AGONIST INDUCED INTERNALISATION AND DOWN REGULATION OF β_2-ADRENOCEPTORS ON INTACT HUMAN PLATELETS.

N Cook, S R Nahorski and D B Barnett. Department of Pharmacology and Therapeutics, University of Leicester, Leicester, LE2 7LX

Agonist-induced desensitisation of the beta-adrenoceptor has been extensively studied in cultured cells. Recently internalisation of cell surface beta-adrenoceptors following agonist incubation has also been demonstrated in human lymphocytes (De Blasi et al, 1985). Coupling of the receptor to adenylate cyclase as a prerequisite for the process of internalisation is disputed, as receptor redistribution has been demonstrated with cyc$^-$ S49 lymphoma cells which lack functional N_S. The present study examines agonist induced redistribution of beta-adrenoceptors which are poorly coupled to cyclase on intact human platelets.

Platelet rich plasma was collected, incubated with (treated), or without (control) 10μM (-) isoprenaline (I) for 30 min at 37°C and whole platelets prepared at 4°C using modifications of previously described methods (Cook et al, 1985). ^{125}I(-) pindolol (PIN) binding assays were performed for 40 min at 37°C or 5 hours at 4°C (to prevent recycling of internalised receptors during assay incubation) as previously described (Barnett et al, 1986). Using saturation analysis we assessed maximal binding capacity of ^{125}I-PIN in the presence of 1μM (-) propranolol (P) or 1μM (±) CGP 12177 (C) which were taken to represent total or cell surface beta-adrenoceptors respectively (Staehelin et al, 1983).

I treatment resulted in a highly significant loss of ^{125}I-PIN binding sites (assayed at 4°C) which was much greater when defined by C ($\simeq$ 70%) than with P ($\simeq$ 25%) when compared with control (Table 1). Thus, 40-50% of the receptors remaining following agonist treatment were no longer accessible to C at 4°C due to removal away from the immediate cell surface. ^{125}I-PIN binding performed at 37°C to treated cells showed a 25% loss of binding sites only that was identical whether defined by either antagonist.

Table 1

Apparent Equilibrium Dissociation Constants (K_D*) and Receptor Density (B_{max}*) of ^{125}I(-)PIN Binding at 4°C to Intact Human Platelets

Cells	Agent Used to Define Non-Specific Binding	B_{max} (fmols/mg protein)	K_D (pM)
Control**	1μM (-) propranolol	2.05 ± 0.14	13.28 ± 1.0
Treated	1μM (-) propranolol	1.46 ± 0.12	12.83 ± 1.04
Treated	1μM (±) CGP 12177	0.556 ± 0.06	13.96 ± 0.32

* Values are the means ± SEM (n = 3).

** P and C identify same number of sites on control cells at 4°C (Barnett et al, 1986).

We conclude that I treatment of intact human platelets at 37°C causes redistribution of beta$_2$-adrenoceptors. A proportion are sequestered away from the cell surface (internalised) becoming inaccessible to the hydrophilic ligand CGP. A further proportion defined by propranolol are totally lost from the cell (down regulated).

Cook N, Nahorski SR and Barnett DB (1985) Eur J Pharmacol, 113, 247-254
Barnett DB, Cook N and Nahorski SR (1986) Br J Pharmacol, 87(suppl), 222P
De Blasi A, Lipartiti M, Motulsky HJ, Insel PA and Fratelli M (1985) J Clin End and Metab, 61(6), 1081
Staehelin M, Simons P, Jaeggi K and Wigger N (1983) J Biol Chem, 258, 3496

β_2-ADRENOCEPTOR DENSITY IS INCREASED IN HEART, LUNG AND KIDNEY OF SPONTANEOUSLY HYPERTENSIVE RATS

J.J. Beckeringh, O.-E. Brodde, D. Büttner, M.C. Michel*, X.L. Wang.
Biochem. Research Labs., Med. Klinik & Poliklinik, Div. Renal & Hypertensive Diseases, University of Essen, D-4300 Essen, Fed.Rep.Ger.

In spontaneously hypertensive rats (SHR) the development of elevated blood pressure can markedly be attenuated by treatment with the highly selective β_2-adrenoceptor antagonist ICI 118,551 (Borkowski&Quinn, 1985). To study the role of β_2-adrenoceptors for the elevation of blood pressure, we investigated the development of β_1- and β_2-adrenoceptors in heart, lung and kidney of SHR in comparison with that of normotensive rats (WKY) at the age of 5, 10 and 20 weeks.

β-Adrenoceptor density was determined by (-)-^{125}I-iodocyanopindolol (ICYP) binding at 6 concentrations ranging from 10-150 pM; unspecific binding was defined as binding in the presence of 1 µM (±)-CGP 12,177 (Wang et al.,1985).The β_1:β_2-adrenoceptor ratio was assessed by non-linear regression analysis of competition curves of ICI 118,551(heart and kidney) or the selective β_1-adrenoceptor antagonist bisoprolol (lung) with ICYP (40-60 pM) binding (Engel et al., 1981).

While β-adrenoceptor density was similar for both strains in the heart at each age, it was significantly higher in lung and kidney of SHR than of WKY ($p<0.05$ at 10 and 20 weeks of age). In kidney, the β_1:β_2-adrenoceptor ratio did not change with ageing neither in SHR nor in WKY; however, with development of hypertension the β_1:β_2-adrenoceptor ratio was shifted towards β_2-adrenoceptors in heart and lung of SHR: thus, β_2-adrenoceptor density was higher in all tissues of SHR at all ages. Destruction of presynaptic nerve terminals by intravenous treatment of 20 weeks old WKY with 6-hydroxydopamine (2x50 mg/kg on day 1, 2x100 mg/kg on day 7, experiment on day 8) resulted in a significant 24% loss in cardiac β_2-adrenoceptors ($p<0.05$) while β_1-adrenoceptor density was unaffected.

It is concluded that with the development of hypertension in SHR the density of β_2-adrenoceptors increases.The fact that after 6-hydroxydopamine treatment 24% of cardiac β_2-adrenoceptors are destroyed favours the idea that at least part of rat cardiac β_2-adrenoceptors is presynaptically located.Therefore, an increase in presynaptic noradrenaline release facilitating β_2-adrenoceptors might be involved in the development and/or maintenance of elevated blood pressure in SHR. However, it can not be decided from the present investigation whether these changes occur primary or secondary to the elevation of blood pressure.

Supported by the Landesamt für Forschung Nordrhein-Westfalen and the SANDOZ-Stiftung für Therapeutische Forschung.

Borkowski & Quinn (1985). J. Auton. Pharmacol. 5:89-100
Engel et al. (1981). Naunyn-Schm. Arch. Pharmacol. 317:277-285
Wang et al. (1985). Eur. J. Pharmacol. 114:157-165.

HAEMODYNAMIC AND LYMPHOCYTE β_2-ADRENOCEPTOR CHANGES DURING PROCATEROL TREATMENT IN MAN

J.J. Beckeringh, O.-E. Brodde*, A. Daul, M.C. Michel, X.L. Wang.
Biochem. Research Labs., Med. Klinik & Poliklinik, Div. Renal & Hypertensive Diseases, University of Essen, D-4300 Essen, Fed.Rep.Ger.

β-Adrenoceptor (R) agonists are commonly used in the therapy of asthma and other pulmonary diseases.Recently,we have shown that in healthy volunteers treatment with the β_2-R agonist terbutaline caused a rapid decrease in lymphocyte β_2-R density (Brodde et al., 1985). To study whether such lymphocyte β_2-R changes are accompanied by similar changes in β_2- and/or β_1-R mediated haemodynamic effects,in the present study we investigated the effects of oral treatment of healthy volunteers with the β_2-R agonist procaterol (Yabuuchi, 1977) on lymphocyte β_2-R density and on changes in heart rate and blood pressure induced by dynamic exercise or isoprenaline(Ipn)-infusion.

10 male healthy volunteers (aged 24.7±0.75(20-27) years) participated in the study after having given informed written consent;all were drug free. Before treatment the volunteers performed on 2 successive days the exercise- and Ipn-infusion test.Thereafter procaterol (2x50 µg/d) was administered orally for 9 days. At day 8 and 9,when the volunteers were still on treatment,the exercise- and Ipn-test were repeated. Lymphocyte β_2-R density was assessed by (-)-^{125}iodocyanopindolol binding as recently described (Brodde et al., 1985).

Procaterol treatment led to a rapid decrease in lymphocyte β_2-R density (maximum after 9 days: 35%).In such treated volunteers the Ipn-induced decrease in diastolic blood pressure, which is mediated by β_2-R stimulation, was markedly attenuated.On the other hand,the Ipn-induced increase in systolic blood pressure, which is mediated by β_1-R stimulation, was not affected. In the procaterol treated volunteers the Ipn-evoked tachycardia,which has been recently shown to be mediated by β_1- and (predominantly) β_2-R stimulation (Arnold et al.,1985; Daul et al.,1986), was significantly suppressed.In contrast, dynamic exercise-induced tachycardia, which is mediated solely by β_1-R stimulation (Arnold et al.,1985;Daul et al.,1986)was not affected by procaterol treatment. The present results indicate that the procaterol-induced decrease in lymphocyte β_2-R is accompanied by similar reductions in β_2-R mediated haemodynamic effects (Ipn-induced tachycardia, Ipn-induced decrease in diastolic blood pressure). On the contrary, β_1-R mediated haemodynamic effects (Ipn-induced increase in systolic blood pressure, exercise-induced tachycardia) are not affected by this treatment.

It is concluded that procaterol is a highly selective β_2-R agonist that acts in therapeutically effective doses only at β_2-R. The subsensitivity of β_2-R mediated effects developing during procaterol administration might be an explanation for the tachyphylaxis of asthmatic patients against therapy with β_2-R agonists.

Supported by Landesamt f. Forsch. NRW and SANDOZ-Stift.f.Ther.Forsch.

Arnold,J.M.O. et al. (1985). Br.J.Clin.Pharmacol. 19: 619-630
Brodde, O.-E. et al. (1985). J.Clin.Invest. 76: 1096-1101
Daul,A. et al. (1986). Acta Pharmacol.Toxicol. 59 (Suppl.V): II-52.
Yabuuchi, Y. (1977). Br.J.Pharmacol. 61: 513-521

REGULATION OF SKELETAL MUSCLE β-ADRENOCEPTORS IN VIVO BY CHRONIC ADMINISTRATION OF ADRENOCEPTOR AGONISTS AND PROPRANOLOL

M.S. Elfellah, J.L. Reid, Department of Materia Medica, Stobhill General Hospital, Glasgow, G21 3UW.

In skeletal muscles, beta adrenoceptors are proposed to maintain physiological levels of potassium during exercise and psychological stress and to mediate the hypokalaemic response to endogenous adrenaline. The hypokalaemic effect of adrenaline may be involved in the genesis of arrhythmias in acute illnesses including myocardial infarction (Struthers & Reid, 1984). The beta adrenoceptors in skeletal muscle have been identified as $beta_2$ subtype (Elfellah et al, 1986). Buur et al (1982) have reported that chronic pretreatment of guinea pigs with terbutaline desensitises the function of beta adrenoceptors in the skeletal muscles observed as reduced responsiveness of adenylate cyclase to beta adrenoceptor agonists. We have investigated the regulation of beta adrenoceptors in the skeletal muscle using radioligand binding techniques.

Groups of male guinea pigs weighing 250-350 gm were pretreated with saline, isoprenaline (50 ug/kg), adrenaline (150 ug/kg) terbutaline (125 ug/kg) or d,l propranolol (20 mg/kg) subcutaneously, thrice daily for one week. Approximately 16 hours after the last injection the animals were sacrificed and the gastrocnemius muscles were isolated and tissue membranes were prepared (Elfellah et al, 1986). The equilibrium dissociation constant, K_D (pM) and maximum number of binding sites, Bmax (fmol/mg protein) were estimated from Scatchard plots using (-) [^{125}I] iodocyanopindolol (ICYP) as the radioligand.

The table shows that pretreatment of the guinea pigs with beta adrenoceptor agonists reduced the binding sites for ICYP. There was a trend of slight reduction in K_D following agonist pretreatment. However, only in the case of the higher dose of adrenaline did this reach a statistically significant level. In contrast pretreatment of animals with propranolol significantly increased the binding sites and this was accompanied by slight but statistically significant increase in the K_D (Bmax = 71.1 ± 9.1 and 123.3 ± 9.3, $p < 0.002$; K_D 9.2 ± 0.7 and 13.9 ± 0.5, $p < 0.0001$ in gastrocnemius muscles from saline, n=6 and propranolol n=8 pretreated animals).

Table: Effect of pretreatment with agonists on binding characteristics of ICYP in gastrocnemius muscles (mean ± S.E. mean)

Pretreatment	(n)	Bmax	K_D
Saline	(20)	77.9 ± 5.0	9.7 ± 0.9
Isoprenaline	(6)	55.8 ± 3.2 *	7.6 ± 0.4
Adrenaline	(7)	49.7 ± 2.0 **	4.6 ± 0.3 **
Terbutaline	(8)	43.3 ± 2.7 **	6.9 ± 0.7

* $p < 0.05$, ** $p < 0.01$ in comparison to saline pretreated group.

In conclusion chronic exposure of the guinea pig to beta adrenoceptor agonists leads to down regulation of beta adrenoceptors in the skeletal muscle while chronic administration of propranolol causes up regulation of the receptors.

References

Buur, T. et al (1982) Br. J. Pharmacol. 76, 313-317.
Elfellah, M.S. et al (1986) Br. J. Pharmacol. 88, 317p.
Struthers, A.D. & Reid, J.L. (1984) Clin. Sci. 66, 377-382.

A PHYSIOLOGICAL ROLE FOR MYOCARDIAL α-ADRENOCEPTORS IN HYPOTHYROIDISM

R. Chess-Williams* and D.J.P. Critchley, Department of Pharmacology & Therapeutics, University of Liverpool, P.O. Box 147, Liverpool, L69 3BX.

The existence of myocardial α-adrenoceptors is well established but their physiological role is unknown since, in the absence of β-blockade, neither adrenaline nor noradrenaline induce a response that is α-mediated in normal myocardial tissues (Schüman, 1984). However, we would like to present the hypothesis that α-adrenoceptors may be involved in the response to endogenous amines in conditions where α- and β-adrenoceptor responsiveness is altered. To test this hypothesis, the susceptability of noradrenaline responses to prazosin and propranolol was examined in cardiac tissues from normal and hypothyroid animals a condition in which β-mediated responses are depressed and α-mediated responses enhanced (Chess-Williams & Critchley - this meeting).

Left atria and left ventricular papillary muscles were isolated from untreated rats and 6-propylthiouracil-pretreatment rats (15 $mgKg^{-1}$ daily in the drinking water for 8-10 weeks). Tissues were set up in oxygenated Krebs at 32°C and paced at $1H_2$. Isometric developed tension was recorded and cumulative concentration-response curves to noradrenaline constructed in the absence and presence of antagonists. Metanephrine (10μM) and desipramine (1μM) were present throughout the experiment.

Table 1: Noradrenaline EC_{50} values (μM) for untreated animals

Antagonist	Left atria	N	Papillary Muscle	N
none	0.19 (0.05-0.74)	6	0.68 (0.26-1.8)	6
Praz (10nM)	0.18 (0.07-0.48)	6	0.80 (0.22-2.9)	6
Prop (0.1μM)	2.6 (0.91-7.5)**	5	6.5 (2.4-17.3)*	5
Praz (10nM) + Prop (0.1μM)	2.1 (1.5-2.9)	5	7.9 (5.7-10.9)	5

Table 2: Noradrenaline EC_{50} values (μM) for hypothyroid animals

Antagonist	Left atria	N	Papillary Muscle	N
none	0.11 (0.04-0.26)	7	0.41 (0.11-1.4)	6
Praz (10nM)	0.11 (0.06-0.19)	7	1.1 (0.24-4.9)*	6
Prop (0.1μM)	0.67 (0.29-1.6)*	7	4.3 (2.1-8.9)**	7
Praz (10nM)) + Prop (0.1μM)	1.3 (0.52-3.3)*	5	11.0 (6.0-20.2)**	6

* $P<0.05$ ** $P<0.01$

In tissues from untreated animals, propranolol antagonised noradrenaline responses but prazosin had no effect in either the absence or presence of β-blockade (Table 1). In tissues from hypothyroid rats propranolol again antagonized noradrenaline, however prazosin was also effective (Table 2). In these tissues prazosin caused a significant increase in left atrial noradrenaline EC_{50} values when examined in the presence of propranolol, and in papillary muscles, prazosin antagonised noradrenaline in both the absence and presence of β-blockade.

These results indicate that myocardial α-adrenoceptors may have a physiological role to play in disease where adrenoceptor responsiveness may be altered.

This work is supported by the British Heart Foundation and Bristol-Myers

Schümann, H.J. (1983). Eur. Heart J. 4 (Suppl A): 55-60.

ACTIVATION OF BROWN FAT THERMOGENESIS IN THE RAT BY CRF

R. Le Feuvre, N.J. Rothwell* & M.J. Stock, Department of Physiology, St. George's Hospital Medical School, Tooting, London SW17 ORE

Brown adipose tissue (BAT) is an important effector of thermogenesis, allowing mammals to raise metabolic rate in response to cold and hyperphagia. BAT is controlled mainly by the sympathetic nervous system but its activity can be modified by a number of hormonal factors, and is markedly stimulated by either adrenalectomy or hypophysectomy, and inhibited by corticosterone. Since these treatments are associated with large changes in the release of CRF, and CRF is thought to modify sympathetic outflow, we have now tested its effects on BAT thermogenesis.

Interscapular BAT and rectal temperatures were recorded using implanted thermocouples, in urethane-anaesthetized, male, Sprague-Dawley rats (age 45-60d). Intravenous injection of noradrenaline (20nmol/rat) provoked a transient rise (peak at 5min) in BAT temperature (0.25±0.10°C) and a smaller, delayed increase in rectal temperature. Stereotaxically-located injections of rat CRF-41 (2-5nmol in 1µl buffer) into the third ventricle (i.c.v.) produced a significant rise in BAT (peak 0.92±0.11°C at 9.5±1.1 min) and rectal (peak 0.70±0.6°C at 12.5±1.9 min) temperatures, which were almost completely abolished by prior treatment of the animals with propranolol (5µmol/kg i.v.). Central injections of vehicle alone or arginine vasopressin (3nmol), or intravenous injection of ACTH (5nmol) failed to affect BAT and rectal temperatures.

Thermogenic activity of BAT, assessed from the binding of guanosine diphosphate (GDP) to isolated mitochondria, was significantly elevated (85±10pmol/mg mitochondrial protein) in interscapular brown fat isolated from rats injected with CRF (4nmol, i.c.v.) 30 minutes previously, compared to those given vehicle alone (45±6pmol/mg protein, $p<0.01$). Sectioning the sympathetic nerves supplying the interscapular BAT depot did not affect GDP binding in controls (52±5), but inhibited levels in CRF-treated animals (72±6, NS vs control denervated).

Regional tissue blood flow was estimated from the distribution of radio-labelled microspheres in anaesthetized rats, 30 minutes after injection (i.c.v.) of vehicle or CRF (4nmol). Flows to epididymal fat, testes, kidney, brain, liver (arterial supply) and skeletal muscle were unaffected by CRF, whereas flow to adrenals was increased by 60%, and to all BAT depots by almost 8-fold (control = 0.45±0.04; CRF = 3.43±0.18ml/min, $p<0.05$).

These data indicate that central administration of CRF can activate BAT thermogenesis in the rat, probably via the sympathetic nervous system. It seems unlikely that ACTH release is responsible for the effects of CRF since they are not mimicked by exogenous ACTH. It is possible that increases in CRF are responsible for the activation of BAT thermogenesis following hypophysectomy.

INHIBITORY EFFECT OF MUCOPOLYSACCHARIDE POLYSULPHATE ON LASER-INDUCED MICROVASCULAR THROMBOSIS

P.Görög, Department of Pharmacology,King's College,Strand,London.

Platelet thrombus formation was induced by helium-neon laser irradiation in the microcirculation of hamster cheek pouch or in the mesoappendix of rat (Kovacs & Görög,1979). The time until occlusion of the irradiated small vein and the duration of occlusion were measured. Mucopolysaccharide polysulphate (Hirudoid^{R},MPS), administered intravenously to hamsters at doses of 1,5 or 10 mg/kg body weight inhibited thrombus formation. The time until occlusion of the irradiated vessel was significantly prolonged at 5 and 10 mg/kg doses ($p<0.001$). Upon topical application of a solution of 1, 2 or 25% the occlusion time was also significantly prolonged by MPS >2% ($p<0.001$).

Application of MPS as a 0.3% cream topically on the cheek pouch increased the occlusion time significantly over the vehicle-controls ($p<0.001$). In rat, MPS cream (0.3%) was applied on the shaved skin of the back for twenty-four hours. Control rats were treated identically except using placebo cream. Percutaneous MPS treatment significantly inhibited thrombus formation in the mesoappendix microcirculation ($p<0.001$). In all cases, reperfusion of the occluded blood vessels occured much earlier in animals treated with MPS (iv. or topical/percutaneously) than in the relevant controls ($p<0.001$). The time until reopening of the vessel was sometimes reduced to about half of controls.

Kovacs I.B. & Görög P. (1979) Microvasc. Res. 18, 403-412.

EFFECT OF MPS ON THE MICROVASCULAR THROMBUS FORMATION IN HAMSTER CHEEK POUCH

Administration	Treatment	No. of hamsters	Time (sec., mean ± s.e.m.) Initial	Occlusion	Reopening
Intravenous	saline	7	155	268	108
	1 mg/kg	5	150	275	42 ***
	5 mg/kg	5	235 **	444 ***	44 ***
	10mg/kg	6	229 **	445 ***	41 ***
Topical (solution)	saline	5	119	217	128
	1%	5	167	299 *	74 **
	2%	5	256 **	399 ***	47 ***
	25%	5	407 ***	554 ***	56 ***
Topical (cream)	placebo	6	137	231	133
	Hirudoid	5	232 ***	393 ***	67 ***

* $p<0.05$; ** <0.01; *** <0.001;

AN ANALYSIS OF THE RESPONSES TO ELECTRICAL FIELD STIMULATION CAPSAICIN OR FORMALIN IN THE RABBIT IRIS SPHINCTER

A. Jamieson* and N.J.W. Russell. Bioscience Department II, ICI Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire SK10 4TG. (Introduced by M.J. Rance).

The isolated iris sphincter muscle is a useful in vitro prepraration to study the miotic response associated with ocular inflammation. Previous evidence has suggested that the non-cholinergic, non-adrenergic response produced by electrical stimualtion of this muscle is mediated by a tachykinin released from the peripheral terminals of primary afferent fibres (Ueda et al. 1982). The present study has examined the neurogenic nature of the responses to electrical stimulation and application of the sensory nerve stimulants capsaicin and formalin.

Electrical transmural stimulation, (60v, 20H_z, 0.3msecs, for 10 secs), produced fast and slow components of contraction which were abolished by tetrodotoxin ((0.2μM), lignocaine (1mM), and cold storage of the tissue (4°C, 7 days). The fast component of the response was selectively suppressed by atropine (0.1μM), and the slow component by the tachykinin antagonist (DPro4,DTrp7,9,10)-$SP_{(4-11)}$ (10μM). Desensitisation by prolonged application of capsaicin (10μM for 2 hours) attenuated only the slow component of the response.

Capsaicin (1-4μM) and formalin (0.002-0.008%) produced dose related contractions of prolonged duration and tachphylaxis developed rapidly. Cumulative dose responses to capsaicin and formalin were not suppressed by tetrodotoxin, lignocaine or atropine whereas cold storage, capsaicin desensitisation and the tachykinin-antagonist attenuated responses to application of capsaicin and formalin.

Responses to carbachol to mimic the fast contraction component were unaffected by cold storage, capsaicin desensitisation and the tachykinin antagonist. Only atropine and high doses of lignocaine attenutated the response to carbachol.

In confirmation of previous findings in the iris sphincter muscle (Ueda et al. 1984) we have shown that the slow response to electrical transmural stimulation is mediated by release of a tachykinin-like peptide from sensory nerves. In addition the responses to the sensory nerve stimulants capsaicin and formalin are also mediated by release of a tachykinin-like peptide from sensory nerves. These pain producing substances act on the sensory nerve elements to release a mediator without requiring Na^+ flux or axonal conduction to produce the response.

These data provide further evidence for a role of tachykinin-like peptides in the non-adrenergic, non-cholinergic miotic response associated with ocular inflammation.

Ueda, N., Muramatsu, I., Hayashi, H. and Fujiwara, M. (1982) Life Sci 31, 369-375.

DECREASED BIOAVAILABLE COPPER IN RAT AND HUMAN ARTHRITIC PLASMA - A RATIONALE FOR COPPER THERAPY

Patricia C. Freeman & P.O'Callaghan[1*], Department of Paramedical Sciences, North East London Polytechnic, Romford Road, London E15 4LZ, and [1]Biological Monitoring Service, Institute of Naval Medicine, Alverstoke, Gosport, Hants.

Inflammation is accompanied by major changes in copper metabolism (Milanino et al, 1985); much of the increase in plasma copper being due to increased synthesis of the acute phase protein, ceruloplasmin. However, ceruloplasmin-bound copper is non-exchangeable, the bioavailable copper being bound reversibly to albumin and low molecular weight complexes (Harris, 1983).

Blood samples (10ml) were collected by venepuncture from recently-diagnosed arthritics (both sexes, ages 36 - 73 years) attending an out-patient rheumatoid clinic, and from age and sex-matched controls. Arthritics were rheumatoid factor positive and not currently receiving anti-inflammatory medication. Copper-binding proteins in these plasma samples were separated by pseudo-ligand chromatography on Blue Sepharose-CL-6B (Pharmacia) for albumin followed by gel filtration on Sephadex G-100 (Pharmacia) for ceruloplasmin and low molecular weight complexes. Copper in each fraction was measured by atomic absorption spectrophotometry with electrothermal atomisation (Perkin-Elmer Model 5000 with HGA-500 graphite furnace) (detection limit 3.03 n mol l^{-1} Cu). Table 1 shows an increase in total serum copper in arthritics compared with controls, reflecting an increase in ceruloplasmin-bound copper. However, bioavailable copper was decreased in these subjects.

Table 1 Concentrations of copper complexes in plasma from both normal and arthritic subjects. Values are mean ± 1 standard error in μmol l^{-1}.

Parameter	Control (n=8)	Arthritic (n=8)
Total Cu	16.86 ± 0.42	22.09 ± 1.30
Ceruloplasmin-Cu	14.69 ± 0.45	20.13 ± 1.36
Albumin-Cu	1.71 ± 0.06	1.53 ± 0.07
Low-MW-Cu	0.46 ± 0.03	0.37 ± 0.04

Arthritis was induced in female Wistar rats (200-230g) by the method of Newbould (1963). Inflammation was monitored by measuring paw depths using a micrometer. Blood samples (1ml) were taken by cardiac puncture under ether anaesthesia on Day 0 and weekly until Day 20. Plasma fractions were separated and analysed for copper as above. Total and ceruloplasmin-bound copper increased while bioavailable copper decreased progressively, the earliest changes being seen prior to the appearance of arthritis.

Given the essential roles of copper in the repair of arthritic cartilage and the maintenance of a competent immune system, both processes which are impaired in rheumatoid arthritis, these results suggest a rationale for the paradox of elevated plasma copper levels in rheumatoid arthritis and the anti-arthritic properties of copper compounds (Sorenson, 1981).

Harris, E.D. (1983) in 'Trace Elements in Health' (ed. J. Rose), pp 44-73, Butterworths, London.
Milanino, R. et al. (1985) Agents & Actions 16, 504-513.
Newbould, B.B. (1963) Br. J. Pharmac. 21, 127-136.
Sorenson, J.R.J. (1981) Agents & Actions Suppl. 8, 305-325.

50P

ACUTE HYPERINSULINAEMIA FOLLOWING ETHER EXPOSURE AFTER α-ADRENOCEPTOR BLOCKADE OR ADRENAL DEMEDULLATION

B.L. Furman, Fiona M. Sidey* & A.C. Wardlaw[1], Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G1 1XW and Department of Microbiology, University Of Glasgow[1]

Marked hyperinsulinaemia following acute stress is seen in B.pertussis infected or pertussis toxin treated mice (Sidey et al 1986). The mechanism is unknown and the present work was undertaken to examine the effect of various drugs or adrenal demedullation on the hyperinsulinaemic response to ether stress in pertussis toxin-treated or B.pertussis-infected mice. 3 week old mice were infected intranasally with B.pertussis and used 2 weeks later (Sidey et al, 1986). Purified pertussis toxin (150 ng/mouse i.v.) was given to adult mice 5 days before the experiment. Exposure of infected mice to ether (90 sec followed by 30 sec recovery before blood collection by decapitation) produced marked hyperinsulinaemia (serum immunoreactive insulin (IRI), ng/ml, geometric mean (95% CL) in infected animals exposed to room air, 1.8 (1.7,2.0) in infected animals exposed to ether, 13.3 (9.7,18.1) $p < 0.01$). A similar response was seen in pertussis toxin treated mice exposed to ether. Bilateral adrenal demedullation four weeks before the experiment did not significantly modify ether induced hyperinsulin-aemia in pertussis toxin treated mice. The hyperinsulinaemia in infected mice was reduced by propranolol (20 mg/kg i.p. 20 min before ether) which produced hypoinsulinaemia. However, two way ANOVA showed that there was no significant interaction between ether and propranolol suggesting that the effect of propranolol was explained by its own hypoinsulinaemic action. This, together with the failure of adrenal demedullation to abolish the response makes it unlikely that pertussis is unmasking a β-adrenoceptor mediated hyperinsulinaemia exerted by adrenaline. Bilateral adrenal demedullated mice showed a marked hyperinsulinaemia after ether, similar to that seen in pertussis toxin-treated or infected animals (Sham-operated mice; serum IRI (ng/ml) without ether 1.2 (1.07,1.36), with ether, 1.3 (1.13,1.48). Demedullated mice; serum IRI without ether 1.28 (1.07,1.54), with ether 6.37 (3.56,11.4), $n=8$, $p<0.01$ for interaction). Alpha$_2$ adrenoceptor blockade in normal mice, using idazoxan (5mg/kg i.p., 20 min before ether) also resulted in a marked hyperinsulinaemic response to ether (control mice; serum IRI (ng/ml) without ether 1.27 (1.19,1.36), with ether, 2.09 (1.74,2.5); idazoxan-treated mice; without ether, 1.68 (1.45,1.94), with ether, 8.31 (5.92,11.65), $n=14$, $p<0.01$ for interaction). A similar pattern was seen with phentolamine (5-40 mg/kg). These results suggest a role for adrenal medullary catecholamines in preventing hyperinsulinaemia in response to acute stress. This action of catecholamines may be prevented by pertussis toxin.

This work was supported by the Scottish Home & Health Department.

Sidey, F.M., et al. (1987) J. Endocrinol. 112: 113-122

THE INFLUENCE OF SPONTANEOUS AND DRUG-INDUCED DIABETES ON MOUSE PANCREATIC AND SUBMANDIBULAR TISSUE KALLIKREIN-LIKE ENZYMES

Mary Assimakopoulos, K.D. Bhoola*, Fiona Dawrant and P.V. Taberner, Department of Pharmacology, Medical School, University Walk, Bristol BS8 1TD.

In the mouse, tissue kallikrein and its related proteases are expressed by 25 genes linked together on chromosome 7, and the diversity of physiological actions of these enzymes suggests a variety of functional roles (Schachter, 1980). Kallikrein injections have been shown to reduce the blood sugar in pancreatectomised dogs (Frey et al., 1930) and in rats (Rohen and Haberland, 1977). This, together with evidence from human diabetics (Mayfield et al., 1984) suggests that disorders of such kallikrein-like enzymes may be associated with diabetes. The present work describes the age and sex-related changes in kallikrein levels in mice from normal (LACG, Bhoola et al., 1973) and inherently diabetic strains (C57Bl/10ScSn and CBA/CA). The latter show insulin-independent maturity onset diabetes in the male only (Connelly & Taberner, 1986).

The pancreas (PG) and submandibular glands (SMG) were dissected and homogenised in TRIS buffer pH 7.4 at 4. The homogenate was centrifuged (4000 x g, 5 min), the pellet washed once and the supernatants combined. Kallikrein-like activity was measured using the synthetic substrate H-D-Val-Leu-Arg-pNA in the presence of soya bean trypsin inhibitor. Protein and plasma glucose levels (PGL) were also measured.

Results are shown in Table 1. Both sexes of C57 mice had higher enzyme levels (and PGL) than LACG mice. In female CBA mice, SMG and PG kallikrein-like activity was similar to that in LACG mice, but in the males activity was significantly higher ($p < 0.01$) and increased with age, in parallel with the PGL.

TABLE 1. Values are means ± SEM, n = 6-8 mice, aged 105-122 days

		Kallikrein-like activity (mU/μg protein)		PGL (mM)
		Submandibular gland	Pancreas	
LACG	male	9.23 ± 0.13	0.400 ± 0.09	9.78 ± 0.61
	female	0.41 ± 0.10	0.03 ± 0.003	9.40 ± 0.66
C57	male	43.09 ± 3.13	0.216 ± 0.06	11.22 ± 0.84
	female	104.6 ± 21.3	0.396 ± 0.032	11.88 ± 0.90
CBA	male	33.75 ± 3.95	0.520 ± 0.15	13.37 ± 0.69
	female	6.18 ± 0.66	0.370 ± 0.005	11.00 ± 0.44

Alloxan (250 mg/kg ip) and streptozotocin (70 mg/kg ip) produced high PGL in male LACG mice after 72h, and the kallikrein levels were also raised relative to controls. The PGL is raised in C57, male CBA and alloxan and streptozotocin-treated mice, all of which show increased kallikrein levels. Since the male CBA mice are hyperinsulinaemic (Taberner & Connelly, 1986) whereas the drug-treated mice are hypoinsulinaemic, it is concluded that the kallikrein levels may be linked to the PGL rather than circulating insulin. The results present further evidence for a possible relationship between diabetes and tissue kallikrein-like enzymes.

Bhoola, K.D., Dorey, G. & Jones, C.W. (1973) J. Physiol. 235, 503-522
Connelly, D.M. & Taberner, P.V. (1986) Br.J.Pharmac. 87, 120P
Frey, E.K. et al. (1930) Arch.exp.Pathol. 158, 334
Mayfield, R.K. et al. (1984) J.Clin.Endoc.Metab. 59, 278-286
Rohen, J.W. & Haberland, G.L. (1977) Med.Welt. 28, 30
Schachter, M. (1980) Pharmac.Rev. 31, 1-17.

POSSIBLE PHYSICAL BASIS FOR ADAPTIVE CYTOPROTECTION

N K Bhaskar, J Davis, B Filipowicz, C J Hawkey*, R T Kemp, R P Walt. Dept of Therapeutics, University Hospital, Nottingham NG7 2UH

Exposure of gastric mucosa to mildly irritating stimuli enhances its subsequent resistance to damage (adaptive cytoprotection). We have investigated whether this is due to stimulation of endogenous prostaglandin synthesis or to formation of a physical barrier over the surface epithelium.

Fasted male Wistar rats (200-220g) were dosed orally with indomethacin (2.5 mg/kg or 10 mg/kg) in 0.9% sodium chloride containing Tween 80 or with vehicle alone. After one hour a priming dose of 1ml of 20% ethanol or water (controls) was introduced into the stomach. Fifteen minutes later ex vivo immunoreactive prostaglandin (iPG)E_2 release and morphological appearances were assessed. iPGE_2 release was measured by radioimmunoassay after incubation of fragments of gastric corpus in Krebs solution for 15 minutes at 37°C. In other rats macroscopic evidence of haemorrhagic necrosis was measured gravimetrically. Histologically, the perpendicular height from muscularis to mucosal surface and proportion of surface epithelium that was disrupted or covered by cellular debris were measured in haematoxylin and eosin sections using a graticule.

In another series of experiments the protective effect of these manouvres was investigated. Fifteen minutes after the priming with 20% ethanol or water (controls), 1ml of 100% ethanol or water (controls) was introduced into the stomach and morphological changes assessed 1 hour later.

Twenty per cent ethanol caused extensive epithelial disruption, with formation of a covering of cellular debris, but did not affect release of iPGE_2. Indomethacin caused dose dependent inhibition of iPGE_2 release but did not significantly affect epithelial disruption (Table 1).

Table 1 - Effects of indomethacin and 20% ethanol

Indomethacin	Nil		2.5 mg/kg		10 mg/kg	
Ethanol	0	20%	0	20%	0	20%
iPGE_2 release (pg/15 min)	301±55	293±50	155±30**	136±35**	59±11**	60±22**
% epithelium disrupted	10±5	50±9**	-	-	20±5	55±11**
% covered by debris	15±5	53±8**	-	-	32±7	48±9*

* $p<0.05$ ** $p<0.01$ compared to control values n = 6 for all values

Priming with 20% ethanol protected the mucosa against subsequent challenge with 100% ethanol regardless of indomethacin pretreatment (Table 2).

Table 2 - Protection against 100% ethanol

Indomethacin	Nil			10 mg/kg		
Ethanol %	0/0	0/100	20/100	0/0	0/100	20/100
% macroscopic necrosis	1±1	37±8**	13±5**	1±0	42±6**	20±3**
Mucosal height (μm)	676±20	555±29**	607±28**	653±31	499±27**	588±31**
% epithelium disrupted	11±4	69±5**	52±9*	29±8	74±6**	53±7**

** $p<0.01$ * $p<0.05$ compared to preceding value n = 9 for all values

Formation of a surface layer of debris is more likely than changes in prostaglandin to account for mucosal protection in these experiments.

PROTECTIVE EFFECT OF PROSTACYCLIN ON PARACETAMOL INDUCED LIVER INJURY

G.J. Blackwell, N.K. Boughton-Smith, F. Guarner* and S. Moncada
The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS.

Prostacyclin has been shown to protect isolated hepatocytes (Guarner, 1985) and white blood cells (Lynch, 1985) against carbon tetrachloride-induced necrosis. We have now investigated the effect of prostacyclin on paracetamol-induced liver injury in the mouse model. Two structurally unrelated thromboxane synthetase inhibitors, OKY 1581 and benzyl imidazole, were also tested in order to evaluate the role of the prostacyclin-thromboxane balance in the development of the hepatic lesions.

Groups of 20 male CFLP mice, which had been fed for a week with phenobarbitone (3 g/l) added to the drinking water, received paracetamol orally (400 mg/kg). Twenty-four hours later blood samples were obtained by cardiac puncture from the surviving mice and their livers removed for histopathological studies. Prostacyclin (10 µg/kg) given intraperitoneally two hours after paracetamol prevented mortality and significantly reduced liver necrosis as assessed by serum glutamic pyruvic transaminases and liver histology. Similar results were obtained in animals treated with OKY 1581 (50 µg/kg i.p.) two hours after paracetamol. Benzyl imidazole (10 mg/kg, i.p.), however, was only effective if given prior to paracetamol. Paracetamol treatment enhanced the generation of prostaglandins and thromboxane in vitro by liver homogenates when measured by specific radioimmunoassays (Salmon, 1983). There was a 20-fold increase of thromboxane B_2 generation ($p < 0.01$) and a 10-fold increase of 6-keto-$PGF_{1\alpha}$ and PGE_2 ($p < 0.01$). In mice treated with OKY 1581 thromboxane production was inhibited while endogenous prostacyclin synthesis, as assessed by 6-keto-$PGF_{1\alpha}$ levels, was significantly higher than in animals receiving paracetamol only. Indomethacin pretreatment (2.5 mg/kg, i.p.) blocked both the generation of prostacyclin by liver homogenates and the protective effect of OKY 1581 against paracetamol-induced liver necrosis. On the other hand, the protective effect of benzyl imidazole, which also inhibited thromboxane synthesis but did not enhance prostacyclin production, was not altered by indomethacin pretreatment. Finally, benzyl imidazole dose dependently increased the sleeping time induced by pentobarbitone (50 mg/kg, i.v.) in mice. The protective effects of benzyl imidazole on paracetamol-induced liver injury were therefore apparently due to an effect on hepatic drug metabolism. Neither prostacyclin nor OKY 1581 altered the pentobarbitone sleeping-time.

In conclusion, prostacyclin may prevent liver necrosis after paracetamol overdose. Enhancement of endogenous prostacyclin synthesis by selective inhibition of thromboxane synthetase also exerts a protective role in this model.

Guarner, F., Freont-Smith, M., Prieto, J. (1985). Liver, 5:35-39.
Lynch, T.J., Blackwell, G.J., Moncada, S. (1985). Biochem. Pharmacol., 34:1515-1522.
Salmon, J.A., Simmons, P.M. and Moncada, S. (1983). J. Pharm. Pharmacol., 35:808-811.

EFFECTS OF SULPHASALAZINE AND PHCL28A ON GASTRIC ULCER FORMATION AND PROSTAGLANDIN METABOLISM IN THE RAT.

C.N. BERRY* and K.G. LLOYD Department of Biology, L.E.R.S. 23 - 25, Ave. Morane Saulnier, 92360, Meudon la Forêt, France

The activity of Sulphasalazine (SZP) in ulcerative colitis, has been related to inhibition of 15-hydroxy prostaglandin dehydrogenase (PGDH, Hoult and Moore, 1978). Also SZP has an antiulcer (stress) activity in the rat (Ogle and Cho, 1985). We investigated SZP in ethanol (ETOH) and phenylbutazone (PBT) induced gastric ulcers, and explored the role of PGDH in these models using a highly potent PGDH inhibitor, PhCL28A (Berry et al, 1985).

Fasted (22 hrs), female Wistar rats (180-250 g ; 6 per group) received either SZP, PhCL28A or vehicle 30 minutes before oral injection of the ulcerogen or vehicle. Two hours after PBT or one hour after ETOH injection the animals were killed and stomachs removed and the ulcers scored. The stomachs were stored at - 80°C for cytosolic PGDH assays as in Berry et al, 1985.

Orally, neither PhCL28A up to 30 mg/kg nor SZP up to 100 mg/kg had any effect on ETOH or PBT ulcers. However, both compounds were active after i.p. injection (ED_{50} values of 13 and 41 mg/kg vs ETOH ulcers and 3 and 38 mg/kg vs PBT-induced ulcers for PhCL28A and SZP respectively). Both compounds produced small statistically significant decreases in PGDH activity irrespective of the route of administration at doses which almost completely inhibited ulcer formation when given i.p. (Table). Moreover, a marked decrease in PGDH activity in both ulcer models was reduced still further by SZP and PhCL28A in some cases.

$PGF_{2\alpha}$ BREAKDOWN (PGM, ng/hour/gm tissue) IN ETHANOL AND PHENYLBUTAZONE ULCERS

Treatment	50 % Ethanol 1 ml/kg po		Phenylbutazone 200mg/kg po			
	Dose mg/kg ip	PGM	Dose mg/kg po	PGM	Dose mg/kg ip	PGM
Vehicle		904 ± 94		1153 ± 39	-	977 ± 62
PhCL28A alone	10	1048 ± 29	30	937 ± 53[1]	30	798 ± 57[2]
SZP alone	50	676 ± 52[1]	100	938 ± 47[1]	100	833 ± 51[2]
Ulcerogen		586 ± 35[3]		805 ± 80[1]		594 ± 29[3]
" +PhCL28A	30	548 ± 66[3]	30	478 ± 67[3,4]	30	408 ± 48[3,5]
" + SZP	100	673 ± 38[3]	100	570 ± 76[3,4]	100	574 ± 59[3]

[1] $p < 0.01$ [2] $p < 0.02$ [3] $p < 0.001$ w.r.t. vehicle
[4] $p < 0.02$ [5] $p < 0.001$ w.r.t. ulcerogen alone

From these results, we conclude that the anti PGDH activity in vitro of SZP and PhCL28A is unlikely to contribute to their anti-ulcer activity.

The authors thank Dr. T. Purcell Dept of Chemistry L.E.R.S. for synthetising PhCL28A and Mr P. Gaubert and Melle S. Hamon for expert technical assistance.

Berry C.N. et al (1985), J. Pharm. Pharmacol., 37, 622-628.
Hoult J.R.S. and Moore P.K. (1978), Br. J. Pharmacol., 64, 6-8.
Ogle C.W. and Cho C.H. (1985) Eur. J. Pharmacol., 112, 285-286.

POTENTIATION OF PROSTAGLANDIN (PG) SYNTHESIS BY AN INHIBITOR OF PG DEHYDROGENASE (PH CL^{28} A) IN RAT ISOLATED LUNG

Y.S. Bakhle* and J.J. Pankhania, Department of Pharmacology, Royal College of Surgeons, Lincoln's Inn Fields, London WC2A 3PN

The lung both synthesizes and inactivates PGs. We have argued that inhibitors of PG inactivation ought to potentiate PG synthesis from exogenous or endogenous arachidonic acid (AA), but have been unable to demonstrate this effect so far (Bakhle & Pankhania, 1985). We have now used a potent inhibitor of PGDH, Ph CL28A, which has no direct effect on PG synthesis in vitro (Berry et al, 1985).

Rat isolated lungs were perfused via the pulmonary artery at a flow of 8ml/min with warmed, oxygenated Krebs solution (Bakhle, 1980). Inactivation of PGE_2 and $PGF_{2\alpha}$, given as bolus injections (0.1ml; 500ng) into the pulmonary circulation, was measured by bioassay on hamster stomach strip and rat colon superfused with lung effluent. The inhibitor was dissolved in the Krebs solution perfusate entering the lung. The survival of both PGs was markedly increased during perfusion with Ph CL28A (300nM); PGE_2 more than 2-fold and $PGF_{2\alpha}$ 10-fold (n=5,6). Survival was also measured by radioimmunoassay (RIA); Ph CL28A increased PGE_2 survival 2.6-fold and that of $PGF_{2\alpha}$ 4.4-fold (n=4,5). Increased PG survival in isolated lung can reflect either decreased uptake or decreased PGDH activity and we have earlier differentiated between these possibilities by measuring the rate of efflux of radioactivity from lung following injection of ^{14}C-PGs (Bakhle, 1980). The efflux rate was expressed as T1/2 (the time for 50% of injected ^{14}C to appear in the effluent). Ph CL28A increased the T1/2 for PGE_2 from 37±2s to 63±6s (n=8; $p<0.05$, mean ± s.e.) and for $PGF_{2\alpha}$ from 40±3s to 68±10s(n=7).

The output of PGE_2, $PGF_{2\alpha}$ and PGI_2 (as 6-oxo-$PGF_{1\alpha}$) in lung effluent following a bolus injection of exogenous AA (10μg) was measured by RIA. PGE_2 and $PGF_{2\alpha}$ output was increased during Ph CL28A 1.8-fold and 2.6-fold respectively. However, 6-oxo-$PGF_{1\alpha}$ was not changed (16.8+4.0 vs 18.9±3.3 ng/ml, n=4). Synthesis of these three PGs from endogenous AA was stimulated by the calcium ionophore A23187 (3μg). Before treatment with Ph CL28A, ionophore induced the release of very low amounts of PGE_2 and $PGF_{2\alpha}$, below our assay limit of 0.2ng/ml, and a larger amount of 6-oxo-$PGF_{1\alpha}$ (2.0±0.4ng/ml). During Ph CL28A perfusion, ionophore-induced output of PGE_2 and $PGF_{2\alpha}$ increased to 0.9±0.1 and 1.4±0.2ng/ml respectively and now 6-oxo-$PGF_{1\alpha}$ increased over 4-fold to 9.3±1.7ng/ml.

In agreement with Berry et al (1985), we found Ph CL28A to inhibit the inactivation of PGs in isolated lungs. The increased survival of PGE_2 and $PGF_{2\alpha}$ following Ph CL28A did not reflect decreased PG uptake as seen with other PGDH inhibitors (Bakhle, 1980). We were also able to demonstrate, using this compound, the postulated increase in PG output from lung. The difference in potentiation of PGI_2 output from exogenous and endogenous AA supports previous suggestions that the cells involved in the two conditions are differently located within the lung (Bakhle et al, 1985). Lastly, our work suggests that the effects of drugs on PG synthesis may not be adequately described if only exogenous substrate is used.

We thank Dr H Agback (Pharmacia) for a generous gift of Ph CL28A.

Berry,C.N. et al. (1985) J.Pharm.Pharmacol. 37: 622-628
Bakhle,Y.S. (1980) Eur.J.Pharmac. 68:493-496
Bakhle,Y.S. et al. (1985) Br.J.Pharmac. 86:55-62
Bakhle,Y.S. and Pankhania,J.J. (1985) Br.J.Pharmac 85:275P

THE EFFECT OF PGI_2 ON POLYMORPHONUCLEAR LEUKOCYTE (PMNL) ACCUMULATION IN RABBIT SKIN

M. Rampart* & T.J. Williams, Vascular Biology Section, MRC Clinical Research Centre, Harrow, HA1 3UJ, Middlesex.

In rabbit skin plasma leakage induced by chemotactic agents such as C5a, FMLP and LTB_4, is critically dependent on a rapid interaction (<5 min) between circulating PMNL and venular endothelial cells (Wedmore & Williams, 1981). Recently, we have reported that PGI_2 (50ng/Kg/min i.v. infusion) selectively suppresses PMNL-dependent increase in vascular permeability, without affecting plasma leakage induced by non-chemoattractants such as bradykinin and histamine (Rampart & Williams, 1986). In vitro, PGI_2 inhibits PMNL activation, including adherence to endothelial cells (Zimmerman et al., 1985). In the present study we have investigated the effect of local and systemic PGI_2 on PMNL accumulation in the rabbit skin using ^{111}Indium labelled neutrophils.

Rabbit PMNL's (>95% pure) were harvested from citrated blood by a two layer discontinuous Percoll-plasma gradient after initial red cell sedimentation with hydroxy ethyl starch. PMNL's were incubated with ^{111}In chelated to 2-mercapto-pyridine-N-oxide for 15 min at room temperature. The labelled cells were washed twice, resuspended in autologous plasma and injected i.v. into rabbits anaesthetised with sodium pentobarbital (30mg/kg, i.v). Local PMNL accumulation in response to i.d. injection (in 0.1 ml volumes) into the clipped dorsal skin of C5a, FMLP (5.10^{-13}-5.10^{-11}moles) and LTB_4 (10^{-12}-10^{-10}moles) was measured over a 30 min period.

Of the injected cells 40 ± 4% were circulating after 5 min, and they disappeared from the circulation with a half-life of 6.5 ± 1h. Free ^{111}In in plasma was always less than 3% (typically 0.5-2.5%). When injected alone, C5a, FMLP and LTB_4 induced only small responses. The simultaneous i.d. injection of PGI_2 (3.10^{-12}-3.10^{-10}moles) greatly potentiated PMNL accumulation, an effect also observed for other vasodilator substances (PGE_2, PGE_1, VIP, CGRP) but not with 6-oxo-$PGF_{1\alpha}$. In contrast, an i.v. infusion of PGI_2 at a sub-vasodepressor dose (50ng/kg/min) almost completely abolished PMNL accumulation induced by i.d. mixtures of chemoattractants plus PGI_2. The results (expressed as number of ^{111}In-PMNL per site) for C5a (5.10^{-11}moles) were: C5a alone: 376 ± 77; C5a + PGI_2 (3.10^{-10}moles): 2118 ± 154; PGI_2 alone: 257 ± 27; saline: 205 ± 40. In animals given PGI_2 infusion the results were as follows: C5a alone: 312 ± 68; C5a + PGI_2: 367 ± 81; PGI_2 alone: 273 ± 70; saline: 235 ± 70. All results are mean ± SEM for 6 animals.

These experiments indicate that the effect of prostaglandins in an inflammatory reaction is critically dependent on their site of generation and action. Thus, PGI_2 can either potentiate or attenuate the inflammatory response in our models, with PGI_2 generated extravascularly enhancing cell infiltration and oedema by increasing blood flow, and intravascular PGI_2 suppressing PMNL accumulation and oedema by inhibiting intravascular PMNL's.

This work was supported by the British Heart Foundation.

Rampart, M. & Williams, T.J. (1986). Am.J.Pathol., 124, 66-73.
Wedmore, C.V. & Williams, T.J. (1981). Nature, 289, 646-650.
Zimmerman, G.A., Wiseman, G.A. & Hill, H.R. (1985). J.Immunol., 134, 1866-1874.

STUDIES ON THE ROLES OF PG ENDOPEROXIDES/THROMBOXANE A_2 AND DIACYLGLYCEROL IN 'WEAK' AGONIST-INDUCED PLATELET SECRETION

V.V. Kakkar & S. Krishnamurthi,* Thrombosis Research Unit, King's College School of Medicine & Dentistry, Denmark Hill, London SE5 8RX.

It is well known that secretion of granule constituents induced by 'weak' platelet agonists such as ADP, adrenaline or platelet-activating factor (PAF) is mediated entirely via endogenously formed prostaglandin (PG) endoperoxides/thromboxane A_2 (TxA_2) (Charo et al, 1977; Krishnamurthi et al, 1984). However, the mechanism by which endogenously formed PG endoperoxides/TxA_2 stimulate secretion in response to a weak agonist is still not clear. Earlier views that they act as Ca^{2+} ionophores (Gerrard et al, 1981) have been disputed by the work of Rink and Hallam (1984) who have shown that $[Ca^{2+}]i$ elevations in response to ADP and PAF is not significantly affected by blockage of prostanoid synthesis using indomethacin. These workers have therefore suggested that endogenously formed PG endoperoxides/TxA_2 may act via inducing formation of 1,2-diacylglycerol (DAG), which is known to activate the enzyme protein kinase C and induce secretion.

In the present study, we have tested this hypothesis and compared the abilities of exogenously added U46619, the PG endoperoxide analogue and, sn-1-oleoyl 2-acetyl glycerol (OAG) and sn-1,2-dioctanoylglycerol (diC_8), the membrane-permeant DAG analogues at restoring weak agonist-induced secretion in indomethacin-treated platelets, in the absence of endogenous PG/Tx synthesis. $[^{14}C]$-5HT secretion from pre-loaded, washed human platelets was correlated with the levels of $[Ca^{2+}]i$, using platelets loaded with quin 2, the fluorescent Ca^{2+} probe. All results are means of 12 determinations from 3 separate experiments.

Concentrations of OAG (62-125μM) and diC_8 (15-30μM), which have previously been shown to be fully effective at activating protein kinase C, failed to significantly enhance $[^{14}C]$-5HT secretion in combination with ADP (10μM) or adrenaline (10μM), although they potentiated platelet aggregation, when added 10-30 sec after these agonists to indomethacin (10μM)-treated platelets. A small but significant potentiation of $[^{14}C]$-5HT secretion was observed with combinations of OAG (62μM) and PAF (0.2μM) and, diC_8 (30μM) and PAF (0.2μM), with the effect of these pairs of agents being greater than additive (diC_8-9.8%, PAF-1.2%, PAF+diC_8-20.2% 5HT release). In contrast, a low concentration of U46619 (0.2μM), that induced nc aggregation, $[^{14}C]$-5HT secretion or rise in $[Ca^{2+}]i$ levels on its own, was able to synergize strongly at potentiating secretion in combination with all three weak agonists examined, as well as in combination with OAG and diC_8 in indomethacin-treated platelets (U46619-0%, 0.2μM PAF+U46619-33%, U46619+30μM diC_8-48% 5HT release). The greater effectiveness of U46619 at potentiating secretion in combination with the weak agonists, compared with that of OAG or diC_8, was not related to different degrees of $[Ca^{2+}]i$ mobilisation, as PAF-induced rise in $[Ca^{2+}]i$ occurred to a similar degree in the presence of U46619 and diC_8, and ranged between 500 and 600nM. At a higher concentration of U46619 (0.6μM), which was maximally effective at inducing secretion and elevating $[Ca^{2+}]i$ levels, addition of the weak agonists or OAG or diC_8, along with U46619, resulted in a further enhancement of secretion which was independent of changes in $[Ca^{2+}]i$ levels. The results demonstrate that U46619 but not OAG or diC_8, is able to fully restore weak agonist-induced secretion in indomethacin-treated platelets, suggesting that the actions of endogenously formed PG endoperoxides/TxA_2 cannot be substituted by DAG and raised $[Ca^{2+}]i$ levels and, may be mediated via a mechanism additional to that involving these mediators.

Charo, I. et al (1977) J. Clin. Invest. 60, 866-873.
Gerrard J.M. et al (1981) in: Platelets in Biology and Pathology (Gordon JL ed) vol.2, pp407-436, Elsevier/North-Holland.
Krishnamurthi, S. et al (1984) Biochem. Pharm. 33, 3025-3035.
Rink, T.J. & Hallam, T.J. (1984) Trends Biochem. Sci. 9, 215-219.

THE CONTRIBUTION OF EICOSANOIDS TO THE ACUTE INFLAMMATORY REACTION INDUCED BY ARACHIDONIC ACID IN RABBIT SKIN

D.M. Aked* and S.J. Foster, Bioscience I Department, ICI PLC, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire SK10 4TG.

Injection of arachidonic acid (AA) into rabbit skin causes a polymorphonuclear leukocyte (PMNL)-dependent acute inflammatory reaction which is attenuated by inhibitors of AA metabolism suggesting that both cyclo-oxygenase and 5-lipoxygenase products contribute to the reaction (Aked et al, 1986). In order to substantiate this, experiments have been carried out to determine the prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) content of inflamed skin sites following AA treatment.

AA (100µg) in 100µl physiological saline or vehicle were injected intradermally into the preshaven backs of female New Zealand White rabbits (2.5-3.0Kg). At various times later the rabbits were killed with a lethal i.v. dose of pentobarbitone sodium and the skin on the back removed. The areas around the injection sites (8mm) were punched out, plunged into liquid nitrogen and homogenised in 50% ice-cold methanol in 0.1M sodium acetate pH 4.2. Eicosanoids were extracted from a 12000 xg supernatant using C_{18} bond elute mini columns by a modification of the procedure described by Powell (1980). PGE_2 and LTB_4 in the column eluate was measured by radioimmunoassay and authenticated by HPLC analysis. Overall eicosanoid recovery, monitored with radiolabelled standards, was 65.0 ± 2.8% and 41.9 ± 1.7% for PGE_2 and LTB_4 respectively.

The i-PGE_2 and i-LTB_4 content (mean ± s.e.m. ng/site) of skin at various times after AA or saline injections are shown for a typical experiment in the table.

TIME(MIN)	AA		SALINE	
	i-PGE_2	i-LTB_4	i-PGE_2	i-LTB_4
5	46.8±10.3	2.15±0.20	3.1±1.2	0.80±0.08
15	29.1± 8.5	1.61±0.20	–	–
20	40.6± 8.2	1.90±0.08	–	–
35	14.2± 3.0	1.46±0.12	–	–
65	13.5± 4.8	1.32±0.17	4.5±1.7	0.71±0.12

Maximal levels of both i-PGE_2 and i-LTB_4 were observed at 5 min after AA injection, the earliest practical sampling time point. In 4 separate experiments the levels of PGE_2 and LTB_4 at 5 min were 56.6±10.9 and 5.74±1.76 ng/site respectively. The levels of both eicosanoids following AA injection decayed almost to saline control values by 65 min. Likewise, the levels of PGE_2 and LTB_4 in skin injected with authentic PGE_2 (200ng/site) and LTB_4 (100ng/site) decayed at a similar rate. We investigated whether the levels of eicosanoids measured following AA treatment caused inflammation. Intradermal injections of LTB_4 (1-10ng) caused a time-dependent PMNL infiltration, assessed histologically and when combined with PGE_2 (50-100ng) induced oedema as measured by extravasation of ^{125}I-albumin.

This data further supports a role for both PGE_2 and LTB_4 in the mechanism of AA-induced inflammation in rabbit skin. The model therefore has utility for evaluating the anti-inflammatory efficacy of inhibitors of AA metabolism.

Aked, D., Foster, S.J., Howarth, A., McCormick, M.E. and Potts, H.C. (1986). Br. J. Pharmacol. In press.
Powell, W.S. (1980). Prostaglandins, 20, 947-957.

ACTIVATION AND INACTIVATION OF 5-LIPOXYGENASE BY ARACHIDONIC ACID

M. P. Dicken, D. J. Masters*, R. M. McMillan and V. C. Vickers, Bioscience I Dept., ICI Pharmaceuticals Division, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG.

Inhibitors of arachidonic acid (AA) metabolism via 5-lipoxygenase (5-LPO) have therapeutic potential in allergic diseases, inflammatory diseases and dermatology. Assays for 5-LPO have been reported using enzymes from various species of leukocytes. These generally employ single time point assays to determine total formation of products rather than true enzyme rates. In this study we have employed a kinetic assay of 5-LPO and have investigated the regulation of enzyme activity by AA and its products.

5-LPO was prepared by sonication of rat basophilic leukaemia cells. Metabolism of [^{14}C]-AA was studied using the 105,000g supernatant and was quantified by mini column fractionation (Masters et al, 1985). Metabolism of unlabelled AA was monitored spectrophotometrically (Aharony & Stein, 1986).

Metabolism of [^{14}C]-AA (1-50μM) at 25°C occurred rapidly and was essentially complete within 60 seconds by which time 80% substrate conversion had occurred. Lowering reaction temperatures to 10°C or 15°C reduced enzyme rates but overall substrate conversion was also decreased to 45% and 60% respectively. These slower reactions were preceded by extensive lag phases which showed considerable inter-experiment variation; in 4 separate experiments at 15°C with different enzyme batches consistent substrate conversion of 57-63% was obtained with 15μM [^{14}C]-AA but lag phases ranged from 30 - 120 sec and the time at which maximum conversion was reached varied from 2-5 min. The variable lag times led to misleading assessments of lipoxygenase inhibitor potencies especially with compounds that extend lag phases (e.g. AA861). Continuous spectrophotometric assessment of 5-LPO revealed that enzyme reactions at 37°C were preceded by brief lag phases which were directly proportional to substrate concentrations. At lower temperatures (10-25°C) lag phases increased whilst the reaction rates and end points decreased. A second addition of AA, after enzyme reactions were complete, failed to stimulate further metabolism which was consistent with formation of an inactivator. Pre-incubation of 5-LPO with AA led to time-dependent enzyme inactivation. Dose response studies demonstrated that AA was more potent as an inhibitor of 5-LPO (IC_{50} = 0.1 -0.8μM) than as a substrate for the enzyme (Km = 5-10μM).

These data are consistent with a model of 5-LPO which requires activation by substrate and inactivation by low concentrations of product. The complex kinetics of 5-LPO, including variable lag times and product inactivation, limit the utility of single time point assays for mechanistic studies on lipoxygenase inhibitors.

Aharony, D. & Stein, R. L. (1986). J. Biol. Chem. IN PRESS.

Masters, D. J., Spruce, K. E., Vickers, V. C. & McMillan, R. M. (1985) Br. J. Pharmacol. 84, Proc. Suppl., 45P.

THE NEGATIVE INOTOPIC ACTIONS OF LEUKOTRIENES ARE SECONDARY TO CORONARY VASOCONSTRICTION

M.A. TREVETHICK, C.J. WHELAN and G. WRIGHT, Dept of Respiratory Pharmacology and Biochemistry, Glaxo Group Research, Ware, Herts. U.K.

Leukotrienes (LT) decrease cardiac output in many species in vivo (Kennedy et al, 1985), and cause coronary vasoconstriction together with decreases in force of ventricular contraction in the isolated perfused guinea-pig heart (Letts and Piper, 1982). These effects may be due to either a direct action on the myocardium, or to coronary vasoconstriction (Feuerstein, 1984). We have attempted to distinguish between these two mechanisms by studying the actions of LT on a range of preparations from cat and guinea-pig hearts.

Hearts were removed from pentobarbitone/chloralose anaesthetised cats or from freshly killed guinea-pigs. Rings from the left anterior descending coronary artery of the cat were mounted for isometric recording, under a resting tension of 0.2g (Trevethick et al, 1986). Papillary muscles and ventricular strips were dissected from cat and guinea-pig hearts under oxygenated Krebs solution at 32°C. Preparations were stimulated in Krebs solution at 32°C by square wave pulses of 2Hz frequency, 1 msec duration and threshold voltage. Guinea-pig isolated hearts were perfused with a modified Krebs solution as described previously (Kennedy et al, 1983).

LTC_4, LTD_4, or LTE_4 caused concentration-dependent contractions of cat coronary artery rings from 50% of the cats studied. The rank order of agonist potency for this effect was LTD_4 (EC_{50} $5x10^{-8}M$) > LTC_4 > LTE_4. The force of contraction of cat electrically-paced, right papillary muscles or ventricular strips was not reduced by either LTC_4, LTD_4 or LTE_4 (10^{-9}-$10^{-5}M$). Verapamil ($10^{-7}M$) consistently reduced the force of contraction by 42.5 ± 4.3% (mean ± S.E.M, n=10) and 65.6 ± 5.9% (mean ± S.E.M, n=7) in right ventricle and papillary muscle preparations respectively.

In guinea-pig perfused hearts, LTC_4 and LTD_4 (10^{-10}-$10^{-5}M$) caused concentration-related increases in perfusion pressure and decreases in the force of ventricular contraction. LTC_4-induced decreases in ventricular contraction were linearly-related to the increases in perfusion pressure (correlation coefficient 0.805). LTC_4, LTD_4 or LTE_4 (10^{-9}-$10^{-5}M$) did not decrease the force of contraction in either guinea-pig electrically-paced, left papillary muscles or right ventricular strips. In contrast, verapamil ($10^{-6}M$) consistently decreased contraction by 74.9 ± 3.2% (mean ± S.E.M, n=4; left papillary) and 78.7% ± 5.3% (mean ± S.E.M, n=5; right ventricle).

These results demonstrate that LTs have no apparent direct negative inotropic action on either cat or guinea-pig papillary muscles or ventricular strips. In contrast, LTs constrict the cat coronary artery and increase perfusion pressure in the guinea-pig heart. Thus, we conclude that the negative inotropic action of LTs is a consequence of coronary vasoconstriction.

Feuerstein, G., (1984), Prostaglandins, 27, 781
Kennedy I. et al, (1983), Br. J. Pharmac., 79, 218p
Kennedy, I. et al, (1985), Br. J. Pharmac., 86, 523p
Letts, L.G. and Piper, P.J., (1982), Br. J. Pharmac., 76, 169
Trevethick, M.A. et al, (1986), Br. J. Pharmac., 88, 393p

1,2-DIOCTANOYLGLYCEROL BUT NOT 1-OLEOYL-2-ACETYLGLYCEROL INHIBITS AGONIST-INDUCED PLATELET RESPONSES

Joseph, SK*, Kakkar VV, Krishnamurthi S & Patel Y, Thrombosis Research Unit, King's College School of Medicine & Dentistry, Denmark Hill, London SE5 8RX.

The role of the enzyme protein kinase C (PrkC) in supporting platelet secretion is now well documented (Nishizuka, 1984). Recent studies though (Drummond & MacIntyre 1985; Krishnamurthi et al, 1986) have demonstrated that a number of agonist-induced platelet responses can be inhibited by prolonged exposure to an activator of PrkC, phorbol 12-myristate 13-acetate (PMA). These results have led to the idea that PrkC may be involved in the termination of agonist-induced platelet activation. However, whether the endogenous activator of PrkC in platelets, 1,2-diacylglycerol (DAG), which is structurally and metabolically distinct from PMA, displays the same inhibitory tendency is unclear. In this study, we have examined the effects of two membrane permeable DAG analogues, 1-oleoyl 2-acetyl glycerol (OAG) and 1,2-dioctanoylglycerol (diC_8), as well as PMA on intracellular Ca^{2+} ($[Ca^{2+}]i$) mobilisation and dense-granule secretion induced by thrombin, collagen and the thromboxane (Tx) mimetic, U46619.

All studies were performed using plasma-free suspensions of human platelets suspended in a Hepes-tyrodes buffer pH7.4, pre-labelled with either Quin 2 (a Ca^{2+} indicator) or [^{14}C]-5HT (a dense-granule marker) and concentrations of agonists that were maximally effective at inducing [^{14}C]-5HT secretion. In response to thrombin or U46619, $[Ca^{2+}]i$ increased from resting levels of 100nM to 758±108nM and 712±58nM respectively. As previously shown (Rink et al, 1983) no significant change in resting $[Ca^{2+}]i$ levels was detected upon addition of collagen or the PrkC activators examined. Addition of diC_8 (30-60μm) or PMA (16nM) 10sec before or after thrombin or U46619 however, reduced the control response by 10-15% and 30-80% respectively, with the degree of inhibition increasing with longer times of preincubation (5min). In contrast to these effects, both diC_8 and PMA had biphasic effects on agonist-induced [^{14}C]-5HT secretion. With short exposure times (<1min pre- or post-agonist addition), secretion in response to thrombin or collagen was unaffected by either activator, and in the case of U46619, was potentiated 1.4-1.6 fold over control levels. On the other hand, with long pre-incubation times (5min) an inhibitory effect (10-40%) of PMA or diC_8 on agonist-induced secretion was evident. This was accompanied by a 40-70% reduction of thrombin and collagen-induced TxB_2 generation. As collagen-induced but not thrombin-induced [^{14}C]-5HT secretion was significantly inhibited (50-60%) by indomethacin, the cyclooxygenase inhibitor, the inhibition of the former but not the latter by diC_8 or PMA may be related to the inhibition of TxB_2 formation. Unlike PMA or diC_8, OAG (63μM) had no significant inhibitory effect on agonist-induced $[Ca^{2+}]i$ mobilisation and [^{14}C]-5HT secretion even with long pre-incubation times (5min).

Two points emerge from our work: firstly as diC_8 and PMA have been shown to be more potent activators of PrkC than OAG and share similar effects on agonist-induced platelet functions, diC_8 rather than OAG may be a better tool as a mimic of endogenously-produced DAG. Secondly, the results with diC_8 suggest that DAG-activated PrkC is capable of exerting an inhibitory effect on agonist-induced phenomena. Further, as the addition of diC_8 after agonist mimics the <u>in-vivo</u> situation where DAG is produced within seconds of agonist stimulation, the inhibitory effects on $[Ca^{2+}]i$ mobilisation may constitute a physiologically relevant phenomenon mediated by PrkC. On the other hand, as [^{14}C]-5HT secretion is not similarly affected by diC_8, this response may not like-wise be regulated by PrkC.

Drummond, A.H. & MacIntyre, D.E. (1985) Trends Pharmacol. Sci. 6, 233-234.
Krishnamurthi, S., Joseph, S.K. & Kakkar, V.V. (1986) Biochem. J. 238, 193-199.
Nishizuka, Y. (1984) Nature 308, 693-698.
Rink, T.J., Sanchez, A. & Hallam, T.J. (1983) Nature 305, 317-319.

EFFECT OF PROTEIN KINASE C ACTIVATORS ON AGONIST INDUCED ARACHIDONATE RELEASE IN HUMAN PLATELETS

S.K. Joseph, V V. Kakkar & S. Krishnamurthi* Thrombosis Research Unit King's College School of Medicine & Dentistry, Denmark Hill, London SE5 8RX.

Agonist binding to platelet receptors leads to the generation of two principle second messengers, Ca^{2+} and 1,2 diacylglycerol (DAG) (Nishizuka, 1984; Rink & Hallam 1984). DAG, an activator of protein kinase C. can stimulate platelet secretion without a rise in intracellular Ca^{2+}, $[Ca^{2+}]i$, levels although raised $[Ca^{2+}]i$ can potentiate its action. While the role of DAG in mediating platelet secretion is now well established, its role in the induction of arachidonate release from membrane phospholipids is not known. We have examined the effect of the DAG analogue 1,2 dioctanoylglycerol (diC_8) and phorbol 12-myristate 13-acetate (PMA), the phorbol ester, which mimics the actions of DAG on protein kinase C and platelet secretion on arachidonate release induced by thrombin, collagen and the Ca^{2+} ionophore, ionomycin, and correlated this with the levels of $[Ca^{2+}]i$ in quin 2-loaded platelets.

Experiments were carried out using phenidone (200μM, a combined lipoxygenase and cyclooxygenase inhibitor)-treated washed, human platelets pre-labelled with $[^3H]$-arachidonic acid or quin 2 and, sub-maximal concentrations of thrombin, collagen and ionomycin. Incubation of $[^3H]$-arachidonate-labelled platelets with diC_8 (30 or 60μM) or PMA (16nM) over a 15 min period, resulted in no significant release of label compared to that in resting platelets. However, thrombin (0.2U/ml) induced a 6-8% release of labelled arachidonate and, addition of diC_8 (30-60μM) or PMA (16nM) 10 sec-5 min before or 10-20 sec after thrombin, resulted in a significant reduction (20-40%) of this response. On the other hand, $[^3H]$-arachidonate release in response to collagen (20μg/ml) and ionomycin (6μM) were significantly potentiated by 1.2-1.9 fold and 1.5-30 fold over control respectively by pre- or post-agonist additions of diC_8 (30-60μM) or PMA (16nM). With very long pre-incubation times of 10-15 min, diC_8 (30-60μM) but not PMA (16nM) had a slight inhibitory effect (15-20%) on collagen-induced arachidonate release.

Dose-response curves of ionomycin-induced $[Ca^{2+}]i$ mobilisation and arachidonate release revealed that potentiation of the latter by 10 sec pre-incubation of diC_8 or PMA, only occurred at $[Ca^{2+}]i$ levels >1uM and ionomycin concentrations >4μM, indicating a dependence on critical levels of $[Ca^{2+}]i$. Addition of diC_8 or PMA to quin 2-loaded platelets did not alter the resting $[Ca^{2+}]i$ levels or $[Ca^{2+}]i$ mobilisation induced by ionomycin. On the other hand, recent reports (MacIntyre et al, 1985; Krishnamurthi et al, 1986) have demonstrated an inhibition of thrombin-induced $[Ca^{2+}]i$ mobilisation by PMA and, in the present study, we found diC_8 to have similar inhibitory effects. The inhibition of thrombin-induced arachidonate release, which is known to be a Ca^{2+}-dependent phenomenon, can therefore be interpreted as being related to the inhibition of $[Ca^{2+}]i$ mobilisation. Interestingly, collagen has been shown to induce no rise in $[Ca^{2+}]i$ levels even though it induces significant arachidonate release (Pollock et al, 1986), and this may explain why diC_8 and PMA had differential effects on collagen-induced, compared with thrombin-induced arachidonate release. In conclusion, our results suggest that the effect of protein kinase C activators such as diC_8 or PMA on agonist-induced arachidonate release, may depend on two factors: firstly, on whether arachidonate release is dependent on agonist-induced $[Ca^{2+}]i$ mobilisation and secondly, whether the latter is inhibited by protein kinase C activation.

Krishnamurthi, S., Joseph, S.K. & Kakkar, V.V. (1986) Biochem. J. 238, 193-199.
MacIntyre, D.E., McNicol, A. & Drummond, A.H. (1985) FEBS Lett. 180, 160-164.
Nishizuka, Y. (1984) Nature 308, 693-698.
Pollock, W.K., Rink, T.J. & Irvine, R.F. (1986) Biochem. J. 235, 869-877.
Rink, T.J. (1984) Trends Biochem. Sci. 9, 215-219.

RECOVERY OF ENDOTHELIAL PROSTACYCLIN SYNTHESIS AFTER INHIBITION BY ASPIRIN, EFFECTS OF CYCLOHEXIMIDE AND EPIDERMAL GROWTH FACTOR

C.E. Frazer & J.M. Ritter*, Department of Clinical Pharmacology, Royal Postgraduate Medical School, Ducane Road, London W12 OHS

Aspirin acetylates fatty acid cyclo-oxygenase (Roth & Majerus, 1975) thereby inhibiting prostaglandin (PG) synthesis. Cultured cells recover the ability to synthesise prostacyclin (PGI_2) after treatment with aspirin by new protein synthesis (Czervionke et al, 1979). Recovery depends on culture conditions and is accelerated by epidermal growth factor, EGF (Bailey et al, 1985). Heavey et al (1985) argued that different tissues recover from aspirin at different rates in vivo. They postulated that endothelium recovers more rapidly than other tissues, particularly vascular media in which PGI_2 synthesis may be substantially inhibited more than 48 h after a single dose of aspirin (Hanley et al, 1981). We investigated this in groups (n = 6 for each condition) of male New Zealand white rabbits (2.4-3.8 kg) injected with vehicle, or with aspirin (10 mg/kg iv) 0.5, 6 or 24 h before death. Some animals were treated with cycloheximide or with EGF, to determine if these influence the partial recovery of PGI_2 synthesis found 6 h after aspirin. Animals were killed with pentobarbitone and portions of aorta, heart (left ventricle), gastric mucosa, ileum, pericardium and peritonium removed rapidly and placed in Hanks' solution on ice. A length of aorta was opened and incubated (15 min) in a template chamber with wells containing Hanks' solution at 37°C overlying the endothelium, to estimate endothelial PGI_2 synthesis (Eldor et al, 1981). Endothelium was removed by abrasion from a separate portion of aorta, which was used to determine PGI_2 synthesis by vascular media and adventitia. This and the other tissues were mechanically chopped into 1 mm cubes before incubation in Hanks' solution (37°C, 1 h). PGI_2 synthesis was assessed by radioimmunoassay of 6-oxo-$PGF_{1\alpha}$, its stable hydrolysis product, in the incubation media (Orchard et al, 1982). Results after aspirin were expressed as percent control ± s.e.mean. Differences were evaluated by the rank sum test and considered significant when $P < 0.05$. At 0.5 h inhibition was greater than 95% in all tissues. Aortic endothelial PGI_2 synthesis recovered to 24 ± 7% control 6 h after aspirin, significantly more than chopped aorta which produced 4.2 ± 0.9% of control 6-oxo-$PGF_{1\alpha}$ at this time (other tissues 2-11%). At 24 h endothelial PGI_2 synthesis was 64 ± 8% control, while chopped aorta produced 33 ± 8% control, and other tissues 19-47%. Cycloheximide (100 mg/kg iv before aspirin or vehicle, 50 mg/kg at 2 h and 4 h) augmented PGI_2 synthesis by all tissues 6 h after vehicle (by 1.2-12.9 fold), but abolished the partial recovery 6 h after aspirin (6-oxo-$PGF_{1\alpha}$ < 2.5% control for all tissues). EGF (2 µg/kg iv at 3 h and 1 µg/kg at 4 h) significantly increased recovery of aortic endothelial PGI_2 synthesis after aspirin to 64 ± 9% control at 6 h (other tissues 9-26%). It is concluded that recovery of the capacity to synthesise PGI_2 following inhibition by intravenous aspirin occurs faster in aortic endothelium than in deeper vascular layers, depends on new protein synthesis and is accelerated by EGF.

This work was supported by the British Heart Foundation.

Bailey, J.M. et al (1985) J.Lipid Research 26, 54
Czervionke, R.L. et al (1979) J.Clin.Invest. 63, 1089
Eldor, A. et al (1981) J.Clin.Invest. 67, 735
Hanley, S.P. et al (1981) Lancet 1, 969
Heavey, D.J. et al (1985) Nature (London) 318, 186
Orchard, M.A. et al (1982) Biochem.Soc.Trans. 10, 241
Roth, G.J. & Majerus, P.W. (1975) J.Clin.Invest. 56, 624

64P

CHEMOATTRACTANT PROPERTIES OF PROSTAGLANDIN E_2 AND LEUKOTRIENE B_4 FOR EQUINE POLYMORPHONUCLEAR AND MONONUCLEAR LEUCOCYTES

J.Dawson*,P.Lees and A.D.Sedgwick, RVC Inflammation Unit, The Royal Veterinary College, North Mymms,Hatfield, Herts., AL9 7TA.

Polymorphonuclear leucocytes (PMN,94% purity) and mononuclear cells (MN, 99% purity) were separated from heparinised equine blood samples on discontinuous Percoll gradients. Two in vitro systems were used to assess the actions of PGE_2 and LTB_4, the Boyden chamber assay (Sedgwick et al.,1982) and the agarose microdroplet assay (Smith and Walker, 1980). In both assays 100 uM N-formyl-methionyl-leucyl-phenylalanine (FMLP) was used as a standard chemoattractant for MN cells and zymosan activated plasma (ZAP) was used as the standard for PMN leucocytes.

The effects of each eicosanoid were investigated at three concentrations, selected to include two concentrations within the range of those detected in equine acute inflammatory exudate (Lees and Higgins, 1985) and one higher concentration (Table 1).

Table 1. Chemoattractant properties of PGE_2 and LTB_4 expressed as percentage increases±s.e.mean (n=6)from controls

Chemoattractant (ng/ml)		Boyden chamber		Agarose microdroplet	
		PMN	MN	PMN	MN
ZAP		190±2**	-	54±7**	-
FMLP		-	165±5**	-	20±8**
PGE_2	5	42±8**	6±2*	25±7**	0.3±5
	50	59±5**	21±1**	29±7**	5±6
	500	89±2**	69±3**	32±6**	22±8**
LTB_4	0.1	47±4**	16±1**	13±7*	6±4
	1.0	90±3**	46±1**	43±10**	8±6
	10.0	118±7**	91±3**	35±8**	12±5*

*p<0.05; **p<0.01

LTB_4 was a potent chemoattractant for equine PMN leucocytes and the similar action of PGE_2 is of interest since E-type prostaglandins have not, in general, been found to increase the locomotion of PMN cells. Both eicosanoids also increased MN cell movement at all concentrations tested in the Boyden chamber assay, but a significant effect was only obtained with high concentrations in the agarose microdroplet assay. This evidence suggests that PGE_2 and LTB_4 are chemokinetic and chemotactic for equine PMN cells at concentrations occurring in vivo, while both eicosanoids are predominantly chemotactic for MN leucocytes.

Supported by the Horserace Betting Levy Board (Project 334).

Lees,P. and Higgins, A.J. (1985). Equine Vet.J.,17,83-96.
Sedgwick,A.D., Edwards,J.C.W. and Willoughby,D.A. (1982). Inflammation,6,13-19.
Smith,M.J.H. and Walker,J.R. (1980). Br.J.Pharmac.69,473-478.

MECHANISM OF IL-1-INDUCED PGE_2 FORMATION IN HUMAN SYNOVIAL CELLS

M.L.Barrett, G.P.Lewis and L.A.J.O'Neill*, Department of Pharmacology, Hunterian Institute, Royal College of Surgeons, Lincolns Inn Fields, London WC2A 3PN.

Interleukin 1 (IL-1) is a potent mediator of inflammation produced by macrophages and, among its many effects, it causes release of prostaglandin E_2 (PGE_2) from synovial cells (Dayer et al, 1979). We have attempted to characterise this effect using cells cultured from synovia obtained from rheumatoid patients undergoing surgery.

Collection of tissue, culture of cells and radioimmunoassay have been described previously (Gordon and Lewis, 1984). Cells (grown to confluence on 24-well macrowell plates; 6 x 10^4 cells/well) were incubated with IL-1 (Genzyme Ultrapure) for fixed time periods and the level of PGE_2 determined in the supernatant. IL-1 (2.5 u/ml) caused a time-dependent production of PGE_2 increasing from a control level of $<$3ng/ml in unstimulated cells to 12±2ng/ml (n=4)after 6-8 h incubation and 54±3ng/ml (n=4) after 24 h.

The following findings have led us to the conclusion that IL-1 increases PGE_2 output by causing the induction of cyclo-oxygenase (CO).

1. Addition of arachidonic acid (AA, 10 μM) to unstimulated cells did not increase PGE_2 production (3±1ng/ml, n=3), indicating that CO activity was minimal in the synovial cells.
2. On the other hand, addition of the AA to IL-1-stimulated cells caused approximately a two-fold increase in PGE_2 production compared with cells treated with IL-1 alone (87±10 ng/ml, n=4), indicating that phospholipase A_2 activation by IL-1 was not the primary event.
3. The PGE_2 production caused by IL-1 alone and IL-1 + AA was prevented by treatment of the cells with cycloheximide 10μg/ml (5±1ng/ml and 4±1ng/ml, n=3) or actinomycin D 10μg/ml (2±1ng/ml and 3±1ng/ml, n=3), indicating that IL-1-induced PGE_2 production was dependent on protein synthesis.
4. Addition of aspirin (100 μM) to the cell cultures abolished PGE_2 production induced by IL-1. However, if the cells were pretreated with aspirin and the free aspirin removed by washing, subsequent addition of IL-1 (2.5u/ml) caused PGE_2 production, restoring it to 12±2ng/ml (n=4) after 6-8 h incubation. This finding indicated that the residual CO would be acetylated irreversibly by the aspirin and free aspirin in the culture medium would acetylate most of the newly-formed CO. However, if the free aspirin was removed, new CO would be available to metabolise AA.
5. Using incubation conditions under which [acetyl-^{14}C]-aspirin specifically acetylated CO, thereby giving a measure of the amount of CO in the cells (Roth, 1982), IL-1, in the presence of labelled aspirin, increased the radioactivity in the cell protein from 30±10dpm at zero time to 750±30dpm at 24 h,with a time-dependent relationship similar to that found for PGE_2 production. This finding indicated an increased amount of CO following incubation with IL-1.

It is therefore concluded that, in human rheumatoid synovial cells, IL-1 causes PGE_2 production primarily as a result of induction of the enzyme, cyclo-oxogenase. The involvement of phospholipase A_2 activity in IL-1 action is at present under investigation.

We gratefully acknowledge the support of CIBA-GEIGY, UK.

Dayer,J-M., Beard,J., Chen,L. and Krane, S.M.(1979) J.Clin.Invest., 64, 1386-1392
Gordon,D. and Lewis,G.P. (1984) Inflammation, 8, S87-102
Roth,G.J. (1982) Methods in Enzymology, 86, 392-393

ANTINOCICEPTIVE ACTIVITY OF WIN 48098.

E.R. Baizman, M.R. Bell, J.C. Bradford, R.A. Ferrari, D.R. Haubrich, D. Luttinger, M.S. Miller, M.H. Perrone, A.K. Pierson, J.K. Saelens and S.J. Ward*. Sterling-Winthrop Research Institute, Rensselaer, New York 12144, USA.

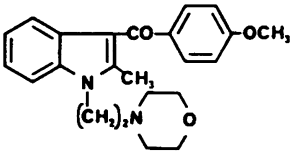
Table 1

NOCICEPTIVE STIMULUS	SPECIES	ED_{50} mg/kg (route)
ACh	mouse	40 (po); 7 (iv); 21 (sc); 2.4 (icv)
PGE_2	mouse	15 (sc)
55°C water	mouse	300 (sc)
Acetic acid	rat	12 (po)
Adjuvant arthritis	rat	100 (po)
Pressure	rat	1 (po)*
i.a. bradykinin	rat	120 (po)

*minimally effective dose

Figure 1

WIN 48098



Win 48098 (Figure 1) inhibited PGE_2 formation in mouse brain both in vitro (IC_{50} = 4.9 μM), and in vivo (ED_{50}) = 22 mg/kg, p.o.; 0.8 mg/kg i.v.). However, in contrast to most reference CO inhibitors, Win 48098 did not produce gastric lesions or irritation in four rodent models. Thus, in doses up to 10 times the antinociceptive dose (see Table 1), Win 48098 produced no gastric lesions in i) mice 2-4 h following oral administration, ii) mice following 1 day b.i.d. dosing, iii) pyloric-ligated rats 1.5 h after oral dosing, or iv) rats following 14 days of once daily dosing. In contrast, reference cyclooxygenase (CO) inhibitors such as zomepirac produced gastric lesions at antinociceptive doses in all four models.

The antinociceptive profile of Win 48098 also differed from that of reference CO inhibitors. Thus, Win 48098 demonstrated efficacy greater than that of reference CO inhibitors (e.g. zomepirac) and comparable to that of opioids (e.g. morphine) in the rat adjuvant arthritis and mouse tail immersion assays. Moreover, whereas reference CO inhibitors were significantly less active when PGE_2 was used as nociceptive stimulus (to bypass peripheral CO), Win 48098, like opioids, was antinociceptive against both ACh- and PGE_2-induced writhing in mice (Table 1). These data suggest that Win 48098 may produce antinociception by a mechanism other than inhibition of peripheral CO.

Win 48098 may possess a central component of action since Win 48098 was more potent i.c.v. than i.v. in the ACh writhing assay (Table 1). Moreover, whilst antinociceptive doses (i.v.) of reference CO inhibitors inhibited PGE_2 formation in brain and the periphery (stomach) equally, the ED_{50} for Win 48098 to inhibit PGE_2 formation in brain (ED_{50} = 0.78 mg/kg i.v.) was 11 times lower than that in stomach (ED_{50} = 8.6 mg/kg i.v.).

Although Win 48098 demonstrated several similarities to opioids, it is not an opioid, since its antinociceptive actions were not antagonized by naloxone (1 mg/kg s.c.), and Win 48098 did not bind to opioid binding sites in vitro (12% inhibition at 10 μM in a 3H-etorphine binding assay).

Thus, Win 48098 is a non-opioid analgesic that does not produce gastric irritation, may possess a central component of action, and which may be capable of managing more severe pain than is currently achieved with CO inhibitors.

THE INTERACTION BETWEEN TWO PUTATIVE SPINAL ANTINOCICEPTIVE AGENTS IN RATS ANAESTHETIZED WITH ALPHAXALONE-ALPHADALONE.

S.Barasi* and J.H. Williams, Department of Physiology,
University College, Cardiff. P.O. Box 78, Cardiff CF1 1XL.

Intrathecally applied noradrenaline (NA) and 5-hydroxytryptamine (5-HT) have both been reported to increase response latencies in various nociffensive tests in rats. Recently, it has been shown that depletion of spinal NA reduces the antinociceptive action of 5-HT (Minor, Post & Archer 1985), while both monoamines appear to interfere with the spinal anti-nociceptive action of dopamine (DA)(Jensen & Smith 1983). The aims of the present study were to investigate further the possible interactions between the putative spinal antinociceptive agents NA and 5-HT.

Male Wistar rats were initially anaesthetised with halothane and subsequently maintained in a lightly anaesthetised state with a constant infusion of Alphaxalone-Alphadalone (Saffan 7-10 mgs/Kg/h i.v.). Drugs were applied intrathecally in a volume of 10 µl. NA bitartrate (15nM/rat) increased the tail flick latency (TFL) elicited by radiant heat applied to the blackened underside of the tail, to a cut-off value (6s) for 120 $\pm$ 5.5 m(n = 12). In contrast, a similar injection of NA in rats lightly anaesthetised with sodium pentobarbital (6-12 mg/Kg/h) increased the TFL for 60.8 $\pm$ 3.2 mins (n = 5).

Intrathecal injections of 5-HT (260nM/rat) increased the TFL for 21 $\pm$ 3 m(n = 7). In these interaction studies the TFL returned to control levels for at least 16 minutes before a subsequent injection of NA (15nM/rat) was made. Following the 5-HT injection, the increase in TFL in response to NA lasted only 64.6 $\pm$ 6.7m (N = 7).

In other experiments electrical stimulation of the nucleus raphe magnus (NRM) (Rhodes SNEX-100, 3Hz, 1 ms pulse width, 10-35 µA) resulted in a transient increase in TFL. Intrathecal injections of NA (15nM) during the period of stimulation, increased the TFL for 44 $\pm$ 14m (n = 6).

Thus both electrical stimulation of the NRM or pretreatment with intrathecal 5-HT both reduce the ability of NA to increase the TFL. These results suggest that activity in the descending tryptaminergic pathway can reduce the antinociceptive action of intrathecally administered NA in rats lightly anaesthetised with Saffan.

Supported by the Wellcome Trust.

Jensen, T.S. and Smith, D.F. (1983) European J.Pharm 86, 65-70.
Minor, B. G., Post, C. and Archer, T. (1985) Neurosci.Lett.54, 39-44.

ANTININOCICEPTIVE EFFECTS OF A NOVEL, PERIPHERAL ACTING OPIOID PEPTIDE, TYR.D.ARG.GLY.PHE[4-NO_2].PRO.NH_2 (443C)

R.L.Follenfant, G.W.Hardy*, L.A.Lowe* and T.W.Smith, Departments of Pharmacology I and Medicinal Chemistry*, Wellcome Research Laboratories, Beckenham, Kent, BR3 3BS.

Opioid antinociceptive activity that is mediated peripherally has been described, for example in writhing models in the mouse, using either local administration of tertiary opiates (Bentley, Newton & Starr, 1981) or systemic administration of quaternary opiates (Smith et al. 1982; 1985). As a further development in this area, we have investigated the properties of a novel analogue of enkephalin, Tyr.D.Arg.Gly.Phe(4-NO_2).Pro.NH_2 (443C), in which the incorporation of the polar amino acid D-arginine might encourage exclusion of the peptide from the CNS.

In the antinociceptive models, mice (22-30g) of various strains were used. Drugs were administered in saline 10-30 min prior to the noxious stimulus and the number of writhes over 5 min or the number of animals responding to the hotplate (55°C) within 30 sec counted. ED_{50}s were calculated by linear regression or probit analysis. In guinea-pigs (Dunkin-Hartley strain, 375-550g) respiratory parameters were measured from a head out body plethysmograph by a Buxco pulmonary mechanics analyser.

On subcutaneous administration 443C, opiates and non-steroidal anti-inflammatory drugs inhibited in a dose-dependent manner irritant-induced writhing in mice, as shown in Table 1. The activity of 443C, however, was low in the hotplate test (Table 1) and in an intravenous study in the guinea-pig of respiratory depression (13.1 ± 6.8% depression of minute volume at 443C 30mg/kg), two models used to investigate central opioid activity.

Table 1: Antinociceptive effects in writhing models and hotplate

	ED_{50} mg/kg sc			
	PBQ	Acetic Acid	Carbacyclin	Hotplate
443C	4.0	1.2	2.5	94.8
morphine	0.1	0.9	0.2	1.8
naproxen	11.7	5.1	10.1	not tested

The inhibitory effects of subcutaneous 443C in writhing models were not reversed by intracerebroventricular (icv) administration of the quaternary opiate antagonist N-methyl nalorphine (0.3µg/mouse), but were antagonised by subcutaneous (sc) administration of this compound (15mg/kg, dose-ratio = 7.8). In comparison, morphine was antagonisted by N-methyl nalorphine icv (0.3µg/mouse), but not sc (11.5mg/kg, dose-ratio = 1.0). Correspondingly, icv naloxone (0.3µg/mouse) also antagonised morphine sc, but not 443C sc.

These studies support the hypothesis that the polar opioid peptide 443C exerts antinociceptive effects in a writhing model in the mouse via peripherally mediated opioid mechanisms.

Bentley, G.A., et al (1981) Br.J.Pharmac. 73, 325.
Smith, T.W., et al (1982) Life Sci. 31, 1203.
Smith, T.W., et al (1985) Int.J.Tiss. Reac. 7, 61.

ON THE MODE OF ANALGESIC ACTION OF TYR.D.ARG.GLY.PHE[4 -NO_2].PRO NH_2 (443C)

B.B.Lorenzetti and S.H. Ferreira*, Department of Pharmacology, Medical School of Ribeirao Preto, 14.049-Ribeirao Preto, SP. - Brazil.

Our demonstration (Ferreira & Nakamura, 1979) that opiates have a peripheral analgesic effect suggested the possibility of the development of a new class of analgesics: derivatives of opiates or enkephalins with restricted ability to cross the blood brain barrier. Our modification of the Randall-Selitto method (Ferreira et al. 1978) was used in this series of experiments to investigate the effects on hyperalgesia of the novel opioid peptide Tyr.D.Arg.Gly.Phe[4NO_2].Pro.NH_2 (443C).

Administration of 443C produced a dose dependent inhibition of the hyperalgesia induced by intraplantar (i pl) injection of 100ng of prostaglandin E_2 (ED_{50} = 18.0 ± 0.4mg/kg, ip; 24.2 ± 0.5mg/kg, sc; 18.5 ± 0.4µg/paw i pl), 100µg of isoprenaline (ED_{50} = 21.0 ± 0.5mg/kg, ip) and 200µg of calcium chloride (ED_{50} = 38.5 ± 0.75mg/kg, ip). However, systemic administration (ip) of 443C did not affect dibutyril cAMP- (100µg) induced hyperalgesia, a test which is sensitive to the central action of opiates (Lorenzetti & Ferreira, 1985). When 443C (up to 50µg) was given into the cerebral ventricles (icv) it did not antagonise PGE_2-induced hyperalgesia, an effect which was observed with morphine (ED_{50} = 4.3 ± 0.3µg).

The analgesic effect of icv administration of morphine was blocked by the sc administration of nalorphine, but not by its quaternary analogue N-methyl nalorphine. Local (i pl) and systemic (sc, ip) administration of N-methyl nalorphine, however, abolished the analgesic affect of sc, ip or i pl administration of 443C indicating a peripheral action of 443C. Also unlike morphine (4mg/kg, ip), 443C (80mg/kg, ip) did not cause tolerance when given for 5 consecutive days.

In summary, 443C may be classified as a peripherally acting opioid which may act upon established hyperalgesia, conditions in which aspirin-like drugs are devoid of analgesic effects.

Ferreira, S.H. et al (1978) Eur.J.Pharmacol. 53, 39.
Ferreira, S.H. & Nakamura, M. (1979) Prostaglandins 18, 191.
Lorenzetti, B.B. & Ferreira, S.H. (1985) Eur.J.Pharmacol. 114, 375.

DEPRESSANT RESPONSES OF CORTICAL NEURONES TO ISOPRENALINE: INVOLVEMENT OF β_1-ADRENOCEPTORS

C.M. Bradshaw, R.D. Sheridan & E. Szabadi*, Department of Psychiatry, University of Manchester, Stopford Building, Oxford Road, MANCHESTER M13 9PT.

α- and β-adrenoceptors on cortical neurones mediate functionally opposite effects on neuronal excitability: microelectrophoretically applied β-adrenoceptor agonists (e.g., isoprenaline) exert a depressant action while α-adrenoceptor agonists (e.g., phenylephrine) are excitatory in action (Bevan et al, 1977; Bradshaw et al, 1984). In the present experiments we have further analysed the β-adrenoceptor-mediated depressant response in an effort to resolve the subtype of β-adrenoceptor involved.

Spontaneously active single neurones were studied in the parietal cortex of the halothane-anaesthetized rat. Our techniques for the extracellular recording of neuronal activity and for the microelectrophoretic application of drugs are described elsewhere (Bradshaw et al, 1985). Statistical comparisons were made using Student's t-test with a criterion of $P < 0.05$.

In the first series of experiments we examined the actions of the selective agonists, prenalterol (β_1) and procaterol (β_2), and compared them with those of the mixed agonist, isoprenaline. Prenalterol, applied on to 18 isoprenaline-sensitive cells with ejecting currents of up to 200 nA for 1 min, failed to affect the firing rate of 11 cells, produced weak depressant responses on 3 cells, and weak excitation of the remaining 4 cells. Procaterol, similarly applied, failed to modify the firing rate of 9 isoprenaline-sensitive cells. Isoprenaline, applied to 60 cells (5-50 nA), evoked both depressant (58 cells) and excitatory responses (2 cells).

In the second series of experiments we examined the effects of the selective antagonists, practolol (β_1) and ICI 118551 (β_2), on the depressant response to isoprenaline. Phenylephrine and acetylcholine were used as control agonists. Practolol (5-10 nA) significantly and selectively attenuated the response to isoprenaline [percentage change in the size of the response in the presence of practolol, mean ± s.e.mean for 6 cells: isoprenaline, -90.1 ± 3.0; phenylephrine, +5.6 ± 11.9; acetylcholine, +19.1 ± 7.6]. ICI 118551 (0-10 nA), however, had no selective effect: either the responses were unaffected or the responses to all three agonists were significantly attenuated [isoprenaline, -32.5 ± 12.3; phenylephrine, -53.9 ± 7.1; acetylcholine, -77.4 ± 5.9] (7 cells). We have also examined the ability of prenalterol, continuously applied with an ejecting current of 10 nA, to antagonize the depressant response to isoprenaline; phenylephrine was used as the control agonist (7 cells). Prenalterol significantly attenuated the response to isoprenaline [-93.0 ± 3.5] without affecting the response to phenylephrine [+8.9 ± 19.1].

These findings indicate that β_1-adrenoceptors are involved in mediating the depressant response of cortical neurones to isoprenaline. Prenalterol appears to be a weak partial agonist in this test system; it is of interest that a similar action of prenalterol has been described in peripheral tissues (Aldridge et al, 1984).

This work was supported by the Sir Jules Thorn Charitable Trust.

Aldridge, JM et al (1984) In Pharmacology of Adrenoceptors ed. Szabadi, E et al, pp. 271-272
Bevan, P et al (1977) Br J Pharmac, 59, 635-641
Bradshaw, CM et al (1984) Br J Pharmac, 82, 453-458
Bradshaw, CM et al (1985) Br J Pharmac, 86, 483-490

CH-38083, A HIGHLY SELECTIVE α_2-ADRENOCEPTOR ANTAGONIST

J. Gaal[2], L.G. Harsing Jr.[1], G.T. Somogyi[1], L. Szabo[3], Cs. Szantay[3], I. Toth[3] and E.S. Vizi[1]*. [1]Institute of Experimental Medicine, Hungarian Academy of Sciences H-1450 Budapest, P.O.B. 67., [2]Chinoin Pharmaceutical and Chemical Works Ltd., H-1502 Budapest and [3]Central Research Institute for Chemistry, Hungarian Academy of Sciences, H-1525 Budapest, Hungary.

Pharmacological evidence has been obtained that berbanes are a new class of alpha2-adrenoceptor antagonists. In this study the selectivity and specificity of CH-38083, [7,8-(methylenedioxi)-14-alpha-hydroxy-alloberbane HCl] for alpha-adrenoceptors have been studied (Vizi et al., 1986) and compared with yohimbine and idazoxan (Doxey et al., 1983) in peripheral tissues and in the central nervous system. In isolated tissue experiments CH-38083 was a competitive antagonist at presynaptic alpha2-adrenoceptors on the axon terminals of the rat vas deferens (pA2 against xylazine=8.17±0.06) and of the longitudinal muscle strip of guinea-pig ileum (pA2 against xylazine=8.07±0.20). CH-38083 and idazoxan produced parallel rightward shifts in the log concentration-response curve. The linearity of the Schild plot with dose ratios over 100 affords confidence that the antagonism is competitive. As far as its postsynaptic alpha1-adrenoceptor antagonistic activity is concerned its affinity in rat vas deferens (pA2=4.95±0.11 against l-phenylephrine) and in rabbit pulmonary artery (pA2=5.38±0.33 against l-noradrenaline) was markedly less than that displayed for presynaptic sites. The antagonism was non-competitive in nature. There was not possible to obtain higher dose-ratio than 3.6 with CH-38083. From pA2-values obtained in rat vas deferens the calculated alpha1/alpha2-adrenoceptor selectivity ratios for yohimbine, idazoxan and CH-38083 were 4.7, 117.5 and 1659, respectively. CH-38083 failed to show any affinity for histamine and muscarinic receptors and it even potentiated the effect of serotonin on atropinized longitudinal muscle strip of guinea-pig ileum. It enhanced the release of 3H-noradrenaline from electrically stimulated mouse vas deferens previously loaded with labelled 3H-noradrenaline. The potency order was CH-38083 > idazoxan > yohimbine, the concentrations evoking a 50% increase in overflow being 10-7, 3x10-7 and 1.6x10-6 M, respectively. This finding indicates that CH-38083 is able to remove negative feedback control of NA release and thereby to enhance NA release. In binding studies carried out in rat brain membrane preparations using [3H]prazosin and [3H]-idazoxan, the selectivity ratios (Ki alpha-1/Ki alpha-2) proved to be 32.5, 289.5 and 1368 for yohimbine, idazoxan and CH-38083, respectively. In rats pretreated with alpha-methyl-p-tyrosine, CH-38083 produced a marked increase in the apparent rate of turnover of NA in cerebral cortex and hypothalamus, without affecting the turnover rate of dopamine in the striatum whereas yohimbine increased the turnover rate of both amines.

These results classify CH-38083 as potent and highly selective antagonist of both peripherial and central alpha2-adrenoceptors with practically no effect on alpha-1 adrenoceptors and it may be useful antidepressant drug.

Doxey, J.C. et al.,(1983) Br.J.Pharmac. 78:489-505
Vizi E.S. et al.,(1986) J.Pharmacol.Exp.Ther. 238:(in press).

INTERACTION BETWEEN FORSKOLIN AND THE α_2-ADRENOCEPTOR-MEDIATED INHIBITION OF THE ELECTRICALLY-EVOKED RELEASE OF [^{3}H]-5HT AND [^{3}H]-NA

Anne-Marie Galzin, S.Z. Langer and Régine Ramdine, Department of Biology, Laboratoires d'Etudes et de Recherches Synthélabo (L.E.R.S.), 58, rue de la Glacière, 75013 Paris, France

Presynaptic inhibitory α_2-adrenoceptors located on both noradrenergic and serotonergic nerve terminals are involved in the modulation of the stimulation-evoked release of noradrenaline (NA) and serotonin (5HT) (Langer, 1980 ; Göthert et al., 1981 ; Galzin et al., 1983). The presynaptic α_2-autoadrenoceptors which regulate noradrenergic transmission seem to be negatively coupled to an adenylate cyclase (Schoffelmeer et al., 1986). However, it is not known whether a similar interaction exists at the level of the α_2-heteroreceptor modulating 5HT neurotransmission. The aim of our study was to investigate the interaction between adenylate cyclase and the α_2-adrenoceptor mediated modulation of both [^{3}H]-5HT and [^{3}H]-NA release from rat hypothalamic slices.

Male rats (180 - 200 g) were killed by decapitation and hypothalamic slices prepared immediately. The endogenous NA and 5HT stores were labelled with [^{3}H]-(±)-NA or [^{3}H]-5HT, respectively. The slices were superfused with Krebs' solution at 37°C. Two periods (S_1 and S_2) of electrical stimulation were applied (3 Hz, 2 ms, 20 mA during 2 min for [^{3}H]-5HT release and 5 Hz, 2 ms, 26 mA during 2 min for [^{3}H]-NA release) with an interval of 44 min. Drugs were added 20 min before S_1 or S_2.

UK 14304, a selective α_2-adrenoceptor agonist, inhibited in a concentration-dependent manner the stimulation-evoked release of [^{3}H]-NA (S_2/S_1 = 0.31 ± 0.06, n = 5 for 1 µM UK 14304, $p < 0.001$, when compared to the control S_2/S_1 = 0.84 ± 0.04, n = 12). The stimulation evoked release of [^{3}H]-5HT was totally inhibited in the presence of 1 µM UK 14304 (S_2/S_1 = 0.04 ± 0.02, n = 4, $p < 0.001$ when compared to the control value (S_2/S_1 = 0.97 ± 0.05, n = 22).

Forskolin, an adenylate cyclase activator, (1 and 10 µM) significantly enhanced the stimulation-evoked release of [^{3}H]-5HT (S_2/S_1 = 1.75 ± 0.23, n = 8, $p < 0.001$, and 2.83 ± 0.36, n = 7, $p < 0.001$, respectively, when compared with the control value). Forskolin (1 and 10 µM) was less effective to increase the stimulation-evoked release of [^{3}H]-NA (S_2/S_1 = 1.37 ± 0.20, n = 6, and 1.78 ± 0.24, n = 5, respectively). In [^{3}H]-NA release experiments, when forskolin (1 µM) was added to the medium 20 min before S_1, the ratio S_2/S_1 was 0.88 ± 0.05, n = 8, not different from the control value in the absence of forskolin. Under these experimental conditions, UK 14304 still inhibited the electrically-evoked release of [^{3}H]-NA, but the concentration-effect curve in the presence of forskolin was shifted to the right when compared with that in the absence of the drug (IC_{50} = 51 nM and 231 nM, respectively in the absence and presence of forskolin). In contrast, forskolin (1 µM) did not modify the α_2-mediated inhibition of UK 14304 on the stimulation-evoked release of [^{3}H]-5HT (IC_{50} = 17 and 14 nM, respectively in the absence and the presence of forskolin).

In conclusion, the increasing effect of forskolin on [^{3}H]-NA and [^{3}H]-5HT release confirms the existence of an adenylate cyclase, associated with the mechanisms of the release of both NA and 5HT. While presynaptic α_2-autoreceptors inhibiting NA release are negatively coupled with the enzyme (Schoffelmeer et al., 1986), there was no interaction between α_2-heteroreceptors present on 5HT nerve endings and adenylate-cyclase, suggesting that biochemical events following the activation of α_2-auto- and heteroreceptors may not be identical.

Galzin, A.M. et al. (1983) J. Pharmacol. Exp. Ther. 228, 725.
Göthert, M. et al. (1981) Naunyn-Schmiedeberg's Arch. Pharmacol. 317, 199.
Langer, S.Z. (1980) Pharmacol. Rev. 32, 337.
Schoffelmeer, A. et al. (1985) J. Neurochem. 46, 1711.

COMPLEX RAT CORTICAL [^{3}H]-RAUWOLSCINE BINDING: HETEROGENEOUS α_2 ADRENOCEPTORS OR A SEROTONINERGIC INTERACTION ?

A.M. Broadhurst*, M.D.Wood and M.G.Wyllie+ (Introduced by M.Briley). Wyeth Research,Huntercombe Lane S, Berks. Present addresses : * Pierre Fabre Médicament 17 Avenue Jean Moulin, 81100 Castres, France. + Pfizer Central Research, Sandwich, Kent.

Recently it has been proposed that the complex binding of ^{3}H-rauwolscine to rat cortical membranes (see Broadhurst and Wyllie, 1986 for references) is a result of a heterogeneous population of alpha$_2$-adrenoceptors in this species (Bylund, 1985). We have previously established that ^{3}H-rauwolscine binds specifically to two sites on this tissue ; a high affinity interaction pharmacologically resembling the alpha$_2$-adrenoceptor and a lower affinity, spiperone-sensitive, serotoninergic-like component (Broadhurst and Wyllie, 1986). We have therefore investigated, in greater detail, the displacement profile at the lower affinity ^{3}H-rauwolscine site on rat cortical membranes. Binding was studied as previously described (Broadhurst and Wyllie, 1986) and the relative affinities of compounds at the two sites were determined by performing all experiments in the absence and presence of spiperone (300 nM) followed by computer analysis of the competition data (Green et al., 1982).

Table 1 Binding profile at the high and low affinity ^{3}H-rauwolscine binding sites

Compounds	IC_{50} (nM) High	Low
WB 4101	88 ± 9	6 ± 0.5
Methysergide	2500 ± 300	25 ± 0.5
Spiperone	2500 ± 290	35 ± 8
Propranolol	4200 ± 1100	48 ± 26
Rauwolscine	8 ± 1	320 ± 120
UK 14304	76 ± 12	10,000

All results are the means ± sem from 3-5 experiments and show that the two ^{3}H-rauwolscine binding components have markedly different displacement profiles. Thus the high affinity site is not unlike that expected for an alpha$_2$-adrenoceptor whereas the alpha$_2$-agonist, UK 14304 and antagonist, rauwolscine show no and reduced affinity respectively for the low affinity site. The rank order, and magnitude, of affinity seen at the lower affinity component is remarkably similar to that found by Peroutka (in press) for the 5HT$_{1A}$ site on rat cortical membranes (WB 4101 > methysergide > spiperone). In addition the reduced but relatively high affinity of rauwolscine for the second site would support our earlier finding that rauwolscine is a potent inhibitor of 5HT binding to the 5HT$_{1A}$ site (Alexander et al., 1986). These results would not support the existence of heterogeneous alpha$_2$-adrenoceptors on rat cortical membranes.

Alexander B. et al.(1986) Br.J.Pharmac. 88, 348P
Broadhurst A.M. & Wyllie M.G. (1986) Neuropharmac. 25, 287-295
Bylund D.B. (1985) Pharmac.Biochem.Behav. 22, 835-843.
Green S. et al. (1982) Nucleic Acids Res. 10, 1411-1421
Peroutka S.J. J.Neurochem. (in press)

THE EFFECT OF ZOLPIDEM ON [^{35}S]-TBPS BINDING TO RAT CORTEX MEMBRANES.

K.G. Lloyd and F. Thuret., Laboratoires d'Etudes et de Recherches Synthelabo (L.E.R.S.) 31, Av. P.V. Couturier, 92220- Bagneux, France.

Benzodiazepines (BZD) may act by modulating the GABA-linked chloride ionophore ^{35}S-TBPS binding(TBPS) is a recently developed tool for in vitro studies of this ionophore, and is regulated differentially by BZD receptor ligands according to their pharmacological activity (Supavilai and Karobath,1984). Zolpidem (Z) is an imidazo-pyridine hypnotic with specificity for BZD_1 binding sites(Arbilla et al,1985). We have compared Z with flunitrazepam (Flu) on TBPS binding.

TBPS binding was carried out on extensively washed rat cerebral cortex membranes according to Honoré and Dreyer (1985). TBPS specific activity (90 ci/mmol,NEN), study compound and membrane suspension were incubated at 25° for 90 min followed by dilution with cold buffer and rapid filtration (Whatman GF/B filters). Nonspecific binding was estimated in the presence of 10µM picrotoxinin;specific binding normally was 85% of the total.

At all concentrations tested (Table) Z significantly enhanced TBPS binding($p<0.01$ vs control) to well-washed rat cortical membranes. The EC_{50}(50% maximum effect) was 84±9nM(n=5). Scatchard analysis of TBPS binding(0.5-0.50nM TBPS) indicated that this was due to an increased affinity(34.3nM in the absence and 23.1 nM in the presence of 0.5µM Z; $p<0.01$,n=7) rather than a change in Bmax.The BZD antagonist RO15-1788(10^{-5}M) apparently competitively inhibited the effect, as high concentrations of the hypnotic overcame this inhibition. The EC_{50} for Z alone was 0.084±0.009µM(n=5) whereas in the presence of RO it was 176±86µM(n=5;$p<0.05$). The maximal effect of Z alone occurred at 5±1.6µM whereas in the presence of RO it was at 260±98µM(n=5 $p<0.05$).

Flu also increased TBPS binding (table). In contrast to Z, Flu markedly decreased TBPS binding at high concentrations (500µM); As for Z, the Flu enhancement of TBPS binding was competitively reversed by RO, whereas the decrease in TBPS binding was not reversed by the BZD antagonist. The maximum effect of Flu alone occurred at 1.5±0.9µM, and at 50(±0)µM in the presence of RO.

Mean Alteration in ^{35}S TBPS binding to Rat Cortical Membranes (% control).

Compound	Concentration of Z or Flu (µM)								
	.05	.1	.5	1	5	10	50	100	500
Z(n=5-6)	114	120	130	133	135	135	131	128	124
Z+RO (n=5)	101**	101**	102**	103**	105**	105**	112**	116*	120
Flu(n=5)	127*	129	134	133	135	134	130	121	42+
Flu+RO (n=3)	100**	101**	103**	103**	107**	110**	120	110	41

Z=Zolpidem; Flu=Flunitrazepam;RO=RO15-1788 (10^{-5}M). *$p<0.05$;**$p<0.01$ vs compound alone;+$p<0.01$ vs Z.

Thus, the new hypnotic Z interacts with the $GABA_A$ receptor linked chloride ionophore. The EC_{50} for his effect (84nM) is lower than its IC_{50} for mixed cortical BZD receptors (141nM vs ^{3}H-RO15-1788 binding)consistent with the observation that Z is selective for BZD_1 receptors (Arbilla et al,1985). The significance of the differences between Z and Flu at high concentrations is not clear, but may be related to the lower incidence of high dose secondary effets observed with Z than with Flu(Depoortere et al,1986).

Arbilla,S. et al(1985) N.S. Arch Pharmac.330,248-251.
Depoortere H. et al(1986)J. Pharmac. Exp Therap. 237,649-658.
Honoré T. and Dreyer,J.(1985) J. Pharm. Pharmac. 37,928-929.
Supavilai,P. and Karobath,M.(1984) J. Neurosci. 4,1193-1200.

CHOLECYTOKININ AND MUSCARINIC RECEPTORS IN FLOW 9000 CELLS ACTIVATE PHOSPHOINOSITIDE TURNOVER IN A GTP-SENSITIVE MECHANISM

C.R. Clarke, J. Hughes & W.W.Y. Lo*, Parke-Davis Research Unit, Addenbrookes Hospital Site, Hills Road, Cambridge, CB2 2QB.

Flow 9000 cells are derived from a human embryonic pituitary cell line. These cells contain both cholecystokinin (CCK) and muscarinic receptors as measured by [^{125}I]CCK-8 and [^{3}H]QNB binding experiments respectively. High affinity [^{125}I]CCK-8 and [^{3}H]QNB binding sites were both shown to be saturable (Bmax = 12fmol/mg prot. for [^{125}I]CCK-8 and 185fmol/mg prot. for [^{3}H]QNB) and reversible (IC_{50} = 0.46nM for CCK-8 and 501μM for carbachol respectively).

We have recently shown that CCK-8 potently activates phosphoinositide (PI) hydrolysis (EC_{50} = 0.79nM) in Flow 9000 cells (Lo et. al., 1986). In the present study, we have shown that muscarinic agonists also stimulate PI turnover, as measured by inositol phosphate formation, in a time- and dose-dependent manner. Cholinergic stimulation of PI turnover was inhibited by atropine but not by curare indicating the muscarinic nature of the response (EC_{50} = 21.15, 25.15, 31.62, 89.05 & 1.58μM for acetylcholine, carbachol, muscarine, bethanechol & oxotremorine). Time course studies showed that a significant amount of $InsP_3$ (176% of control) was formed as early as 5 sec following carbachol treatment (1mM) suggesting an initial breakdown of $PtdIns(4,5)P_2$ as the principal mechanism operating during receptor activation. Recent studies indicate that a guanine-nucleotide binding protein is involved in PI turnover in the Flow 9000 cell line (Lo et. al., 1986a). To this end, the effects of GTP[S] on CCK-8 and muscarinic stimulations of PI breakdown were investigated in saponin-permeabilized Flow 9000 cells. Both CCK-8 and acetylcholine stimulation of inositol phosphate formation were abolished upon saponin treatment, but were restored to significant levels when GTP[S] was introduced.

In combination experiments, maximal effects of CCK-8 and muscarinic agonists on inositol phosphate production were found to be additive suggesting that these agents were acting on independent receptor-linked PI pools.

Taken together, we have shown the presence of functional CCK and muscarinic receptors in the human embryonic pituitary cell line Flow 9000, which are coupled to the activation of phospholipase C by a GTP-dependent mechanism.

W.W.Y. Lo is a Commonwealth Scholar and a Bye-Fellow at Downing College, Cambridge.

Lo, W.W.Y., Clark, C.R. & Hughes, J. (1986) Biochem. Soc. Trans. 14:1108-1109
Lo, W.W.Y., Clark, C.R. & Hughes, J. (1986a) Biochem.Soc.Trans 14:1135-1136

5-HT STIMULATED PI TURNOVER DOES NOT REFLECT ALTERED 5-HT_2 FUNCTION AFTER ANTIDEPRESSANTS OR NEUROCHEMICAL LESIONING

P.P. Godfrey*, D.G. Grahame-Smith, D.J. Heal[1], S.J. McClue and M.M. Young, MRC Unit and University Department of Clinical Pharmacology, Radcliffe Infirmary, Oxford OX2 6HE and [1]The Research Department, The Boots Company PLC, Nottingham NG2 3AA.

Godfrey et al (1986) have demonstrated that 5-HT stimulated phosphatidylinositol (PI) turnover in mouse cortex appears to be mediated by the 5-HT_2 receptor subtype. In C57/B1/6O1a mice, both cortical 5-HT_2 receptor number and 5-HT_2-mediated head-twitch responses are increased after 5,7-dihydroxytryptamine (5,7-DHT) lesioning (Heal et al, 1985) and repeated electroconvulsive shock (ECS; Goodwin et al, 1984; Metz and Heal, 1986) and decreased by repeated administration of desipramine (DMI) or zimeldine (Goodwin et al, 1984; Metz and Heal, 1986). We have, therefore, now investigated the effects of these treatments on 5-HT mediated PI responses in mouse cortex.

Adult male C57/B1/6O1a mice, 25-30g, were used; 5-HT stimulated PI turnover was measured in a pooled cortical slice preparation as described previously (Godfrey et al, 1986). Head-twitches to 5-methoxy-N,N-dimethyltryptamine (5-MeODMT; 2 mg/kg) were counted in the 6 min immediately following injection. 5,7-DHT (50µg in 4µl) was administered icv, with controls receiving vehicle (4µl icv). Behavioural and PI responses were assessed 14 and 21 days later, respectively. ECS (100v, 1s) was given 5 times over 10 days, while controls received halothane anaesthesia. DMI and zimeldine were injected 5mg/kg twice daily for 14 days.

After 5,7-DHT lesioning 5-MeODMT head-twitches were enhanced by 210% ($P<0.01$) while cortical 5-HT concentrations were depleted by 88±7%; the 5-HT (10^{-7}-10^{-5}M) dose-response curve for inositol phosphate (IP) formation was, however, totally unaltered. Repeated ECS enhanced head-twitch responses by 85% ($p<0.01$), 24h after the final shock, but also failed to alter the PI response to 5-HT (0.3 and 3µM). Repeated zimeldine injection decreased head-twitches by 60% ($p<0.01$) but did not decrease 5-HT stimulated IP formation. Repeated DMI, however, reduced both head-twitches by 56% ($p<0.01$) and 5-HT stimulated IP formation at 3µM by 33% ($p<0.02$). When added in vitro DMI (10µM) did not affect the PI response. Since all the above treatments have previously been shown to produce identical directional changes in cortical 5-HT_2 receptor number and head-twitch responses (Goodwin et al, 1984; Heal et al, 1985; Metz and Heal, 1986) the results from the present study suggest that 5-HT stimulated PI turnover does not reflect alterations in 5-HT_2 receptor function, even though it has the pharmacological characteristics of a 5-HT_2 receptor-mediated response.

Godfrey, P.P. et al This meeting
Goodwin, G.M. et al (1984) Br. J. Pharmac. 83, 235
Heal, D.J. et al (1985) Neuropharmacology 24, 1201
Metz, A. & Heal, D.J. (1986) Eur. J. Pharmac. In press

ADENOSINE INHIBITS HISTAMINE-INDUCED INOSITOL PHOSPHOLIPID HYDROLYSIS IN MOUSE CEREBRAL CORTEX SLICES

S.J. Hill & D.A. Kendall*, Department of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Clifton Boulevard, Nottingham NG7 2UH, U.K.

Adenosine is an inhibitory modulator of both central and peripheral neurotransmission which can either inhibit or stimulate adenylate cyclase activity via A_1 and A_2 receptors respectively (Snyder, 1985). In a recent communication to the Society we reported that adenosine selectively enhanced histamine stimulated inositol phospholipid hydrolysis in guinea-pig cerebral cortical slices despite having no effect alone (Hill & Kendall, 1986). In marked contrast we now report that adenosine and some of its analogues inhibit inositol phospholipid hydrolysis in cerebral cortical slices from the mouse.

Mouse (C57 black, 20-25 g) cerebral cortical slices were washed and preincubated in Krebs-bicarbonate buffer for 60 min at 37°C. Aliquots of a gravity packed slice suspension (25 µl) were then incubated for 40 min in 300 µl Krebs medium containing [^{3}H]-myo-inositol (0.3 µCi, 0.1 µM) before addition of agonists in 10 µl medium. Antagonists were added 20 min before the addition of agonists when appropriate. Incubations were terminated after 45 min by addition of 10% (w/v) perchloric acid. [^{3}H]-inositol phosphates were then separated by anion exchange chromatography (Donaldson & Hill, 1986).

Histamine (1 mM) stimulated [^{3}H]-inositol phosphate accumulation by 9.3±1.7 fold (n=5) with an EC_{50} of 110±30 µM (n=4). Stimulation was apparently via an H_1 receptor since mepyramine competitively antagonised the response to histamine. Adenosine (0.1 mM) had no effect alone but reduced the maximum response to histamine by 72±5% (n=3) with an IC_{50} of 16±6 µM (n=3). There was no apparent change in the EC_{50} of histamine. The dose/response curve to adenosine was shifted 10-fold to the left in the presence of the adenosine uptake inhibitor dipyridamole (0.5 µM).

The adenosine analogues N^6-(R)-phenylisopropyl adenosine (R-PIA) and N^6-cyclopentyladenosine (CPA) both reduced histamine stimulated [^{3}H]-inositol phosphate accumulation by a similar amount to that produced by adenosine.

The inhibition of the response to histamine in the presence of 0.1 mM adenosine was completely reversed by co-incubation with the adenosine antagonist isobutylmethylxanthine (1 mM) but not by theophylline (1 mM) or 8-phenyltheophylline (1 µM).

Adenosine did not affect the stimulation of [^{3}H]-inositol phosphate accumulation due to carbachol (100 µM), noradrenaline (100 µM), 5HT (300 µM) or elevated KCl (31 mM).

We cannot say on the basis of the data obtained so far whether adenosine acts via separate receptors to enhance or inhibit histamine stimulate inositol phospholipid hydrolysis in different species or whether a common recognition site is coupled to different signal transduction pathways. However it is clear that adenosine might play an important role in the control of histamine mediated processes in the mammalian central nervous system.

We thank the Wellcome Trust for financial support.

Snyder, S.H. (1985) Ann. Rev. Neurosci. 8, 103-124.
Hill, S.J. & Kendall, D.A. (1986) Proceedings of Br. Pharmac. Soc., Hatfield.
Donaldson, J. & Hill, S.J. (1986) Eur. J. Pharmac. 127, 255-266.

POSTNATAL ONTOGENY OF AGONIST AND DEPOLARISATION-INDUCED PHOSPHOINOSITIDE HYDROLYSIS IN RAT CEREBRAL CORTEX

S R Nahorski & T A Rooney*, Department of Pharmacology and Therapeutics, Medical Sciences Building, University of Leicester, University Road, Leicester, LE1 7RH

There is now evidence that several neurotransmitter receptors, including muscarinic and alpha$_1$-adrenoceptors, are closely coupled to inositol phospholipid hydrolysis in rat cerebral cortical slices (Brown et al, 1984; Jacobson et al, 1984). Furthermore, depolarisation of such slices also evokes similar phospholipid responses which can be greatly potentiated by the cholinesterase inhibitor physostigmine (Batty et al, 1985). In the present study we examined the responsiveness of slices prepared from cerebral cortex to these stimuli at different stages in development in order to examine the neurochemical basis for adrenergic and cholinergic induced inositol phospholipid hydrolysis in the immature brain.

Rat brains were dissected and 350 x 350 µM slices were prepared, preincubated with ^{3}H-inositol in the presence of 5 mM Li^+ and ^{3}H-inositol phosphates (^{3}H-IP) separated by anion-exchange chromatography.

The greatest changes in the developmental expression for maximal carbachol-stimulated (^{3}H-IP) accumulation were observed during the period of 2-21 days of age. Thus, very large muscarinic responses were observed in the cortex (about 20-fold) in the first week. After this the maximal response declined with time until it reached adult levels at (> 21 days). During this time there was negligible change in the EC_{50} value for carbachol stimulation of (^{3}H-IP) and no effect on the relative intrinsic activity of arecoline (1 mM), a partial muscarinic agonist. Noradrenaline responses were comparable with adult levels at early ages (2,7 and 14 days) and no changes were observed at later ages (21 and 40 days). However, at the early ages the EC_{50} of noradrenaline for stimulation of (^{3}H-IP) was significantly lower than at later ages and there was a higher relative intrinsic activity for phenylephrine (3×10^{-4}M), a partial alpha$_1$-adrenoceptor agonist. The relative proportions of inositol, mono, bis, tris and tetrakis phosphates were similar throughout development but a higher ratio of phosphatidylinositol-4, 5-bisphosphate to phosphatidylinositol-4-phosphate was detected at early ages.

Elevation of extracellular K^+ to 18 mM resulted in an increase in the production of ^{3}H-IP at all ages and achieved adult levels (about 3-fold) at 7 days. The cholinesterase inhibitor physostigmine (50 µM) was able to enhance the response produced by elevated K^+ at 14 days and reached adult levels by 21 days. No such enhancement was observable at 2 or 7 days of age.

Differences in the development pattern for the potentiation of the K^+ response by physostigmine probably reflects the maturation of cholinergic terminals in cortex. Direct muscarinic receptor stimulation although not showing variability in coupling, does produce markedly enhanced maximal responses at early times. On the other hand, alpha$_1$-adrenoceptors appear to be more efficiently coupled to phosphoinositide metabolism during the perinatal period. The relationship of these responses to the developing cholinergic and noradrenergic terminals and the receptor-linked phosphoinositide pool size remains to be established.

This work was supported by the SERC

Batty I et al (1985) Br J Pharmacol, 84, 108P
Brown E et al (1984) J Neurochem, 42, 1379
Jacobson M et al (1984) J Neurochem, 44, 465

A RECEPTOR RESERVE FOR MUSCARINIC INHIBITION OF ADENYLATE CYCLASE ACTIVITY IN RAT STRIATAL MEMBRANES

M. Keen* and S.R. Nahorski, Department of Pharmacology and Therapeutics, University of Leicester, University Road, Leicester LE1 7RH, U.K.

Muscarinic cholinergic receptor activation leads to several distinct biochemical responses, including inhibition of adenylate cyclase, in many tissues (Harden et al., 1986), which in the central nervous system provides a rare opportunity to study the relationship between muscarinic receptor occupation and response. In the present study we have used a rat striatal membrane preparation to examine muscarinic agonist binding and inhibition of adenylate cyclase.

Rat striata were initially homogenized 1:20 w/v in 20mM HEPES/2mM EGTA, pH 7.4. Adenylate cyclase activity of these membranes was measured by a modification of the method used by Olianas et al. (1983) using a cAMP protein binding assay (Brown et al., 1971). Muscarinic receptor binding activity (as the atropine [1μM] displaceable binding of ^{3}H-N-methylscopolamine) was measured in the same buffer by a centrifugation assay.

The maximal response produced by carbachol, a full muscarinic agonist, was 30% inhibition of basal cyclase activity. The EC_{50} for carbachol was ∿5μM, more than 100 times lower than its apparent binding affinity measured under the same conditions (∿600μM). This suggested that there was a considerable receptor reserve in this system, with carbachol able to produce a maximal response by occupying only 30% of the available receptors. Arecoline produced the same maximal response as carbachol, with an EC_{50} (∿30μM) that was the same as its affinity in binding studies. Thus arecoline seemed to be a full agonist in this system, but with no receptor reserve. Pilocarpine is a partial muscarinic agonist. In this system the pilocarpine EC_{50} was the same as its binding affintity (∿6μM), and the maximal response to pilocarpine was ∿80% of that produced by carbachol and arecoline.

From comparison of the dose/response and binding curves for the various agonists, the effects of altering the number of available receptors could be predicted. These predictions were tested by blocking different proportions of the receptors with the irreversible muscarinic antagonist benzilylcholine mustard (BCM; Gill and Rang, 1966). BCM produced a time dependent blockade of muscarinic binding sites; the antagonist and agonist binding properties of the remaining receptors remaining essentially unaltered. The effects of BCM blockade on the dose/response curves for the various agonists were as predicted, implying that a receptor reserve does exist for muscarinic inhibition of adenylate cyclase activity in this system. This contrasts with muscarinic receptor mediated phosphoinositide hydrolysis in rat striatum (Rooney and Nahorski, 1986) and may provide the basis for agonist selectivity.

The molecular basis for this receptor reserve remains to be elucidated. The linkage between receptor activation and inhibition of adenylate cyclase in this system must be very close, invloving activation of N_i, and the subsequent interaction of this N_i with N_s and/or the cyclase itself. It may be that the apparent 'receptor reserve' is in fact an 'N_i reserve', related to the large amounts of N_i present in these membranes.

M.K. is a Merck Sharpe and Dohme Research Fellow

Brown, B.L. et al. (1971) Biochem. J., 121, 561-563
Gill, E.W. and Rang, H.P. (1966) Mol. Pharmacol., 2, 284-297
Harden, T.K. et al. (1986) Trends Pharmacol. Sci., Suppl., 14-18
Olianas, M.C. et al. (1983) Mol. Pharmacol., 23, 393-398
Rooney, T.A. and Nahorski, S.R. (1986) J. Pharmacol. Exp. Ther. (in press)

NMS/OXO-M RATIO, A BINDING ASSAY DESIGNED TO PREDICT EFFICACY AT MUSCARINIC RECEPTORS

Freedman SB, Elizabeth Harley and Iversen LL Merck Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Harlow, Essex CM20 2QR

Muscarinic binding assays provide information about receptor affinity. In such assays muscarinic agonists recognise both high and low affinity states of the muscarinic receptor whereas antagonists do not recognise such heterogeneity. Biochemical methods for determining efficacy generally involve detailed curve fitting to determine high and low affinity components or guanine-nucleotide shifts.

A simple and more reliable method was developed using [^{3}H]-oxotremorine-M (oxo-M) to label the high affinity state of the cerebral cortex muscarinic receptor and [^{3}H]-N-methyl scopolamine (NMS) to label both affinity states. Membranes were prepared as described by Freedman et al (1981) and resuspended in 20 mM Hepes Krebs buffer pH 7.4 for [^{3}H]-NMS binding and 20 mM Hepes buffer pH 7.4 for [^{3}H]-Oxo-M (1ml total assay volume). Assays were incubated for 60 and 40 minutes respectively and were terminated by filtration through Whatman GF/B and 0.05% P.E.I. presoaked GF/C filters using a Brandel Cell Harvester. Drug displacement curves were assessed using 0.1 nM [^{3}H]-NMS and 3.0 nM [^{3}H]-Oxo-M. The results for selected agonists and antagonists are shown below.

Muscarinic Compound on NMS/Oxo-M Binding

Compound	[^{3}H]-NMS Kapp (μM)	[^{3}H]-Oxo-M Kapp (μM)	NMS/Oxo-M Ratio
Carbachol	24	0.0057	4200
Muscarine	19	0.0046	4100
RS86	5.4	0.039	140
McN-A-343	6.8	0.051	130
Pilocarpine	4.9	0.041	120
AFDX-116	0.54	0.121	4.5
Pirenzepine	0.07	0.024	2.9
Atropine	0.0012	0.00049	2.0

Kapp, apparent affinity constant corrected for ligand occupancy.

Muscarinic agonists displayed high affinity for the [^{3}H]-Oxo-M assay but lower affinity for [^{3}H]-NMS. In contrast M1-, M2-selective and non-selective antagonists displayed similar affinity for both. Full agonists showed an NMS/Oxo-M ratio of > 4000 whereas compounds which displayed partial agonist activity on PI turnover eg pilocarpine (Freedman, 1986) had an NMS/Oxo-M ratio of 100 fold.

These results suggest that the NMS/Oxo-M ratio may be indicative of efficacy at central muscarinic receptors.

We thank Boots, Sandoz and Institute De Angeli for gifts of compounds.

Freedman S.B., Poat J.A. and Woodruff G.N. (1981) J. Neurochem 37 608-612
Freedman S.B. (1986) Br. J. Pharmacol. 87 29P