_2 selective agonist gastrin-releasing peptide GRP (20nM) produced a hyperpolarisation of 32.3 ± 7.2 mV from an initial resting potential of -51.2 ± 3.2 mV that was poorly reversible in nature (n=8). In whole-cell voltage clamp recordings, 20nM GRP induced an outward current of 225.3 ± 16.5pA at -60mV (n=7) which had an estimated reversal potential of -82.2 ± 2.1mV (n=5). The magnitude of this current was significantly and reversibly reduced by the addition of 2mM $BaCl_2$ to the bath solution (by 92.3 ± 4.5 % (n=4), $p<0.001$).

In conclusion, we have demonstrated that activation of bombesin receptors on primary cultures of human astrocytes causes hyperpolarisation of membrane potential and increased mobilisation of calcium from intracellular stores.

Hosli L, Hosli E, Winter T, Kaser H (1993) *Neuroscience Letters.* 163. 145-147.
Enkvist MOK, Holopainen I, Akerman KEO (1989) *Brain Research.* 500. 46-54

285P THE VSV ISOFORM OF THE HUMAN 5-HT$_{2C}$ RECEPTOR COUPLES TO ENDOGENOUS G-PROTEINS IN CHO CELLS

K. Quirk, A. Misra, V. Harvey and A. Knight. Cerebrus, Oakdene Court, 613 Reading Road, Winnersh, Wokingham, RG41 5UA.

The 5-HT$_{2C}$ receptor is expressed in different isoforms as a result of mRNA editing. Both INI (unedited) and VSV (edited) isoforms are abundant in the rat brain (Niswender et al., 1998). The VSV isoform lacks the high affinity recognition site for 5-HT, this may be caused by a low efficiency of coupling to G-proteins (Colleen et al., 1999). We have investigated agonist stimulated [^{35}S]GTPγS binding in Chinese Hamster Ovary (CHO) cells transfected with 5-HT$_{2C\text{-}VSV}$ as a measure of G-protein activation.

CHO cells stably transfected with 5-HT$_{2C}$ receptors were obtained from Euroscreen, Belgium (Expression level: 13.8±1.2 pmoles per mg protein). [^{35}S]GTPγS binding was carried out using the method described by Lazareno and Birdsall (1993). Relative efficacy was calculated as a percentage of the response elicited by 10μM 5-HT. Compounds were tested for their ability to displace [^{3}H]5-HT binding in the same cell line. Cell membranes were labelled with 5nM [^{3}H]5-HT. Assays were incubated for 1 hour at 37°C in 50mM Tris, 0.1% ascorbate before rapid filtration.

Results are shown in Table 1. The EC$_{50}$ values obtained for agonists are 10 fold lower than the affinity of the compounds for the 5-HT binding site of the receptor. The relative efficacies obtained are consistent with the known literature for 5-HT$_{2C}$ agonists. These results suggest that the VSV isoform of the 5-HT$_{2C}$ receptor couples functionally to endogenous G-proteins and confirms that this coupling is relatively inefficient.

Colleen, M. et al., (1999) *J. Biol. Chem* **274**, 14 9472-9478

Lazareno, S. et al., (1993) *Br.J. Pharmacol* **109**, 1120

Niswender, C.M. et al., (1998) *Annals NY Acad Sci* **861**, 38-48

Table 1: Potency of agonists in [^{35}S]GTPγS binding assay and affinity for 5-HT recognition site (n=3 ± SEM)

Compound	EC$_{50}$ (nM)	Relative Efficacy %	Ki (nM)
5-HT	84.1±9.6	100	18.1±1.49
mCPP	408±59	74.4±6.5	40.1±5.27
TFMPP	397±68	64.9±7.8	41.7±5.1
MK-212	4000±1440	97.2±2.3	262±72.1
Ro 600175	217±41	91.8±4.1	12.3±2.9
RU 24969	4570±897	56.2±1.3	307±84.4
DOI	124±79	56.5±5.5	12.9±1.23
DOB	169±12	67.4±5.2	22.9±2.9
Tryptamine	864±96	87.2±1.5	57.8±1.1
8-OH-DPAT	63700±5670	31.0±0.9	13700±5030

286P [^{3}H]-SB-269970 RADIOLABELS 5-HT$_7$ RECEPTORS IN GUINEA-PIG CEREBRAL CORTEX MEMBRANES

P.J. Atkinson, D.R. Thomas, M. Ho, *S.M. Bromidge, J.J. Hagan, D.N. Middlemiss and G.W. Price. Departments of Neuroscience Research and *Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, Harlow, CM19 5AW, U.K.

5-HT$_7$ receptors have been shown to be present in brain, particularly in thalamic and limbic areas (e.g. To *et al.*, 1995). The majority of 5-HT$_7$ receptor binding studies in brain tissue have used the 5-HT$_7$ agonist radioligand [^{3}H]-5-CT. However, since [^{3}H]-5-CT also displays high affinity for 5-HT$_{1A/B/D}$ receptors, blocking drugs must be included to inhibit binding to these non-5-HT$_7$ sites. There is, therefore, a need for a selective 5-HT$_7$ receptor radioligand. [^{3}H]-SB-269970 is a selective, high affinity antagonist radioligand for the human cloned 5-HT$_{7(a)}$ receptor (Price *et al.*, this meeting). In the present study, [^{3}H]-SB-269970 has been used to characterise 5-HT$_7$ receptors in guinea pig cerebral cortex. The profile of binding for [^{3}H]-SB-269970 has been compared with that for [^{3}H]-5-CT.

Radioligand binding to guinea pig cerebral cortex membranes was carried out according to the method of Boyland *et al.*, (1996) using 0.5nM [^{3}H]-5-CT or 1nM [^{3}H]-SB-269970. For [^{3}H]-5-CT binding, WAY100635 (1μM) and GR127935 (10μM) were included to inhibit binding to 5-HT$_{1A}$ and 5-HT$_{1B/1D}$ receptors respectively.

[^{3}H]-SB-269970 (0.1 - 10nM) bound saturably to a single population of receptors in guinea pig cortex membranes. Specific binding defined by the presence of 10μM 5-HT represented 50-60% of total binding. The K$_d$ of 1.7 ± 0.3nM was similar to that determined at the human cloned 5-HT$_{7(a)}$ receptor (1.3 ± 0.1nM).The B$_{max}$ for [^{3}H]-SB-269970 binding to guinea pig cortex membranes was similar to that determined for [^{3}H]-5-CT (125 ± 8.2 and 143 ± 19 fmoles mg^{-1} protein respectively), consistent with both radioligands labelling the same receptor population and suggesting that, as seen for the human cloned 5-HT$_{7(a)}$ receptor, most sites labelled appear to be in the agonist high affinity state. The profile of inhibition of [^{3}H]-SB-269970 (1nM) binding by a range of 5-HT$_7$ receptor agonists and antagonists correlated well with that for [^{3}H]-5-CT (0.5nM) binding (correlation coefficient 0.98) (Table 1). Hill slopes for drug inhibition of both [^{3}H]-SB-269970 and [^{3}H]-5-CT binding were generally close to 1 (data not shown), consistent with binding to a single population of receptors. The overall pharmacological profile was therefore consistent with specific binding to 5-HT$_7$ receptors in guinea pig cerebral cortex membranes.

Table 1. Inhibition of [^{3}H]-5-CT and [^{3}H]-SB-269970 binding to guinea pig cortex membranes

	pK$_i$ (± s.e.mean; n ≥3)	
	[^{3}H]-5-CT	[^{3}H]-SB-269970
5-CT	8.80 ± 0.17	8.79 ± 0.15
SB-269970-A	8.29 ± 0.21	8.74 ± 0.06
5-HT	7.98 ± 0.18	8.25 ± 0.22
Methiothepin	7.40 ± 0.06	7.67 ± 0.13
SB-258719	7.15 ± 0.08	7.56 ± 0.17
Mesulergine	6.85 ± 0.18	7.26 ± 0.22
Clozapine	6.50 ± 0.10	6.94 ± 0.05
8-OH-DPAT	6.57 ± 0.18	6.79 ± 0.14
Sumatriptan	6.19 ± 0.27	5.89 ± 0.04

It can be concluded that [^{3}H]-SB-269970 selectively labels 5-HT$_7$ receptors in guinea pig cerebral cortex and should prove valuable for further studies of 5-HT$_7$ receptors in recombinant and native tissues.

Boyland, P.S., *et al.*, (1996) *Br. J. Pharmacol.*, 117, 132P.
Price, G.W. *et al.*, (1999) *Br. J. Pharmacol* (this meeting).
To, Z.P., *et al.*, (1995) *Br. J. Pharmacol.*, 115, 107-116.

287P RAPID DESENSITISATION OF HUMAN 5-HT$_{2B}$ RECEPTORS MEASURED BY FLUORIMETRY: COMPARISON WITH HUMAN 5-HT$_{2A}$ AND 5-HT$_{2C}$ VSV AND INI RECEPTOR ISOFORMS

Malcolm C.S., Allen N.H., Lamb H., Revell D. and Porter R.H.P. Cerebrus, Oakdene Court, 613 Reading Road, Winnersh, Wokingham, RG41 5UA

This study examined the desensitisation characteristics of human 5-HT$_2$ (h5-HT$_2$) receptor subtypes stably expressed in Chinese Hamster Ovary (CHO-K1) cells. 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors have previously been reported to show agonist mediated desensitisation (Roth *et al.*, 1995; Akiyoshi *et al.*, 1995). We therefore compared the desensitisation characteristics of the h5-HT$_{2B}$ receptor to the h5-HT$_{2A}$ and both edited and unedited variants of the h5-HT$_{2C}$ receptor.
A fluorometric imaging plate reader (FLIPR^{TM}) was used to measure 5-HT induced calcium responses following pre-incubation with 5-HT. The h5-HT$_{2B}$ receptor was most sensitive to desensitisation mediated by pre-exposure to 10μM 5-HT reducing the maximum calcium response to approximately 20% of control values with a $T_{1/2} < 5$ min. The h5-HT$_{2A}$ and h5-HT$_{2C}$ (VSV isoform) exhibited broadly similar desensitisation characteristics with pre-exposure to 10μM 5-HT resulting in a reduction of the response elicited by a subsequent challenge to 50-60% of control. Unedited h5-HT$_{2C}$ receptors (INI isoform) displayed slightly greater levels of desensitisation to approx. 30-40% of control levels.
We also compared the effects of pre-incubating all 5-HT$_2$ receptor subtypes with varying concentrations of 5-HT for 1 hour prior to performing subsequent concentration response curves to 5-HT (0.01nM - 10μM). Pre-exposure to 5-HT for 1 hour caused a concentration-dependent decrease in agonist potency and a decrease in the maximal response measured.

The most dramatic levels of desensitisation were observed with the human 5-HT$_{2B}$ receptor subtype where pre-exposure to 10μM 5-HT reduced the subsequent maximal response by approximately 80%

TABLE 1

Receptor	Pre-treatment	EC_{50} (nM)	R.E.
5-HT$_{2A}$	buffer	27.1	100.8
	10^{-8} M 5-HT	30.6	97.1
	10^{-5} M 5-HT	154.2	62.5
5-HT$_{2B}$	buffer	1.1	101.2
	10^{-8} M 5-HT	3.0	78.1
	10^{-5} M 5-HT	12.8	20.4
5-HT$_{2C\ (VSV)}$	buffer	2.9	100.6
	10^{-8} M 5-HT	6.0	95.9
	10^{-5} M 5-HT	49.5	47.5
5-HT$_{2C\ (INI)}$	buffer	2.9	101.4
	10^{-8} M 5-HT	5.2	91.5
	10^{-5} M 5-HT	15.8	35.8

This study is the first direct demonstration of 5HT$_{2B}$ receptor desensitisation and the first study to compare the desensitisation characteristics of differentially edited isoforms of the human 5HT$_{2C}$ receptor.

Akiyoshi J., Nishizono A., Yamada K. *et al.* (1995) *J. Neurochem.* **64**, 2473-2479.
Roth B.L., Palvimaki E.P., Berry S. *et al.* (1995) *J. Pharmacol. Exp. Ther.* **275**, 1638-1646.

288P ENTACAPONE, A PERIPHERAL COMT INHIBITOR, IMPROVES THE CENTRAL BIOAVAILABILITY OF L-DOPA AND ITS ABILITY TO INCREASE DOPAMINE LEVELS IN RAT BRAIN

E. MacDonald[1], T. Viitamaa[2], I.-B. Linden[2], A. Haapalinna[2], & P. Männistö[1], [1]Dept. Pharmacol. & Toxicol., Univ. Kuopio, PL 1627, 70211 Kuopio, Finland, [2]Orion Corporation - Orion Pharma, Finland.

Entacapone (ENT) is a novel catechol-O-methyltransferase inhibitor (COMT-I) (Kaakkola et al., 1994). Its effects are restricted primarily to the periphery as ENT does not gain access to the brain. Since it is intended as a supplement to regular Parkinson therapy, we decided to examine its effects in rats being dosed with benserazide (BENS) (5 or 10 mg/kg) and L-DOPA (10 mg/kg). In particular we were interested in evaluating whether these drug combinations would inhibit aromatic amino acid decarboxylase (AADC), the enzyme which converts L-DOPA to dopamine (DA). To assess the activity of AADC in the brain, we measured the content of 5-hydroxytryptophan (5-HTP), since AADC inhibition in the brain is reflected as an increase in 5-HTP levels from their normally almost undetectable levels.

A total of 36 male Sprague-Dawley rats (219 - 250 g) were divided into six groups. They were fasted overnight and given the following drugs per os (p.o); group 1= control (carboxy-methylcellulose 2%); group 2 = L-DOPA (10 mg/kg) group 3 = BENS (5 mg/kg) + L-DOPA (10 mg/kg); group 4 = BENS (10 mg/kg) + L-DOPA (10 mg/kg); group 5 = ENT (10 mg/kg) + BENS (5 mg/kg) + L-DOPA (10 mg/kg); group 6 = ENT (10 mg/kg)+ BENS (10mg/kg) + L-DOPA (10 mg/kg). The drugs were given in rapid succession, each rat received a volume of 3 x 2 ml/kg. Two hours later the rats were decapitated and whole brain dissected and stored at -80°C prior to HPLC with electrochemical detection analysis of biogenic amines and their metabolites as well as brain levels of DOPA and its metabolite o-methyldopa (OMD). Statistical analysis was ANOVA followed by Scheffe's test with $P < 0.05$ considered significant.

L-DOPA caused a slight but non-significant change in brain 5-HTP levels. However, in both groups (i.e. 3 & 4) treated with BENS + L-DOPA, we detected highly significant ($P<0.01$) increases in 5-HTP concentrations. Supplementation with ENT (groups 5 & 6) prevented this increase (Table 1). Since none of the groups showed any change in 5-hydroxyindoleacetic acid (5-HIAA) levels, we postulate that the increase in 5-HTP concentrations represents blockade of synthesis at the AADC stage, rather than elevated turnover. ENT also prevented the increase in OMD levels seen in the two groups treated with BENS + L-DOPA (groups 3 & 4) but only in the group 6 (ENT + BENS/10 + L-DOPA) was the increase in brain DOPA concentrations significant ($P<0.01$). Dopamine (DA) levels were also only elevated ($P<0.01$) in the two groups of rats treated with ENT.

Table 1: Concentrations in nmol/g (± s.e. mean) of 5-HTP, 5-HIAA, DOPA, OMD and DA in rat brain 2h after injection

Group	5-HTP	5-HIAA	DOPA	OMD	DA
1	0.02±.00	5.7±0.1	ND	ND	5.3±0.1
2	0.06±.03	5.5±0.1	0.1±0.0	1.2±0.3	5.4±0.2
3	0.18±.01*	6.4±0.4	1.1±0.2	13.4±1.0#	6.2±0.2
4	0.21±.01*	5.7±0.3	1.9±0.5	14.5±0.8#	6.3±0.3
5	0.06±.01	6.3±0.3	3.8±0.7	2.7±0.5	7.5±0.4*
6	0.08±.01	6.2±0.1	10.2±2.1#	3.3±0.3	8.8±0.4*

ND=nondetectable (* $P<0.01$ vs group 1; # $P<0.01$ vs group 2)

In conclusion, we propose that ENT, by virtue of its COMT-I properties, may be a valuable supplement to conventional Parkinson therapy (BENS + L-DOPA). The methylated metabolite of DOPA, OMD, blocks AADC in serotonergic nerves. Since the same enzyme is responsible for decarboxylation of DOPA in DA nerves, any lessening of OMD formation would be predicted to be therapeutically valuable.

Kaakkola, S., Gordin, A. & Männistö, P.T. (1994) *Gen Pharmacol.* 25:813-824.

289P PHARMACOLOGY OF HUMAN RECOMBINANT ADENOSINE A_{2A} RECEPTORS AND RAT STRIATAL A_{2A} RECEPTORS: POSSIBLE SPECIES DIFFERENCES

V. Harvey, J. Jones, A. Misra, K. Quirk, M. Sheardown, S Weiss and A.R. Knight. Cerebrus Ltd, Oakdene Court, 613 Reading Road, Winnersh, Wokingham, RG41 5UA.

The neuromodulatory actions of adenosine are mediated in part by the adenosine A_{2A} receptor. These receptors are enriched in striatal tissue, and can be specifically labelled by the agonist [^{3}H]-CGS-21680 (Jarvis et al., 1989) and the antagonist [^{3}H]-ZM-241385 (Alexander, 1999). In the present study we have investigated the pharmacology of these binding sites in rat striatal tissue and HEK-293 cells expressing cloned human adenosine A_{2A} receptors.

Binding assays were carried out by the method of Jarvis et al. (1989). Membranes from HEK-293 cells expressing adenosine A_{2A} receptors were obtained from Receptor Biology Inc. Rat striatal membranes were treated with 2 IU/ml adenosine deaminase (ADA) for 30 min at 37 °C prior to assay. Receptors were labelled with [^{3}H]-CGS-21680 (18 nM) or [^{3}H]-ZM-241385 (5nM), in 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 0.1 IU/ml ADA. All data are the mean of three or more independent experiments.

Results are shown in Table 1. Antagonists displaced [^{3}H]-CGS-21680 and [^{3}H]-ZM-241385 with similar potency in both receptor preparations. Agonists were 8 to 14 fold less potent as displacers of the antagonist, [^{3}H]-ZM-241385.

Some xanthines were less potent in displacing binding to rat striatal receptors than to human receptors (both [^{3}H]-CGS-21680 and [^{3}H]-ZM-241385 assays). These were: DPCPX (9.5 and 10.6 fold less potent, respectively); theophylline (7.3 and 7.7 fold) and caffeine (6.3 and 3.3 fold). This shift in affinity may indicate a species difference in A_{2A} receptors.

Alexander, S.P.H. (1999) *Br. J. Pharmacol.* **120** 27P
Jarvis, M.F. et al. (1989) *J. Pharmacol. Exp. Ther.* **251** 888-893
Ongini, E. & Fredholm, B.B. (1996) *TiPS* **17** 364-372
Shimada, J. et al. (1997) *Bioorg. & Med. Chem. Lett.*7 (18) 2349-2352

Table 1. K_i values (nM) for compounds as displacers of [^{3}H]-CGS-21680 and [^{3}H]-ZM-241385 (mean ± s.e. mean, n>3)

	[^{3}H]-CGS-21680 Human	[^{3}H]-CGS-21680 Rat Striatum	[^{3}H]-ZM-241385 Human	[^{3}H]-ZM-241385 Rat striatum
CGS-21680	64 ± 8	53 ± 14	508 ± 143	493 ± 90
NECA	21.1 ± 7.1	37.4 ± 5.9	298 ± 74	306 ± 61
KW-6002	36.5 ± 6.9	35 ± 3.9	34.7 ± 9	38.8 ± 8
ZM-241385	2.37 ± 0.4	1.67 ± 3.9	1.08 ± 0.6	1.11 ± 0.7
DPCPX	250 ± 54	2385 ± 727	216 ± 30	2290 ± 393
Theophylline	2920 ± 716	21400 ± 2672	7190 ± 1880	54600 ± 9080
Caffeine	4350 ± 642	27600 ± 10825	2929 ± 900	9770 ± 875

290P THE EFFECT OF CANNABINOID LIGANDS ON [^{35}S]GTPγS BINDING IN SPINAL CORD SLICES OF NON-INFLAMED AND INFLAMED RATS

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

Cannabinoid CB_1 receptors are present in the dorsal horn of the spinal cord (Tsou *et al.*, 1998), a location which suggests that this receptor may modulate nociceptive transmission. Behavioural and electrophysiological studies have shown cannabinoid agonists to be antinociceptive (Martin & Lichtman, 1998). Alterations in spinal CB_1 receptor activity may contribute to phenomena such as hyperalgesia that is manifested in some chronic pain states. Here we have used [^{35}S]GTPγS autoradiography to study cannabinoid receptor coupling to G-proteins in the spinal cord of untreated rats and those with a local inflammation following intraplantar injection of carrageenan.

Sprague-Dawley rats were stunned and decapitated 3h after being briefly (5 min) anaesthetised with 3% halothane in 66% N_2O / 33% O_2 and injected with either 100µl of 0.9% w/v NaCl solution (n=4) or 100µl of 2% λ-carrageenan in saline (n=4) into the plantar surface of the left hindpaw. A third experimental group received no anaesthetic or injection (n=4). The spinal cord incorporating the lumbar enlargement was rapidly removed from each animal and freeze-mounted in OCT compound using cryospray. Transverse sections of spinal cord (20 µm) were cut on a cryostat at –30°C and thaw-mounted onto gelatin coated microscope slides. Spinal cord slices in each treatment group were assessed for agonist-stimulated [^{35}S]GTPγS binding (Sim *et al.*, 1995) using the cannabinoid receptor agonist, HU210 (1µM). Antagonism of HU210-stimulated [^{35}S]GTPγS binding by the CB_1 receptor antagonist SR141716A (1µM; Rinaldi-Carmona *et al.*, 1994) was also studied. The slides plus a ^{14}C standards strip were exposed to Hyperfilm-βmax (Amersham) for 4-5 days. Autoradiograms were digitised and quantified densiometrically using the National Institutes of Health IMAGE software. Data are presented as mean ± s.e.mean; statistical analysis was performed using ANOVA.

HU210 (1µM) produced an increase in [^{35}S]GTPγS binding in spinal cord slices from untreated, saline-injected and carrageenin-injected rats; in the superficial dorsal horn levels were increased to 116±7% ($p<0.05$), 126±7% ($p<0.01$) and 119±9% of basal binding respectively. In addition, [^{35}S]GTPγS binding was increased in the remainder of the grey matter, where corresponding values were 117±6%, 138±7% ($p<0.001$) and 125±9% ($p<0.05$) of basal binding. Within each treatment group there was no difference in HU210-stimulated [^{35}S]GTPγS binding between ipsilateral and contralateral sides. There was also no significant difference in binding between the 3 treatment groups. HU210-stimulated [^{35}S]GTPγS binding was inhibited by SR141716A (1µM); in the superficial dorsal horn, HU210-stimulated [^{35}S]GTPγS binding in untreated, saline-injected and carrageenin-injected rats was significantly decreased to 86±5% ($p<0.001$), 88±6% ($p<0.001$) and 96±7% ($p<0.05$) of basal levels respectively. HU210-stimulated [^{35}S]GTPγS binding in the remainder of the grey matter was inhibited by 1µM SR141716A to 96±5%, 94±5% ($p<0.001$) and 102±8% ($p<0.05$) of basal binding.

Thus administration of the cannabinoid agonist HU210 to spinal cord slices results in an SR141716A-sensitive coupling to G-proteins in the dorsal horn of the rat spinal cord. A difference in CB_1 receptor binding in non-inflamed and inflamed rats was not found.

Martin, B.R. & Lichtman, A.H. (1998) *Neurobiol. Disease*, 257, 119-122.
Rinaldi-Carmona, M. *et al.* (1994) *FEBS Lett.*, 350, 240-244.
Sim, L.J., Selley, D.E. & Childers, S.R. (1995) *Proc. Natl. Acad. Sci. USA*, 92, 7242-7246.
Tsou, K. *et al.* (1998) *Neurosci.*, 83, 393-411.

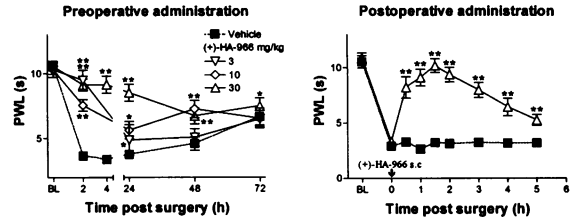
291P EVALUATION OF (+)-HA-966 IN A RAT MODEL OF SURGICAL PAIN

Carnell, A.J., Pitcher, T. & Singh,L. *Parke-Davis Neuroscience Research Centre, Forvie site, Robinson Way, Cambridge,CB2 2QB*

Surgical interventions almost inevitably result in post-operative pain. This is thought to occur as a result of the initial barrage of nociceptive transmission into the spinal cord, resulting in sensitisation of nociceptive pathways. It has been suggested that prevention of this sensitisation process may attenuate the development of post-surgical pain. The concept of pre-emptive analgesia suggests that a drug given before surgery should have a superior analgesic profile than the same drug administered afterwards (Woolf & Chong, 1993). There is a strong line of evidence suggesting that the NMDA receptor plays an important role in the sensitisation of spinal cord during pain. Research into pre-emptive analgesia has been hindered by the lack of good animal models of surgical pain. However, recently a rat model of post-surgical pain has been described (Brennan *et al*, 1996). In the present study, we have examined the activity of the glycine/NMDA site antagonist (+)-HA-966 in this model.

A longitudinal incision of the plantaris muscle of a right hindpaw was carried out in male Sprague-Dawley rats (200-225g) under anaesthesia (2% isofluorane in a 1:2 mixture of O_2 and N_2O) as described previously (Field *et al*, 1997). Drugs were administered either before or following surgery to investigate pre-emptive and post-surgical actions. The observer remained blind to drug administration. Thermal hyperalgesia (Hargreaves model) and static allodynia (using von Frey hair filaments) were assessed as previously described (Field *et al*, 1997). (+)-HA-966 was obtained from Tocris. Pregabalin was synthesised by Parke-Davis research laboratories (Ann Arbor, Michigan) and served as internal control. Both drugs were dissolved in normal saline.

Control animals developed thermal hyperalgesia (decreased paw withdrawal latency, PWL) and static allodynia, which was fully developed at 1h post-surgery (thermal) and 2h post-surgery (static allodynia), the respective first test points. This lasted for at least three days. Pregabalin (30 mg/kg s.c.; 1 h before surgery) caused a complete blockade of the development of both thermal hyperalgesia and static allodynia. These animals did not show either hypersensitivity during the 3 days of the experiment. However, when administered 1h following surgery, pregabalin blocked both responses for only 5h, after which they returned (data not included here for sake of clarity). (+)-HA-966 (3 – 30 mg/kg s.c.; 15min before surgery) caused a dose-dependent blockade of the development of thermal hyperalgesia, remaining significant for up to 3 days, in the case of the highest dose. When administered following surgery, during the maintenance phase, the anti-hyperalgesic action was of much shorter duration. Tactile allodynia was not influenced by (+)-HA-966.



Means ± s.e.mean; n=8-12; *P<0.05, **P<0.01 vs. Vehicle. ANOVA followed by Dunnett's.

These data demonstrate that (+)-HA-966 can block development and maintenance of thermal hyperalgesia-induced by a surgical injury. However, it was somewhat surprising to see its failure to block static allodynia. The reasons for this differential block are unclear. It is tempting to speculate that the two modalities of sensation involve different mechanisms. (+)-HA-966 is a partial agonist at the glycine/NMDA receptor. Thus, it also remains a possibility that a higher level of receptor occupancy is required to block static allodynia. The key observation of this study is, however, that pre-emptive administration of the compound resulted in long lasting anti-hyperalgesic activity, the duration of which was far greater than that observed following post-surgical administration. Recent clinical data suggests that NMDA receptor blockade may also produce preemptive analgesia in man (Wu *et al*, 1999). In conclusion, modulation of NMDA receptors may provide a means of preventing and blocking some components of post-operative pain in the clinic.

Brennan *et al, Pain* (1996) 64: 493 – 501
Field *et al, J.P.E.T.* (1997) 282: 1242 – 1246
Woolf & Chong, *Anaesth.Analg.* (1993) 77: 362 – 379
Wu *et al, Anaesth.Analg.* (1999) 88: 1331 - 1334

292P MAPPING OF THEα5 SUBUNIT CONTAINING $GABA_A$ RECEPTOR IN THE HUMAN HIPPOCAMPUS

O. Howell, J. Atack , R. McKernan & C. Sur. Department of Biochemistry, Merck Sharp and Dohme, Harlow, Essex CM20 2QR

In Alzheimer's disease (AD) the hippocampal formation is the first and most severely disrupted region. Specific changes in GABAergic neurotransmission have been evidenced by reduced benzodiazepine binding and α1 immunolabelling (Mizukami et al.,1998). The α5 subunit containing receptors are enriched in the rat hippocampus, where they comprise 20-25% of the total benzodiazepine receptor population as determined by quantitative autoradiography with [^{3}H]L-655,708, a α5 selective ligand (Sur et al., 1999). Here we describe the distribution by autoradiography of the α5 subunit in human hippocampus and compare its expression in Alzheimer's subjects.

Brains were obtained from control patients with no known history of benzodiazepine treatment and AD confirmed subjects, and matched for post mortem delay (control=9±4;AD=11±5hrs) and age (control=76±5yrs; AD=80±4yrs). Sections were cut at 10µm and washed twice in KH_2PO_4, KCl (10mM, 100mM, pH 7.4) buffer. Incubations were for 1hr in 0.2-20nM [^{3}H]L-655,708 (for saturation studies) or 4nM [^{3}H]L-655,708 for distribution studies (plus 10µM Zolpidem to block other benzodiazepine sites). Flunitrazepam (10µM) defined non-specific binding. Sections were rinsed in buffer, dipped in dH_2O and dried under a current of cold air. Slides were exposed for 8 weeks. Quantification was by MCID image analysis system (Imaging Research) and specific densities expressed as fmole bound/mg of tissue. Kd and Bmax values were calculated (Excel; Microsoft) from saturation plots on 5 controls and 5 AD confirmed cases.

[^{3}H]L-655708 binding sites are distributed heterogeneously throughout all regions of the normal human hippocampal formation in a distinctly laminar density pattern. Saturating α5 containing sites revealed the molecular layer (ML) of the dentate gyrus to have the greatest number of sites, and the underlying polymorphic layer (PmL) to have the lowest. Little variation between the dissociation constant (Kds: 2-5 nM) for [^{3}H]L-655,708 was seen between the two groups. Thus, comparison of α5 receptor expression in a large number of control and Alzheimer's sections were performed with 4 nM [^{3}H]L-655,708 and are presented in Table1.

Table 1. Comparison of the densities (fmole/mg of tissue) of [^{3}H]L-655,708 sites within control (n=9-12) and AD (n=7-9) brain regions.

	Controls	Alzheimer's	Ratio (%)
ML of dentate gyrus	78±3	84±5	107
PmL of dentate gyrus	16±1	17±1	111
CA3	30±2	29±3	97
CA2	42±3	37±3	89
CA1	47±2	35±3**	73
Entorhinal Cx	33±3	23±2*	78

The % values indicate the relative retention of α5 sites. (**) P<0.01 and (*) P<0.04 (t-test).

Levels of [^{3}H]L-655,708 signal were retained or slightly elevated within the ML, PmL and subicular complex. Within the Ammon's horn, a small progressive decrease of α5 receptor density is observed from area CA3 to CA1 and enthorinal cortex. The CA1 field and enthorinal cortex were the only regions where α5 loss attained significance.

These results indicate that the majority of α5 receptors are preserved in AD hippocampus, with only moderate signal depletion in the most severely disrupted regions.

Mizukami K, et al. (1998). *Brain Research. 799, 148-155.*
Sur C, et al. (1999). *Brain Research. 822, 265-70.*

293P EXPRESSION OF A NOVEL $GABA_A$ RECEPTOR SUBUNIT Θ IN RAT BRAIN

A. Chadha[2], T. Bonnert[1], P. Whiting[1], S. Duty[2], R. McKernan[1], J. Atack[1] & C. Sur[1]. [1]Merck, Sharp and Dohme Research Laboratories, Terlings Park, Essex CM20 2QR. [2]Neurodegenerative Disease Research Centre, Kings College London, London SW3 6LX.

The $GABA_A$ receptor is a ligand gated chloride channel through which the majority of inhibitory actions of GABA and various classes of drugs such as benzodiazepines and barbiturates are mediated (Sieghart, 1995). It is a pentameric protein made from the assembly of subunits belonging to different classes; α_{1-6}, β_{1-3}, γ_{1-3}, δ and ε. These subunits have unique expression patterns in the mammalian brain and their combination creates functionally distinct receptor subtypes (McKernan & Whiting, 1996). Here we report the immunocytochemical distribution of a novel $GABA_A$ receptor subunit, θ in rat brain.

Male Sprague Dawley rats (250-300g) were anaesthetised, transcardially perfused with 0.1M phosphate buffered saline (PBS), 4% paraformaldehyde and brains postfixed in 4% paraformaldehyde containing 30% sucrose. Contiguous sections (50μm) were sliced on a freezing microtome throughout the rat brain. Free-floating sections were incubated overnight with an affinity purified θ antibody (0.2μg/ml), raised in rabbit against a 19mer peptide sequence, located in the large putative intracellular loop between TM3 and TM4 of the θ subunit. The presence of the primary antibody was revealed with an avidin-biotin-peroxidase complex (ABC, Vector Laboratories) with 3,3 diaminobenzidine (DAB) as the chromogen. Omission of the primary antibody or pre-absorption with the antigenic peptide prevented DAB immunostaining.

The θ protein was found to have a widespread distribution in the rat brain (Table 1). The highest densities of immunostaining were found in the piriform cortex, striatum, substantia nigra pars compacta and Purkinje cell layer of the cerebellum. At the cellular level, different neuronal structures were labeled. For example, a diffuse intracellular staining was observed in the soma of substantia nigra pars compacta neurones, whereas in the striatum θ immunoreactivity was associated with neuropil.

Table 1. $GABA_A$ receptor θ subunit immunoreactivity in rat brain

REGION	IMMUNOREACTIVITY
Piriform cortex	+++
Frontal and parietal cortex	++
Striatum	+++
Substantia nigra pars compacta	+++
Substantia nigra pars reticulata	++
Globus pallidus	-
Septum	++
Basolateral amygdala	++
Hippocampus	+
Hypothalamus	+
Thalamic relay nuclei	++
Geniculate thalamic nuclei	++
Purkinje layer of cerebellum	+++

(- absent, + low, ++ moderate, +++ intense)

This is the first description of $GABA_A$ receptor θ subunit distribution in rat brain. The high expression of θ in specific brain regions adds additional diversity to native $GABA_A$ receptors. Future studies are needed to reveal the neurochemical nature of neuronal populations which express this subunit in the CNS.

Bonnert T.P., McKernan R., Farrar S. *et al.* (1999). *Proc. Natl. Acad. Sci. USA*, 96, 9891-9896.
McKernan R. & Whiting P. (1996). *Trends Neurosci.*, 19, 139-143.
Sieghart W. (1995). *Pharmacological Rev.*, 47, 181-234.

294P THE EFFECTS OF THE $GABA_A$ RECEPTOR AGONIST MUSCIMOL ON WATER INTAKE ON RATS

A.J. Houston, J.C.L. Wong and I.S. Ebenezer. Neuropharmacology Research Group, School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth, Hampshire, PO1 2DT.

We have previously demonstrated that stimulation of $GABA_B$ receptors inhibits primary drinking in rats (Ebenezer et al., 1992; Houston et al., 1995). The present study was undertaken to determine if stimulation of $GABA_A$ receptors also affects water intake in rats.

Experiment 1. Male Wistar rats (n=8; b. wt. 350 - 450g) that were deprived of water for 16h, were injected s.c. with either physiological saline (control) or muscimol (0.5, 1.0 or 2.0 mg kg^{-1}) 15 min before presentation of water. Water intake was measured for 30 min after presentation, as described previously (Ebenezer et al., 1992). A repeated measures design was used with each rat receiving all doses of saline and muscimol in a random fashion. At least 3 days separated successive drug trials.
Experiment 2. Male Wistar rat (n=8; b. wt. 350 - 400g) that were deprived of water for 16h, were injected with either saline followed by saline, bicuculline (1.0 mg kg^{-1}), followed by saline, saline followed by muscimol (1.0 mg kg^{-1}) or bicuculline (1.0 mg kg^{-1}) followed by muscimol (1.0 mg kg^{-1}). All injections were given s.c. A period of 15 min separated the two injections. Fifteen min after the second injection, the rats were presented with water and intake measured over a 30 min period. A repeated measures design was used with each rat receiving all 4 treatments in a random fashion.

The results obtained in Experiment 1 show that muscimol (0.5 - 2.0 mg kg^{-1}) reduced water intake in a dose-related manner [Mean ± s.e.mean water intake at 30 min: saline 11.1 ± 1.0 ml, muscimol (0.5 mg kg^{-1}) 12.1 ± 1.3 ml; muscimol (1.0 mg kg^{-1}) 5.9 ± 2.1 ml; muscimol (2.0 mg kg^{-1}) 0 ml]. The 0.5 mg kg^{-1} dose was without significant effect on water intake. By contrast, the 1.0 and 2.0 mg kg^{-1} doses of muscimol significantly reduced water intake ($P<0.01$ in each case). The 0.5 and 1.0 mg kg^{-1} doses produced no overt abnormal behavioural effects, whereas the 2.0 mg kg^{-1} dose produced mild ataxia. The results of Experiment 2 show that the inhibitory effect of muscimol (1.0 mg kg^{-1}) was almost completely reversed by pretreatment of the rats with bicuculline (1.0 mg kg^{-1}). Analysis of the data indicated a significant interaction between bicuculline and muscimol ($F_{(1,21)}=25.38$, $P<0.01$). [Mean ± s.e.mean water intake at 30 min: saline+saline 10.9 ± 0.9 ml, muscimol+saline 7.5 ± 0.6 ml; bicuculline+saline 9.8 ± 0.7 ml; bicuculline+muscimol 9.8 ± 0.8 ml). The dose of muscimol and bicuculline used in this experiment produced no overt abnormal behavioural effects.

The results of this study show that the $GABA_A$ receptor agonist muscimol inhibits primary drinking in water deprived rats. Furthermore, the observation that this effect is blocked by pretreating the animals with the $GABA_A$ receptor antagonist bicuculline suggests that the inhibitory effect of muscimol on water intake is mediated via $GABA_A$ receptors. The present finding suggest the possibility that a $GABA_A$ receptor mechanism may play a role in the control of water intake.

Ebenezer, I.S., Houston, A.J. and Crook, T.J. (1992) Gen. Pharmacol., 23, 375-379.
Houston, A.J., Wong, C.L.J. and Ebenezer, I.S. (1995) Br. J. Pharmacol., 114, 305P.

295P EFFECTS OF ACUTE BUSPIRONE ON OPERANT FOOD INTAKE IN FOOD-DEPRIVED AND SATIATED PIGS

I.S. Ebenezer[1], Parrott, R.F. and Vellucci, S.V. Dept. of Neurobiology, Babraham Institute, Cambridge, U.K. and [1]Neuropharmacology Research Group, School of Pharmacy and Biomedical Science, University of Portsmouth, Portsmouth, U.K.

Agonists acting at $5HT_{1A}$ receptors, such as 8-hydroxy-2-(di-N-propylamino)-tetralin and buspirone, have previously been shown to increase food intake in non-deprived rats by an action at $5HT_{1A}$ autoreceptors (Gilbert & Dourish, 1987). By contrast, it has been demonstrated that these agents decrease feeding in food-deprived rats (Ebenezer, 1992); the mechanism for this effect is not known The present study was undertaken to investigate whether a $5HT_{1A}$ agonist would produce similar effects on feeding in a non-rodent species.

Prepubertal Large White boars (b.wt. 30 - 40 kg) were chronically prepared under halothane anaesthesia with jugular vein cathethers and housed in metabolism cages where they were trained to perform operant responses to obtain food and water reinforcements. The pigs were maintained on the following feeding schedule: a buzzer sounded at 10.00h which signalled that the feeder was activated for 120 min and that the pigs could press the food panel during that period. At 16.00h the feeder was activated again for 30 min; water was also available *ad libitum*. Experiment 1. Fifteen min prior to the morning feed, pigs (n=6) were injected iv with either saline (control) or buspirone (0.125, 0.25 or 0.5 mg kg^{-1}) and operant food intake was measured over the subsequent 120 min. A repeated measures design was used, with each pig receiving all treatments in a random fashion. Experiment 2. Pigs (n=5) were injected with either saline (control) or buspirone (0.25 mg kg^{-1}, iv) 60 min after starting their morning feed and operant food intake was measured for a further 60 min. A cross-over design was used with each pig receiving all treatments; 3-4 days separated successive drug trials.

Analysis of the data obtained in Experiment 1 showed that buspirone (0.125 - 0.5 mg kg^{-1}, iv) produced a dose-related reduction in food intake in food-deprived pigs during the first 30 min of their 2h feeding period ($F_{(3,15)}$=11.7696, P<0.01, see Table 1). There were no effects of buspirone on cumulative food intake at 60 or 120 min. By contrast, the results obtained in Experiment 2 showed that buspirone (0.25 mg kg^{-1}, iv) significantly increased operant food intake (P<0.01) in satiated pigs during the 30 min period following administration (number of food reinforcements, mean ± s.e. mean: saline 1.2 ± 0.9, buspirone 31.0 ± 8.2). There was no significant effect on feeding during the successive 30 min period.

These results show that acute iv injection of buspirone produces a reduction in feeding in fasted animals (Exp.1) and increased food intake in satiated pigs (Exp.2). The present findings extend the results of previous studies in rodents (Ebenezer, 1992) and show that buspirone can produce both stimulant and depressant effects on feeding which appear to be dependent on the state of hunger of the animal.

Table 1. Effects of buspirone (B) on operant food intake in food-deprived pigs. Doses are expressed in mg kg^{-1}. Dunnett's t-test, saline vs buspirone: **P<0.05, **P<0.01

	Food Reinforcements ± s.e. mean			
Time (min)	Saline	B (0.125)	B (0.25)	B (0.5)
0-10	37.0±4.1	25.3±4.5*	19.0±3.6**	13.3±4.9**
0-20	68.0±8.2	55.7±8.3	44.8±6.2 *	30.2±9.7 **
0-30	91.5±11.3	79.0±12.6	73.3±9.1	42.3±13.0**

Ebenezer, I.S. (1992) NeuroReport 3: 1019 – 1022

Gilbert, F. & Dourish, C.T. (1987) Psychopharmacol 93: 349 – 352.

296P A70104 AND FOOD INTAKE IN RATS: EVIDENCE AGAINST A ROLE FOR ENDOGENOUS PERIPHERAL CCK AS A SATIETY FACTOR

I.S. Ebenezer. Neuropharmacology Research Group, School of Pharmacy & Biomedical Sciences, University of Portsmouth, Portsmouth, PO1 2DT.

The observation that systemic administration of cholecystokinin (CCK) inhibits food intake in mammals has led to the hypothesis that endogenous peripheral CCK released from the small intestine during a meal acts as a satiety factor (see Baldwin et al., 1998). Furthermore, as CCK cannot cross the blood brain barrier (BBB), it has been proposed that it must act at a peripheral site to elicit its hypophagic actions. It was predicted that if CCK does play an important role in satiety, then administration of a specific peripherally-acting CCK antagonist should block the effects of the endogenous peptide released during a meal and increase food intake (see Ebenezer and Baldwin, 1995, Baldwin et al., 1998). The present study was undertaken to test the CCK-satiety hypothesis by investigating the effects of the CCK_A receptor antagonist, A70104, which is unlikely to cross the BBB (Ebenezer and Parrott, 1993), on food intake in rats.

Experiment 1. Male Wistar rats (n=8, b wt. 225 - 300g) that were fasted for 20h were given access to food for 30 min (oral preload). They were then injected i.p. with A70104 vehicle (veh; see Ebenezer and Parrott, 1993) or A70104 (20, 50, 100 μg kg^{-1}) and 30 min later given further access to food for 120 min. The amount of food consumed during this test-meal period was measured. A repeated measures design was used in which each rat received all treatments in a random fashion; 3 - 4 days separated successive trials. Experiment 2. Male Wistar rats (n=8, b.wt. 260 - 300g) that were fasted for 22h, were injected with either veh followed by saline, veh followed by CCK (5 μg kg^{-1}), A70104 (50 μg kg^{-1}) followed by veh, or A70104 (50 μg kg^{-1}) followed by CCK (5 μg kg^{-1}). Both injections were given i.p. A period of 30 min separated the 2 injections. Immediately after the second injection, the rats were placed separately in experimental cages with access to food and food consumption was measured after 30 min. A repeated measures design was used with each animal receiving all 4 treatments in random order.

The results from Experiment 1 show that A70104 (20 - 100 μg kg^{-1}) had no significant effects on the intake of the test meal in rats [e.g. mean ± s.e.mean. food intake at 120 min: veh. 4.1 ± 0.4g; A70104 (20 μg kg^{-1}) 4.5 ± 0.8g]. However, pretreatment with A70104 (50 μg kg^{-1}) abolished the inhibitory effect of CCK (5 μg kg^{-1}) on food intake (Experiment 2; Fig. 1).

The present findings that A70104 has no effect on food intake when administered on its own but abolishes the suppressant effect of exogenous peripheral CCK, confirm and extend previous observations with A70104 in the pig (Ebenezer and Parrott, 1993) and lends further support to the suggestion (Ebenezer and Parrott, 1993; Ebenezer & Baldwin, 1995) that endogenous peripheral CCK is not a satiety factor.

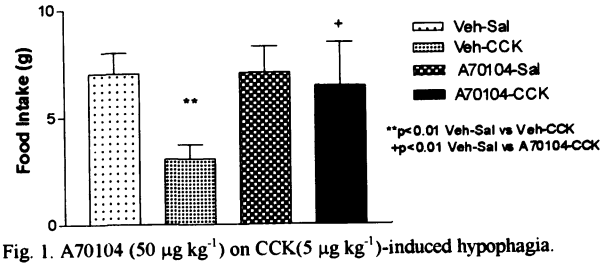


Fig. 1. A70104 (50 μg kg^{-1}) on CCK(5 μg kg^{-1})-induced hypophagia.

Baldwin, B.A., Parrott, R.F. and Ebenezer, I.S. (1998) NeuroBiology, 553, 477 – 507.

Ebenezer, I.S. and Parrott, R.F. (1993) NeuroReport, 7, 495-498.

Ebenezer, I.S.& Baldwin, B.A. (1995) Br. J. Pharmacol., 116, 2371-2374.

297P A PHARMACOLOGICAL INVESTIGATION OF MOTOR DISTURBANCES INDUCED BY MCPP AND MK-212 IN THE RAT

G.C. Wadsworth, S.M. Weiss, M.J. Bickerdike. G.A. Kennett
Dept. of Neuropharmacology, Cerebrus Ltd., Wokingham. RG41 5UA. U.K.

5-HT_{2C} receptor activation in rodents is associated with a variety of behavioural effects including hypophagia, anxiogenesis, and hypolocomotion (Kennett, 1993). There are reports also that 5-HT_{2C} receptor agonists such as 1-(3-chlorophenyl)piperazine (mCPP) and 6-chloro-2-(1-piperazinyl)pyrazine (MK-212) induce specific motor disturbances in the rat, although at relatively high doses. The motor disturbances consist of phenomena such as hindlimb abduction, tremor and forepaw treading (Lucki et al, 1989).

The contribution of 5-HT_{2C} receptor activation to some of the motor disturbances induced by mCPP and MK-212 has been assessed by examining their effects in the absence and presence of the selective 5-HT_{2C} receptor antagonist, SB-242084 (Kennett et al, 1997). During a one min test rats (Male Hooded-Lister, 250-450g, 8/group) were scored, blind with respect to treatment, on a scale of 0-3 for the presence and intensity of any of three behaviours: hindlimb abduction, hunched back posture and retropulsion.

The results are represented as the sum of the group scores (Table 1). Both mCPP and MK-212, administered s.c., 30 mins prior to testing, produced a significant ($p<0.05$) and dose-dependent increase in motor disturbance score, with MED's of 1.0 and 3.0 mg/kg, respectively. Prior s.c. administration (40 mins pre-test) of SB-242084 significantly ($p<0.05$) reversed the effect of MK-212 (10 mg/kg) in a dose-dependent manner (MED 3 mg/kg) with a total abolition of motor disturbances at 10 mg/kg. SB-242084 also reversed the effect of mCPP (10 mg/kg) with an MED of 3 mg/kg.

These data indicate that mCPP and MK-212 induce motor disturbances in rats via 5-HT_{2C} receptor activation. While these effects are seen only at doses higher than required for other reported 5-HT_{2C} receptor mediated behaviours, such as hypophagia (eg Kennett and Curzon, 1988), they nevertheless represent a method for assessing 5-HT_{2C} receptor agonist activity.

Kennett G.A., Wood M.D., Bright F., et al (1997) Neuropharmacology, 36(4-5), 609-620.
Kennett G.A., (1993), Curr. Opin. Invest. Drugs, 2, 317-362
Lucki I., Ward H.R. & Frazer A. (1989), J. Pharmacol. Exp. Ther., 249, 155-164.
Kennett G.A. & Curzon G., (1988), Psychopharm., 96, 93-100.

Table 1

mCPP (mg/kg)	Score	SB-242084 (mg/kg)	mCPP (mg/kg)	Score	MK-212 (mg/kg)	Score	SB-242084 (mg/kg)	MK-212 (mg/kg)	Score
0	2	0	0	0	0	2	0	0	0
0.3	4	0	10	15*	0.1	2	0	10	19*
1	7	3	10	6*^	0.3	3	10	0	1
3	10*	10	10	7*^	1	7*	1	10	15*
10	15*				3	20*	3	10	8*^
					10	23*	10	10	1^

*$p<0.05$ vs. vehicle group; ^$p<0.05$ vs. agonist + vehicle group (Kruskal-Wallis ANOVA/Mann-Whitney U-test).

298P AN INVESTIGATION INTO THE ACTIONS OF 5-HT ON THE RAT ANOCOCCYGEUS MUSCLE

K.N. Green, N.M. Barnes & N.C. Harris, Department of Pharmacology, The Medical School, University of Birmingham, Edgbaston Birmingham B15 2TT.

A range of non-vascular smooth muscle preparations have been found to respond to the monoamine 5-hydroxytryptamine (5-HT). In the relative absence of reports of the actions of 5-HT, via identified 5-HT receptors, on the anococcygeus muscle, the present study investigates effects of 5-HT on this muscle..

The rat anococcygeus muscle was removed from male Wistar rats as described by Gillespie (1972), and mounted under 1 g tension in a 5 ml organ bath filled with Krebs-Henseleit solution (mM: NaCl 118.0; KCl 1.4; $MgSO_4$ 1.2; KH_2PO_4 1.17; $NaHCO_3$ 25.0; glucose 11.0; $CaCl_2$ 2.0) and gassed with 95%O_2/5%CO_2 at 37°C. The muscle state was measured isometrically. The muscle was also subjected to pulses of field stimulation by two electrodes either end of the muscle. A train of 10 pulses, at 10 Hz applied every 20 seconds at a supramaximal voltage (~70 V), results in the "twitching" of the muscle. Experiments were performed both on the resting tissue, and also on the "twitching" preparation. Agonist dose response curves were constructed on all tissues, antagonist were allowed to equilibrate for at least 30 min prior to repeating the agonist dose response curve. Agonist responses were expressed as a percentage of the maximal response observed with that tissue and mean EC_{50} values determined ± SEM. Antagonist affinity (pK_B) was determined from the Gaddum equation (pKB=log(CR-1)-log[B]).

The unstimulated rat anococcygeus muscle contracted in response to 5-HT (5 -100 μM; EC_{50}=13±0.1 μM, n = 11). This contraction was found to be tetrodotoxin (1 μM) insensitive and was also unaffected following depletion of noradrenaline by guanethidine (30 μM). The 5-HT mediated contraction was antagonised by spiperone (25 nM; 5-HT EC_{50}=44±0.25 μM, n=4) giving a pK_B of 8.07. The 5-$HT_{2B/2C}$ antagonist, SB 206553 (500 nM; Barnes and Sharp, 1999), had no effect on the 5-HT mediated contraction.

5-HT (30 nM) inhibited the field stimulated contraction by ~5% ($p>0.05$) but, at a higher concentration (1 μM) the response was augmented 15 ± 5% ($p<0.001$, n=9) 2,5-Dimethoxy-4-iodoamphetamine (DOI; 6-60 nM) evoked the same augmentation in the presence of mesulergine (500 nM; to stop the baseline contraction). Agonist (5-HT or DOI) induced augmentation was not affected by mesulergine (500 nM; Mes), ondansetron (1 μM) or SB 206553 (500 nM).

The "twitch" response was inhibited by sumatriptan (Sum) in a concentration dependent manner (1 - 100 μM; 90% inhibited at 100 μM). 5-Carboxamidotryptamine also evoked a small inhibition in the same concentration range. Sumatriptan-induced inhibition was not antagonised by pindolol (500 nM), naloxone (500 nM), yohimbine (500 nM) or mesulergine (500 nM). See **Table 1.**

Table 1 Agonist and antagonist effects on the "twitching" muscle (n numbers in brackets). Data represents mean ± SEM.

Drugs	EC_{50}/μM	Drugs	EC_{50}/μM
5-HT (11)	0.73±0.1	Sum (17)	25±0.06
5-HT+Mes (4)	1.2±1.2	Sum+Naloxone(4)	18±0.26
5-HT+Ondansetron(3)	1.0±0.35	Sum+Pindolol (4)	23±0.32
DOI+Mes (3)	0.01±0.34	Sum+Yohimbine(4)	25±0.31
DOI+Mes+SB206553(3)	0.01±0.34	Sum+Mes (4)	8.6±0.26

These results suggest that a post-synaptic 5-HT_{2A} receptor is responsible for contraction. Further pharmacological studies are required to define the 5-HT sensitive receptors modulating the "twitching" rat anococcygeus muscle with confidence.

Barnes N. M. and Sharp T. (1999), *Neuropharmacology* 38, 1083-1152
Gillespie J. S. (1972), *Br. J. Pharmac.* 45, 404-416.

299P THE GUANYLYL CYCLASE/cGMP SYSTEM REDUCES TONE BUT NOT CAPACITATIVE CALCIUM INFLUX ACTIVATED BY THAPSIGARGIN IN MOUSE ANOCOCCYGEUS CELLS

P. Wallace, I. McFadzean & A. Gibson, Messengers & Signalling Group, Division of Pharmacology & Therapeutics, King's College London, Guy's Campus, London SE1 9RT.

Capacitative calcium entry is an important source of activator calcium in certain tonic smooth muscles (Gibson et al., 1998). Drugs activating the guanylyl cyclase/cGMP system inhibit capacitative calcium entry, probably by re-filling the depleted sarcoplasmic reticulum (SR; Gibson et al., 1998; Cohen et al., 1999). In vascular smooth muscle, it has been proposed that such inhibition may be the primary mechanism underlying nitrovasodilator-induced relaxations, since the relaxations are prevented by the SR Ca-ATPase inhibitor thapsigargin (TG; Cohen et al., 1999). Here, we investigate whether this proposal also holds true in a non-vascular smooth muscle.

Anococcygeus muscles were dissected from male mice (LACA; 25-35g) and set up for the recording of isometric tension responses. Single smooth muscle cells were isolated and loaded with Fura-2 to determine changes in cytoplasmic calcium (Wayman et al., 1999). All experiments were carried out in the presence of 10μM verapamil and the results were expressed as mean±s.e. IC_{50} values were calculated by linear regression.

TG (0.4-100nM) produced concentration-related, strong and sustained contractions of the mouse anococcygeus. The contraction to 100nM TG was relaxed, in a concentration related manner, by sodium nitroprusside (SNP; 0.1-5μM; IC_{50} 0.34μM), nitric oxide (NO; 3-15μM; IC_{50} 2.1μM), and 8-Br-cGMP (10-400μM; IC_{50} 30μM). Relaxations to SNP and NO were almost abolished in the presence of the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxodiazolo[4,3-a]quin oxalin-1-one (5μM), whereas those to 8-Br-cGMP were unaffected. The general calcium entry blocker SKF96365 (0.4-40μM; IC_{50} 4.4μM) also relaxed TG-induced tone. In single smooth muscle cells bathed in calcium-free medium, TG (100nM) caused only a slight and transient increase in fluorescence ratio ($R_{340/380}$; basal ratio 0.46±0.01, peak ratio 0.49±0.01); subsequent addition of calcium (2.5mM; 10min after addition of TG) resulted in a much larger and sustained increase (basal ratio 0.43±0.01; peak ratio 0.64±0.03). Influx of Mn (200μM), as determined by quenching of Fura-2, was also enhanced by 100nM TG. The effects of the relaxant drugs on the calcium response to TG were determined using concentrations of each drug which had relaxed TG contractions by 80-90%. TG-induced calcium influx was inhibited by 20μM SKF96365 (by 60±10%; n=5), but was unaffected by either 1μM SNP or 100μM 8-Br-cGMP.

The results confirm that capacitative calcium entry is important for excitation-contraction coupling in this non-vascular smooth muscle. The lack of effect of relaxant concentrations of SNP and 8-Br-cGMP on TG-induced calcium influx suggests that inhibition of capacitative calcium entry is not obligatory for relaxation via the guanylyl cyclase/cGMP system in this tissue.

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Cohen, R.A., Weisbrod, R.M., Gericke, M., *et al.* (1999) *Circ. Res.* 84, 210-219.
Gibson, A., McFadzean, I., Wallace, P. *et al.* (1998) *TIPS* 19, 266-269.
Wayman, C.P., Wallace, P., Gibson, A., *et al.* (1999) *Eur. J. Pharmacol.* 376, 325-329.

300P CYANIDE-EVOKED QUANTAL CATECHOLAMINE RELEASE FROM PHAEOCHROMOCYTOMA (PC12) CELLS

S. C. Taylor, S. M. Shaw & C. Peers, Institute for Cardiovascular Research, University of Leeds, Leeds LS2 9JT.

Recent studies have indicated that pheochromocytoma (PC12) cells act as chemoreceptors in a manner that compares well with carotid body chemoreceptor cells, since they release catecholamines in response to acute hypoxia (Taylor & Peers, 1998, 1999). The carotid body has long been known to be excited by metabolic inhibitors such as cyanide, and this involves catecholamine release from chemoreceptor (type I) cells within the carotid body (Gonzalez *et al.*, 1994). Here, we have investigated whether cyanide also evokes catecholamine release from PC12 cells.

Release was monitored from single cells amperometrically as previously described, using polarized (+800mV) carbon fibre microelectrodes which allow resolution of release of individual vesicles (Taylor & Peers, 1998, 1999). Cells were constantly perfused at room temperature (21-24°C) with a HEPES-buffered solution containing 2.5mM Ca^{2+} (see Taylor & Peers, 1998, for composition). When cells were exposed to NaCN in the perfusate, exocytosis was detected in a concentration-dependent manner. For example, at 0.1mM, NaCN evoked the appearance of exocytotic events at a rate of 0.10 ± 0.02Hz (mean ± s.e.m., n=8 cells), and release rate appeared maximal (1.00 ± 0.11Hz, n=9) at a NaCN concentration of 5mM.

Secretion evoked by 2.5mM NaCN (0.79 ± 0.07Hz, n=8) was completely and reversibly abolished by removal of extracellular Ca^{2+} (replaced with 1mM EGTA, n=8) or by application of the non-selective blocker of voltage-gated Ca^{2+} channels, Cd^{2+} (200μM, n=8). These findings suggested that NaCN-evoked secretion was entirely dependent on voltage-gated Ca^{2+} entry.

PC12 cells possess L,- N-, and P/Q-type Ca^{2+} channels (Liu *et al.*, 1996). In the presence of 2μM nifedipine to block L-type channels, 2.5mM NaCN evoked secretion at a rate of 0.60 ± 0.08Hz (n=8). The same concentration of NaCN evoked secretion at 0.79 ± 0.10Hz from 8 cells pretreated for 10min with 200nM ω-Agatoxin GIVA, a blocker of P/Q-type channels. These levels of secretion were not significantly different from those observed in the absence of Ca^{2+} channel blockers. By contrast, pretreatment of cells for 10min with 1μM ω-conotoxin GVIA significantly reduced secretion evoked by 2.5mM NaCN by approximately 75% to 0.20 ± 0.05Hz (n=8, $P<0.0001$ vs control cells, unpaired Student's t-test). These results indicate that NaCN is a potent secretagogue in PC12 cells, and that NaCN-evoked secretion requires Ca^{2+} influx primarily through N-type Ca^{2+} channels.

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Gonzalez, C. *et al.* (1994) *Physiol. Rev.* 74, 829-898.
Liu, H. *et al.* (1996) *J Neurosci.* 16, 7557-7565.
Taylor, S.C. & Peers, C. (1998) *Biochem. Biophys. Res. Comm.* 248, 13-17.
Taylor, S.C. & Peers, C. (1999) *J. Physiol.* 514, 483-491.

301P A SURVEY OF THE SLOPES OF CONCENTRATION-RESPONSE CURVES

R.B.Barlow, Ash Lea Cottage, Ravenstonedale, Kirkby Stephen, Cumbria CA17 4NG

The slopes of concentration (dose)- response curves vary greatly and can conveniently be expressed as the exponent, P, using the empirical (logistic) model of Parker & Waud (1971); this is numerically the same as the Hill coefficient. With weak agonists the "operational model" of Black & Leff (1983) gives curves which are not logistic and the differences in shape could be detected with theoretical data (Barlow, 1997). To see if such differences occurred with experimental results, a search was made of graphs appearing in the British Journal of Pharmacology. Values of response and concentration were recovered from published figures with a digitising tablet. Colquhoun (1998) has pointed out, however, that the operational model makes unjustifiable assumptions about agonist-receptor binding so the aim of the search was lost but it showed the wide range of values of P found experimentally and was extended to see how this varied with the type of response and to what extent experimental reasons might account for flat curves.

In all 365 curves were examined: P ranged from 0.18 to 8.0 (mean 1.03, sd 0.60, median 0.91). Although the selection was arbitrary, a cumulative frequency curve of values of log.P was roughly normal (logarithmic mean 0.91). The distribution of values of P is shown as the full line in Fig 1A: the broken line shows that for responses involving electrical events (e.g. effects on currents) which have higher values (mean 1.23, sd 0.51, median 1.10, n=41). Fig 1B shows the distribution for responses involving effects on tissues, divided into positive effects (e.g. contraction: full line, mean 1.15, sd 0.88, median 0.98, n=89) and negative effects (e.g. relaxation: broken line, mean 1.05, sd 0.44, median 1.01, n=110) which are surprisingly similar, although the relation between muscle force and [internal calcium^{++}] had P=8 (Shiraishi, Y. *et al.*, 1998). Fig 1C shows the distribution for binding experiments

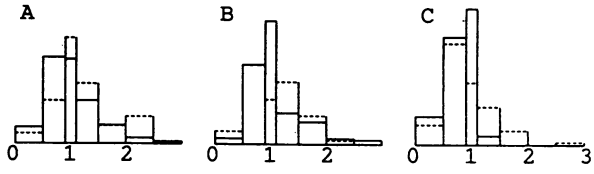

Figure 1 Proportion of results lying within the values of P indicated.

(full line, mean 0.73, sd 0.25, median 0.75, n=49) and for effects on second messengers (broken line, mean 0.98, sd 0.55, median 0.85, n=63). For many of the (competitive) binding experiments P should be 1 and lower values suggest binding to more than one site. The effects on second messengers have a very wide range (0.25 to 3.67).

Curves will appear flat if a positive baseline has been overlooked or effects such as relaxation are expressed as a fraction of a resting value which has been under-estimated: 50% of the curves with P<0.5 (14% for 0.5<P <0.9) showed an improved fit when allowed to find their own baseline. With many flat curves more than one process appeared to contribute to the response: 41% of the curves with P<0.5 (36% for 0.5<P <0.9) fitted better to a 2-site curve. The exponent should indicate the molecularity of an agonist process, e.g. P=2 for electrical responses from nicotinic receptors. Flat curves (P<1) imply fractional molecularity and the very high value, P=8, for muscle force and [internal calcium^{++}], the final step in many responses, suggests the importance of checking that the flatness is genuine and not an artefact of the method of analysis.

Barlow, R.B. (1997) Br.J.Pharmacol., **122**, 351P.
Black, J.M. & Leff, P. (1983) Proc.R.Soc. B, **220**, 141-162.
Colquhoun, D. (1998) Br.J.Pharmacol., **125**, 923-947.
Parker, R.B., & Waud, D.R. (1971) J.Pharmacol. Exp Ther., **177**, 1-12.
Shiraishi, Y., Kanmura, Y. & Itoh T., (1998) Br. J.Pharmacol., **123**, 869-878.

302P OPERATION WINDOWS MAKE FUNCTIONAL ANTAGONISM APPEAR INDEPENDENT

R.B.Barlow[1], Susan M. Bond, Claire Grant, D.S.McQueen, Jennifer Smart & Zeenat Yaqoob, Dept Neuroscience, 1 George Square, Edinburgh EH8 9JZ, Scotland. [1] Ash Lea Cottage, Ravenstonedale, Kirkby Stephen CA17 4NG

Many important effects such as dilatation are produced by compounds which appear to be antagonists of constrictors but are actually agonists at different receptors using different mechanisms to produce the opposite effect. Gaddum (1957) described this as "independent" antagonism: it has also been called "physiological antagonism" and can be represented by:

$$E = M_A \frac{[A]^{PA}}{[A]^{PA} + [A_{50}]^{PA}} - M_B \frac{[B]^{PB}}{[B]^{PB} + [B_{50}]^{PB}} \quad \ldots 1$$

where E is the response to the agonist, [A], in the presence of the independent antagonist, [B], M_A and M_B are their respective maximum effects, $[A_{50}]$ and $[B_{50}]$ are the concentrations producing half these effects and PA and PB are the exponents for the two curves. With this scheme the concentration of antagonist producing 50% inhibition, $[IC_{50}]$, should be constant (unaffected by [A]). With a powerful antagonist, however, the overall effect (E) may be negative and if this is recorded as zero, $[IC_{50}]$ will be less than $[B_{50}]$ with low concentrations of agonist, and rise to this limiting value as [A] is increased (Fig. 1A). If the processes share a common pathway ("functional antagonism", Ariens *et al.*, 1957), the antagonist may not be able to reduce the agonist response below zero (e.g. Van der Graaf *et al.* 1996) so $[IC_{50}]$ is unaffected by increasing agonist (Fig. 1B). With negative responses recorded as zero, shifts as much as 10-fold in agonist (or antagonist) concentration- response curves can be obtained without reducing the maximum. Such effects have been found (Van den Brink, 1973) with drugs and tissues which should share a common pathway (methacholine, (-)isoprenaline and calf trachea; histamine, (-)isoprenaline and guinea-pig ileum). Fig 1C shows an example obtained in our experiments with guinea-pig bronchial muscle, ileum and rat uterus in oestrus.

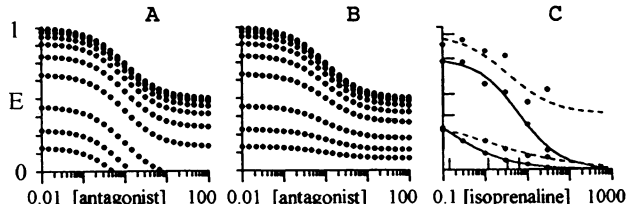

Fig. 1 Relation between E, antagonist (with M_B=0.5) and increasing concentration of agonist for independent (A), functional antagonism (B) and (C) responses (in arbitrary units) from guinea-pig ileum, carbachol and (-)isoprenaline (nM): a 3-fold increase in agonist produces a large rightward shift with isotonic recording (full line) and a 6-fold increase with isometric recording (broken line) reduces the maximum effect.

The steepness of agonist concentration- response curves varied from PA often <1 (bronchial muscle), PA usually 1-2 (ileum) and PA usually >5 (rat uterus) and the aim was to see how this and the method of recording affected antagonist concentration- response curves. Carbachol was agonist (histamine also on ileum): the antagonists were (-) and (±) isoprenaline and (-) adrenaline. With all types of tissue, agonist curves with isometric recording were flatter than those for tissue from the same animal recorded isotonically, so with the smaller window through which isotonic observations can be made there will be a shift in IC_{50} as seen in Fig 1C. The antagonist curves varied greatly and shifts were seen with all types of tissue. The results fit the independent model even though the agonist and antagonist must share a common path: possibly, like the recording, this operates within a window between a baseline and a maximum not set by saturation.

Ariens, E.J., Van Rossum, J.M. & Simonis, A.M. (1957) Pharmacol. Rev., **9**, 218-236.
Gaddum, J.H. (1957) Pharmacol. Rev., **9**, 211-218.
Van den Brink, F.G (1973) Eur.J.Pharmacol., **22**, 270-278, 279-286.
Van der Graaf, P.H., Shankley, N.P. & Black, J.W. (1996) Br.J.Pharmacol., **118**, 1308-1316.