## 1P CHARACTERISATION OF $K^+$ CHANNELS PRESENT IN INTACT FRAGMENTS OF RAT CEREBRAL ARTERIOLES

C.S. Davie, R.A.J. Challiss, N.B. Standen & J.P. Boyle., Department of Cell Physiology and Pharmacology, University of Leicester, University Road, Leicester LE1 9HN

It has long been known that $K^+$ channels in vascular smooth muscle play an important role in the modulation of arteriole diameter and hence resistance and flow. However, although flow is determined by the diameter of small arterioles, very few electrophysiological studies have been performed on smooth muscle cells from small, resistance arteries. Klieber & Daut (1994) demonstrated that intact arteriole segments could be voltage clamped. We have used a similar technique to study $K^+$ currents in intact fragments of small cerebral arterioles from the rat.

Arteriole segments were isolated from the pia mater of adult Wistar rats using a method adapted from Quinn & Beech (1998). Patch clamp recordings were made from arteriole fragments with diameters <25μm. Recordings were made in the perforated patch configuration using amphotericin B. The solutions used contained (mM): pipette; 140 KCl; 0.5 EGTA; 10 HEPES; 1 $MgCl_2$ (pH 7.2); bath; 140 KCl; 0.1(or 1.8) $CaCl_2$; 10 HEPES; 10 glucose; 1 $MgCl_2$ (pH 7.4). Resting membrane potential was recorded in each fragment and ranged from -10 to -60mV (n=31).

Using a holding potential of -60mV, voltage steps positive to -40mV elicited a current that activated in a time-dependent manner and was outward at potentials positive to the $K^+$ equilibrium potential ($E_K$, 0mV) (n=11). Superfusion with 1mM 4-aminopyridine (4-AP) blocked a component of this current in a voltage-dependent manner causing 70 ± 7% (n=4) inhibition of the current evoked by a step to +30mV. The activation curve of this current, derived from the tail currents, could be fitted with a Boltzmann function with a $V_{0.5}$ of −14.4 ± 1.6mV. This value is consistent with the measured current flowing through voltage-activated $K^+$ channels ($K_v$), as is the sensitivity of this current to inactivation by clamping the fragments at 0 mV. After inactivating $K_v$ channels, voltage steps positive to +40mV evoked a large, noisy outward current that was insensitive to 1 mM 4-AP, but which was largely inhibited by 100 nM iberiotoxin (IbTX) (n=9). Currents evoked by stepping to +100mV in the absence and presence of IbTX were 720 ± 25 pA and 49 ± 17 pA respectively. This current was similarly sensitive to inhibition by 1 mM tetraethylammonium and had a $V_{0.5}$ value of +84 ± 8mV indicating that it was carried by large-conductance, calcium-activated $K^+$ channels ($BK_{Ca}$). Stepping from a holding potential of 0mV to voltages in the range −100 to +40mV evoked large inward currents negative to $E_K$ with very little outward current detected positive to $E_K$. This inwardly rectifying current was blocked by $Ba^{2+}$ (0.1 mM) with a maximum inhibition of 52 ± 7% at −100mV (n=5). Glibenclamide was without effect on evoked currents at any concentration tested and likewise $K_{ATP}$ channel opening drugs failed to evoke any increase in current in any fragment tested.

In conclusion, we have demonstrated the presence of $BK_{Ca}$, $K_v$ and inward rectifier $K^+$ channels, but find no evidence of $K_{ATP}$ channels, in intact fragments of rat cerebral arterioles.

Supported by the MRC.

Klieber, HG & Daut, J (1994) *Cardiovasc.Res.* **28**, 823-830.

Quinn, K & Beech, DJ (1998) *Pflügers Arch.* **435**, 564-569.

---

## 2P PAR1 AND PAR2 MODULATE BLOOD PRESSURE IN ANAESTHETISED RATS

Carla Cicala, S. Morello, P. Harriot[1] and G.Cirino
Department Experimental Pharmacology, University of Naples "Federico II", via D. Montesano 49,80131 Naples, Italy.[1]Centre for Peptide and Protein Engineering, Queen's University of Belfast, Ireland, U.K.

In vitro studies have shown that peptides activating either the thrombin receptor PAR-1 (protease activated receptor-1) or the trypsin receptor PAR-2 (protease activated receptor-2) cause endothelium-dependent vasorelaxation (Laniyonu & Hollenberg, 1995; Hwa et al., 1996). PAR-1 activating peptides (PAR1-AP) have also been shown to cause, in vitro, direct contraction of smooth muscle and, recently, this effect has been described also for PAR-2 activating peptides (PAR2-AP) (Moffatt & Cocks, 1998). Studies performed in vivo, on experimental animals, have shown that both PAR1-AP and PAR2-AP cause hypotension, but only for PAR1-AP has a hypertensive effect been shown (Cheung et al., 1997). Aim of our study was to compare the effect on blood pressure (BP) of PAR-1 or PAR-2 activation in anaesthetised rats.

Male Wistar rats (200-280g) were anaesthetised with urethane (sol 10%w/v; 10 ml/kg ip.), the right jugular vein was cannulated for drug administration and the left carotid artery was cannulated and connected to a pressure transducer for a continuos monitoring of BP. After blood pressure stabilisation, PAR1-AP (SFLLRNPND, 1 mg /kg i.v.) or PAR2-AP (SLIGRL, 1 mg/kg i.v.) were administered three consecutive times at 20 min intervals. To eliminate any influence of autonomic nervous system activation, different groups of animals were pretreated with the ganglion-blocking agent, chlorisondamine (2.5 mg/kg ip.), before administration of peptides. In ganglion-blocked rats, L-NAME was administered as infusion through the tail vein (0.3 mg /kg/min iv.) and changes in BP induced by peptides were evaluated before and after L-NAME injection.

In normal rats, injection of PAR1-AP caused a biphasic response characterised by a hypotension of 13.00±1.57 mmHg (n=6) immediately followed by a hypertension of 21.5±3.28 mmHg (n=6). Injection of PAR2-AP caused a marked hypotension (26.75±5.85 mmHg, n=4) followed after about 5-6 min by a hypertension of 20.75±4.17 mmHg (n=4). In ganglion-blocked rats, both PAR1-AP- and PAR2-AP-induced hypotension was not significantly different from values obtained in control rats. On the contrary, hypertension induced by PAR1-AP was significantly increased (58.00±3.29 mmHg, n=4, $p<0.001$ vs. control, Student's t-test) and the hypertension induced by PAR2-AP was reduced to 3.67±1.60 mmHg (n=5, $p<0.01$, vs. control, Student's t-test). When L-NAME was infused in ganglion-blocked rats, hypotension induced by PAR1-AP was unchanged, while hypotension induced by PAR2-AP was significantly increased (42.00±8.3, n=5 $p<0.05$, ANOVA). L-NAME infusion did not affect PAR1-AP-induced hypertension, but significantly prolonged the endurance, when it was evaluated as area under the curve (AUC 9358.25±950 $mm^2$ vs. 4535±515 $mm^2$, n=4; $p<0.01$, ANOVA). After L-NAME infusion, hypertension induced by PAR2-AP was of 13.80 ± 2.80 (n=5; $p<0.05$, ANOVA).

Our results indicate roles for PAR1 and PAR2 in the control of BP or local vascular resistance.

Cheung W. et al. (1998) *Can.J.Physiol.Pharmacol.* 76,16-25.
Hwa J.J. et al., (1996) *Circ. Res.* 78, 581-588.
Laniyonu A.A. et al (1995) *Br.J.Pharmacol* (1995) 114, 1680-1686
Moffatt J.D. et al. (1998) *Br.J.Pharmacol.* 125, 591-594.

## 3P TYROSINE PHOSPHORYLATION AND MYOGENIC RESPONSES IN ARTERIOLES

T.V. Murphy, B.E. Spurrell & M.A. Hill, Microvascular Biology Group, Dept. Of Human Biology and Movement Science, RMIT University, Bundoora, Victoria 3083, Australia.

Skeletal muscle arterioles respond to an increase in transmural pressure with a myogenic constriction, a phenomenon termed the myogenic response. Previous studies have shown an increase in the intracellular [$Ca^{2+}$] and level of myosin light-chain phosphorylation of the vascular smooth muscle cells is requisite for this response (Zou et al., 1995), however other intracellular signalling mechanisms have been suggested to be involved. In the present study the role of tyrosine phosphorylation in myogenic tone of rat cremaster arterioles was further examined. Arterioles (diam approx. 140 μm) were dissected from cremaster muscle of anaesthetised (sodium thiopentone, 100 mg/kg i.p.) Sprague-Dawley rats. The vessels were cannulated on micropipettes, mounted in a 10 ml superfusion chamber, positioned on the stage of an inverted microscope and superfused with Krebs-bicarbonate buffer at 4 ml/min at 34°C. To enable study of myogenic responses in the absence of shear stress, arteriolar segments were pressurised without intra-luminal flow.

Vessels demonstrated significant myogenic vasoconstriction in response to increased intraluminal pressure. In the absence of extracellular $Ca^{2+}$, vessels dilated passively in response to increased pressure. Vessels with myogenic tone were dilated by the tyrosine kinase inhibitors genistein (30 μM; dilated by 19.2±1.9% of maximum diameter at 70 mmHg, n=6) and tyrphostin A47 (10 and 30 μM; 12.7±4.4 and 22.2±4.5% respectively, n=5 for both) and were further constricted by the tyrosine phosphatase inhibitor pervanadate (1 μM, constricted by 30.3 ± 2.1 %, n=5 and 10 μM, 35.9 ± 1.0%, n=5). However the vessels remained myogenically active in the presence of the tyrosine kinase and phosphatase inhibitors; acute increases in intra-luminal pressure still caused myogenic constrictions of similar magnitude to control responses. The effect of the tyrosine kinase inhibitors on arteriolar [$Ca^{2+}$] was measured using the $Ca^{2+}$-sensitive dye Fura-2. A step increase in intra-luminal pressure from 50 to 120 mmHg caused a significant and rapid increase in arteriolar [$Ca^{2+}$], which peaked at 121 ± 5% of the pre-step (50 mmHg) level. Arteriolar [$Ca^{2+}$] then declined to a steady-state level over the following 5 min (113 ± 3% at 5 min post-step). Neither genistein (10 and 30 μM) nor tyrphostin (10 and 30 μM) altered the pressure-evoked increase in [$Ca^{2+}$], either the peak (tyrphostin A47 30 μM, 135 ± 8% of control; genistein 30 μM 118 ± 10% of control) or the steady state [$Ca^{2+}$] (tyrphostin A47 30 μM, 135 ± 10% of control; genistein 30 μM 114 ± 9% of control). Tyrosine phosphorylation was then examined more directly using confocal microscopy. In vessels incubated with pervanadate, to inhibit phosphatase activity, and subsequently labelled with FITC-conjugated anti-phosphotyrosine antibody, arterioles tested at 10 mmHg showed a lower level of pixel intensity (39.6 ± 2.6 flux units, n=6) than vessels tested at 70 mmHg (52.7 ± 7.8, n=6) or 100 mmHg (84.6 ± 9.8, n=7). Tyrphostin A47 (30 μM) greatly reduced the increase in anti-phosphotyrosine fluorescence. In the absence of extracellular $Ca^{2+}$, myogenic responsiveness was abolished but anti-phosphotyrosine fluorescence was increased (70 mmHg, 103.6 ± 1.1, n=6; 100 mmHg, 108.1 ± 1.5, n=7). These data suggest that increased tyrosine kinase activity is not involved in acute myogenic constriction, in particular the increase in intracellular [$Ca^{2+}$] obligatory for myogenic constriction, but may play a role in modulating arteriolar tone directly via a parallel pathway. Prevention of the myogenic response by removing extracellular $Ca^{2+}$ caused a large increase in tyrosine phosphorylation which may represent increased phosphorylation of proteins involved in mechanotransduction or possibly growth.

Zou H, Ratz PH & Hill MA (1995) Am. J. Physiol., 269, H1590-H1596

---

## 4P ATP AND PDGF STIMULATE PROLIFERATION DIFFERENTIALLY IN VASCULAR SMOOTH MUSCLE CELLS FROM THE HUMAN SAPHENOUS VEIN AND INTERNAL MAMMARY ARTERY

P.J. White, R.Kumari, K.E. Porter[1], N.J.M. London[1] & M.R. Boarder,
Department of Cell Physiology and Pharmacology,
Medical Sciences Building, University Road, Leicester LE1 9HN and
[1]Department of Surgery, Robert Kilpatrick Building, Leicester Royal Infirmary, Leicester.

Intimal proliferation of vascular smooth muscle (VSM) cells is an important component of common human vascular diseases. Extracellular ATP and its breakdown product ADP, acting via G-protein coupled P2 receptors, have been shown to stimulate DNA synthesis in VSM cells (Boarder & Hourani, 1998; Erlinge, 1998). This study uses paired explant cultures (from the same patient) of human saphenous vein (SV) and internal mammary artery (IMA) VSM. We have compared activation of the phosphoinositide signalling pathway, the p42/p44 mitogen activated-protein kinase (MAPK) pathway and [3H]-thymidine incorporation following exposure to ATP and platelet derived growth factor (PDGF).

Quiescent, near confluent early passage cells (p2-4) were exposed to either ATP, PDGF or both for 1 hour. After 19h in serum-free medium, incorporation of [$^3$H]-thymidine into DNA was measured over 4 hours as an index of mitogenesis. MAPK activation was indicated by western blot specific for the phosphorylated form of p42/p44 MAPK. Accumulation of inositol 1,4,5 trisphosphate (Ins (1,4,5)$P_3$) was measured by stimulating cells for between 10s and 2min followed by a protein binding assay. To measure cytosolic $Ca^{2+}$, cells were loaded with Fura-2 and the 340/380nm ratio recorded for fields of 15-30 cells.

SV and IMA VSM cells showed differences in the [$^3$H]-thymidine response to PDGF (10pM-30nM). PDGF had a higher potency in IMA cells ($pEC_{50}$ values - SV, 8.45 ± 0.08; IMA, 9.22 ± 0.11; $P<0.001$ n=3) but gave a greater maximal response in the SV cells (SV, 4828 ± 330, IMA 1792 ± 121 d.p.m./ μg protein, $P< 0.05$). ATP (300μM) alone did not induce an increase in [$^3$H]-thymidine incorporation in either cell type. In SV cells, ATP (300μM) in the presence of PDGF (1nM) evoked a significant increase compared to PDGF alone (ATP+PDGF, 1403.0 ± 188.4; PDGF, 710.7 ± 6.4 d.p.m./μg protein $P< 0.01$ n=3). However, there was no such response to ATP in the presence of PDGF in the IMA cells (ATP+PDGF, 1095.3 ± 123.6; PDGF, 1465.3 ± 165.2 d.p.m./μg protein , n=3).

Western blot specific for the phosphorylated form of p42 and p44 MAPK showed a response to ATP (300μM) with both SV and IMA cells, but the response was greater in the IMA cells (n=6).

$Ca^{2+}$ and Ins (1,4,5)$P_3$ data were collected following stimulation of IMA cells with ATP and PDGF. While ATP (300μM) and PDGF (1nM) clearly elevated cytosolic $Ca^{2+}$, there was no detectable Ins (1,4,5)$P_3$ response to either agonist.

These results show that the proliferative response of human VSM cells to both ATP and PDGF is dependant on the vessel of origin, with the SV cells being more responsive than the IMA cells. MAPK activation may be involved, but the greater response in IMA compared to SV cells underlines the importance of further studies on the cell signalling mechanisms which control proliferation in human VSM cells.

Boarder, M.R. & Hourani, M.O. (1998) *TiPS.* **19**, 99-107.
Erlinge, D. (1998) *Gen. Pharmac.* **31**, 1-8.

## 5P POST-JUNCTIONAL $\alpha_2$-ADRENOCEPTOR SUBTYPES IN DOG AND RAT VEINS

M.Q. Paiva, M. Morato, D. Moura & S. Guimarães, Institute of Pharmacology & Therapeutics, Faculty of Medicine, 4200 Porto, Portugal

The majority of prejunctional $\alpha_2$-adrenoceptors belong either to $\alpha_{2A}$- (in rabbit, dog, humans) or $\alpha_{2D}$-adrenoceptor subtypes (in mouse, rat, guinea-pig) (Hieble *et al.*,1996). Furthermore, it is now generally accepted that these subtypes are species orthologs. In contrast to the wealth of data on the sub-classification of prejunctional $\alpha_2$-adrenoceptors, information is lacking on the subtype of postjunctional $\alpha_2$-adrenoceptors. To test whether or not the receptor subtype is the same at pre- and postjunctional level, a characterisation of postjunctional $\alpha_2$-adrenoceptors in two species with different $\alpha_2$-adrenoceptor subtype at prejunctional level (dog and rat) was done.

Cumulative concentration-response curves for the highly selective $\alpha_2$-adrenoceptor agonist UK-14,304 were determined on isolated rings from either the canine mesenteric vein or the rat femoral vein. The $pA_2$ for the antagonism exerted by eight α-adrenoceptor antagonists (rauwolscine, RX821002, yohimbine, phentolamine, idazoxan, WB4101, spiroxatrine and prazosin) on the effect of UK-14,304 were determined and correlated with $pK_i$ values of the same antagonist at tissues expressing only one subtype of $\alpha_2$-adrenoceptors. To selectively eliminate $\alpha_1$-adrenoceptors, the experiments were carried out after pretreatment of the tissues with 30 nM phenoxybenzamine (Guimarães *et al.*, 1987). $pA_2$ and $pK_i$ values for the antagonists used are shown in Table 1. The correlation between these values show that in the canine mesenteric vein postjunctional $\alpha_2$-adrenoceptors resemble more closely $\alpha_{2A}$-adrenoceptors ($r=0.96$; slope=1.03; $p<0.0001$) while in the rat femoral vein postjunctional $\alpha_2$-adrenoceptors are more closely related to $\alpha_{2D}$-adrenoceptors ($r=0.99$; slope=1.24; $p<0.0001$).

*Supported by PECS/C/SAU/80/95 and PRAXIS/2/2.1/SAU/1293/95*

Table 1. Dog mesenteric and rat femoral veins. Shown are potencies ($pA_2$) of the antagonists at postjunctional $\alpha_2$-adrenoceptors obtained in vein rings and affinities ($pK_i$) of the same antagonists for cloned human $\alpha_{2A}$, $\alpha_{2B}$ and $\alpha_{2C}$-adrenoceptors expressed in Chinese hamster lung cells (MacLennan *et al.* 1997) or for $\alpha_{2D}$-adrenoceptors in the bovine pineal gland or in the rat submaxillary gland (O'Rourke *et al.*,1994; Renouard *et al.*,1994).

| Antagonists | $pA_2$ | | $pK_i$ | | | |
|---|---|---|---|---|---|---|
| | Dog mesenteric vein | Rat femoral vein | $\alpha_{2A}$ | $\alpha_{2B}$ | $\alpha_{2C}$ | $\alpha_{2D}$ |
| Rauwolscine | 8.91±0.10 | 7.63±0.18 | 9.02 | 8.78 | 9.24 | 8.02 |
| Yohimbine | 8.40±0.18 | 7.38±0.12 | 8.67 | 8.35 | 7.97 | -- |
| RX821002 | 8.38±0.16 | 8.72±0.08 | 9.36 | 8.69 | 8.84 | 9.28 |
| WB4101 | 7.54±0.21 | 7.43±0.14 | 7.99 | 7.49 | 8.90 | 7.88 |
| Idazoxan | 7.49±0.18 | 7.57±0.12 | 7.89 | 7.41 | 6.69 | 8.15 |
| Phentolamine | 7.36±0.10 | 8.63±0.15 | 7.49 | 7.24 | 6.74 | 9.14 |
| Spiroxatrine | 6.97±0.08 | -- | 7.43 | 9.32 | 9.00 | 7.98 |
| Prazosin | 5.82±0.15 | 6.01±0.20 | 6.12 | 7.38 | 7.23 | 5.86 |

Values are means±e.e.mean; n=4 to 7.

We conclude that postjunctional $\alpha_2$-adrenoceptors in the canine mesenteric vein belong to the $\alpha_{2A}$- and in the rat femoral vein to the $\alpha_{2D}$-subtype, showing an agreement between the subtype expressed pre- and postjunctionally.

Guimarães, S., Paiva, M.Q. & Moura, D. (1987). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **335**, 397-402.

Hieble, J.P., Ruffolo, R.R. & Starke, K. (1996). *$\alpha_2$-Adrenergic Receptors* Harwood Academic Publishers, pp 1-18.

MacLennan, S.J., Luong, L.A., Jasper, J.R., *et al.* (1997). *Br. J. Pharmacol.* **121**,1721-1729.

O'Rourke, M.F., Iversen, L.J., Lomasney, J.W., *et al.* (1994). *J. Pharmacol. Exp. Ther.* **271**,735-740.

Renouard, A., Widdowson, P.S. & Millan, M.J. (1994). *J. Pharmacol. Exp. Ther.* **270**, 946-957.

## 6P CHOLESTEROL DIET AND ARTERIAL NORADRENALINE CONTENT

L. Almeida, M.T. Morgadinho, C.A. Fontes Ribeiro, S. Cabri ta, M.L. Matos Beja, F. Teixeira – Instituto de Farmacologia e Terapêutica Experimental, Faculdade de Medicina, 3049 Coimbra Codex, Portugal

Our group (Teixeira *et al.*, 1991) found that a low cholesterol (0.1 or 0.2%) enriched-diet (CED) induces a precocious (1 and 3 months (M)) noradrenaline (NA) content increase in the femoral (an atherosclerosis-(Ath) prone resistance vessel) artery and vein but not in other vessels e.g. aorta (an Ath-prone conductance vessel), carotid (an Ath- nearly refractory conductance vessel) and mesenteric arteries.

We aim to improve information about NA content, either using other diet times (6 and 9M) or researching some underlying mechanisms in femoral, carotid and aorta arteries.

42 male NZW rabbits (mean weight 2.290 ± 0.035, n = 15, in the control vs. 2.305 ± 0.031 Kg, n=27; 2.5 M old) were fed a normal diet or the same diet + 0.1% cholesterol during 6 or 9M. After sacrifice (sodium pentobarbital) two rings from each arte ry were processed (before and after perifusion) until determina tion of the catecholamines content by HPLC-ECD (Table I).

Table I – NA content ($ng.g^{-1}$ ± SEM) in arterial segments from rabbits fed with (0.1%) or without cholesterol

| Artery | Diet | 6 M | 9 M |
|---|---|---|---|
| Femoral | Normal | 247.1 ± 73.2 (n = 5) | 494.8 ± 315.1 (n = 6) |
| | Cholesterol | 423.0 ± 252.5 (n = 6) | 966.7 ± 793.2 (n = 6) |
| Aorta | Normal | 340.3 ± 81.6 (n = 5) | 440.8 ± 90.5 (n = 6) |
| | Cholesterol | 635.2 ± 101.5 * (n = 7) | 741.9 ± 151.1 + (n = 6) |

Student's t-test p: + < 0.06; * < 0.05

Four aortic rings were taken for characterizing type and degree of Ath lesions. Catecholamines efflux experiment (concentration also determined by HPLC) was performed by four perifusion periods of two segments from each artery, under cocaine (7.5 µM), DOCA (41 µm) and pargyline (1 mM). At the fifth and last period, one segment was perifused under yohimbine (100 nM) and the other was control.

At 6 or 9 M, just for ex.: a) Oppositely to the early series (Teixei ra *et al.*, 1991), the carotid (at 9 M: 2110.4 ± 511.4 $ng.g^{-1}$, n=7, in the CED group *vs.* 1034.9 ± 250.2 $ng.g^{-1}$, n=6, $p < 0.06$) and aorta accumulate NA and the femoral artery not so (Table I); b) Just light Ath lesions (3 at 6 M and 5 at 9 M, in crossa, for instance) or none lesion (4 at 6 M and 1 at 9 M) occur in the CED group. However, in both CED (the early and the present one), NA accumulation seems to parallel hypercho lesterolaemia evolution, sometimes more evidently but never per fectly as NA content from different arteries do not significantly correlate at each time, suggesting other mechanisms may interfe re, for ex: a) As NA (basal efflux 12.2 ± 3.0 $pg.g^{-1}{\cdot}min^{-1}$, n=5 *vs.* 22.9 ± 7.2, n=8, in the control group) spontaneous efflux is significantly diminished in the femoral artery, probably meaning a membrane disturbance under cholesterol accumulation, NA content could increase (719.4 ± 160.3, n=12, in the CED group *vs.* 423.1 ± 116.9 ng.g-1, n=13) (Almeida *et al.*, 1994).

Hypercholesterolaemia, even slight and before light atherosclero sis, increases arterial NA content, more precociously in the fe moral and latter more prominently in the aorta. Besides hyper cholesterolaemia, some mechanisms influence NA content but with such a complexity that it deserves further research.

Almeida *et al.*, (1994) Arq. Inst. Farm. Terap. Exp. Coimbra, 30: 101

Teixeira *et al.*, (1991) J. Lipid Mediators 3: 167-175

## 7P EFFECT OF BRADYKININ AND ANGIOTENSIN II ON NORADRENALINE RELEASE FROM THE RAT VENTRICLE

H. Pinheiro, M. J. Vaz-da-Silva, S. Magina, D. Moura & S. Guimarães, Institute of Pharmacology & Therapeutics, Faculty of Medicine, 4200 Porto, Portugal

Bradykinin enhances noradrenaline release from sympathetic nerves in the rat heart and this effect is markedly reduced by removing the endocardium (Vaz-da-Silva *et al.*,1996). Then, it was postulated that bradykinin might release some factor which is formed in the endocardium and spreads away to reach the sympathetic nerves. To check the hypothesis that angiotensin II might be the factor responsible for the facilitation of noradrenaline release caused by bradykinin, the influence of angiotensin II receptor antagonists ($AT_1$ selective, losartan; $AT_2$ selective, PD123319 (S(+)-1-((4-(dimethylamino)-3-methylphenyl)methyl)-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazol(4,5-c)pyridine-6-carboxylic acid ditrifluoroacetate); and non-selective, saralasin) on the effect of angiotensin II and bradykinin was studied.

Slices of the left ventricle of male Wistar rats were preloaded with $^3$H-noradrenaline (0.2 μM), mounted on perifusion chambers, washed out for 60 min and submitted to three periods of electrical stimulation (2 Hz; 600 pulses) starting at min 60 ($S_1$), 105 ($S_2$) and 150 ($S_3$). From min 90 on the perifusion fluid was collected continuously until the end of the experiment. Radioactivity in the samples of perifusion fluid was measured by liquid scintillation counting. Drugs were added to the perifusion fluid between $S_2$ and $S_3$. Results (geometric mean and 95% confidence limits) were expressed as the ratio of tritium overflow evoked by $S_3$ over that evoked by $S_2$.

Both angiotensin II (1-100 nM) and bradykinin (0.1-100 nM) concentration-dependently increased tritium overflow evoked by electrical stimulation [$EC_{50\%}$ values of 12.0 nM (3.5;41.1), n=5 and 3.5 nM (1.2;10.2), n=5, respectively]. The maximum increase expressed as the percentage of control was 205 ± 24% and 208 ± 18% (arithmetic mean±s.e.mean, n=5), respectively. Saralasin (10 nM-1 μM) had no effect on tritium overflow induced by electrical stimulation. However, saralasin at a concentration of 100 nM abolished the effect of both drugs. Neither losartan (up to 1μM), nor PD123319 (up to 1μM) changed the facilitatory effect of either bradykinin or angiotensin II.

These results suggest that locally formed angiotensin II mediates the facilitatory effect of bradykinin. Additionally, it can be concluded that the receptor involved in the prejunctional action of angiotensin II in the rat left ventricle is similar to that described in other vascular tissues (Guimarães *et al.*, 1998) because it is blocked neither by $AT_1$ nor $AT_2$ antagonists.

Guimarães, S., Paiva, M.Q. & Moura, D. (1998). *Br. J. Pharmacol.* **124**,1207-1212.
Vaz-da-Silva, M., Magina, S., Domingues-Costa, A., *et al.* (1996). *Br. J. Pharmacol.* **118**, 364-368.

Supported by JNICT (project 80/95) and Praxis/2/2.1/SAU/1293/95

---

## 8P A POTENTIAL ROLE FOR ENDOTHELIN-1 IN PERIPHERAL VASCULAR DISEASE

M.R. Dashwood[1], I.A. Jagroop[1], D. Gorog[2] and P. Bagger[2]. Department of Molecular Pathology & Clinical Biochemistry, Royal Free & University College Medical School, London NW3 2QG[1] and Dept of Cardiology, Hammersmith Hospital, London W12 0HS[2].

There is evidence of increased circulating levels of endothelin-1 (ET-1) in peripheral vascular disease (PVD). ET-1 is a potent vasoconstrictor peptide acting via $ET_A/ET_B$ receptors located on vascular smooth muscle cells (VSMCs). Here we have used a combination of immunohistochemistry and *in vitro* autoradiography to identify ET-1 and its receptors on proximal and distal regions of femoral arteries obtained from patients undergoing vein grafting for PVD.

Slide-mounted sections of proximal and distal femoral artery wall were incubated in [$^{125}$I]-ET-1 and receptor subtypes identified using [$^{125}$I]-PD151242 ($ET_A$) and [$^{125}$I]-BQ3020 ($ET_B$). Autoradiographs were generated on film and receptor binding was quantified by densitometric analysis. Microscopic localisation of binding was achieved using nuclear emulsion and ET-1 and specific cell types identified on tissue sections using immunomarkers.

There was dense binding of [$^{125}$I]-ET-1 and subtype-selective radioligands to VSMCs of the tunica media of vessels obtained from PVD patients. Densitometric analysis indicated that there was no difference in binding between proximal (control) and distal ("diseased") vessel regions (see table 1.)

Table 1. ET receptor binding to vessels from PVD patients.

| $ET_A$ | | $ET_B$ | |
|---|---|---|---|
| Proximal | Distal | Proximal | Distal |
| 10.1±1.5 | 10.1±1.4 | 6.9±0.4 | 7.5±0.7 |

Specific [$^{125}$I]-PD151242 ($ET_A$) and [$^{125}$I]-BQ3020 ($ET_B$) binding to tunica media of proximal and distal segments of femoral artery from PVD patients undergoing vein grafting. Binding expressed as mean ± sem radioligand binding ($dpmx10^3$) per $mm^2$ (n=6 patients).

High resolution autoradiographs, generated on nuclear emulsion, revealed $ET_B$ binding to the vasa vasorum and vascular nerves and ET-1 immunoreactivity was associated with the endothelium and vasa vasorum.

These results show that there are no regional variations (proximal vs distal) in ET receptor binding in femoral arteries from PVD patients. However, the medial $ET_A/ET_B$ receptors identified in this study are ideally situated to be affected by the raised circulating levels of ET-1 described in PVD. Our data regarding the cellular localisation of $ET_B$ receptors indicates that, in PVD, ET-1 may cause microvascular ischaemia, by an action on the vasa vasorum, and may also have a neural role in claudication via the vascular nerves. These results underscore the possible therapeutic potential of ET antagonists in PVD.

9P ENDOTHELIN BLOCKS L-TYPE CALCIUM CHANNELS IN MICROVASCULAR SMOOTH MUSCLE OF THE RABBIT CHOROID

Tim M. Curtis & C. Norman Scholfield

Department of Physiology, Queen's University, 97 Lisburn Road, Belfast BT9 7BL.

It is now well established that the peptide endothelin-1 (ET-1) is an important regulator of vascular function. The effects of ET-1 on L-type Ca-channels of microvascular smooth muscle cells (MVSMCs) have not been clarified. In this study, using the whole cell perforated patch technique, we have examined the effects of ET-1 on L-type Ca-channels in MVSMCs of the rabbit choroid.

The retinae were removed from freshly killed adult NZ white rabbits, the choroids were then peeled away and digested in collagenase/20μM $Ca^{2+}$ solution. The digestate was washed in medium containing 100μM $Ca^{2+}$ and placed into a recording bath. MVSMCs were identified by their short (35 μm × 5 μm) spindle shape and their resemblance to those still attached to microvessels. Whole cell perforated patch recordings were made using K+-electrodes containing amphotericin and connected to a conventional patch recording amplifier. Because L-type calcium currents are difficult to resolve in MVSMCs a divalent free cation bathing medium was used. This solution contained (mM): NaCl (143.3), KCl (5.9), Hepes (10), glucose (5), EGTA (5), pH 7.3. During the recording, the solution flowing over the cell also contained TEA and 4-AP.

50% of the cells successfully patched displayed an inward conductance which activated at -50 mV and had a maximal current of 90-600 pA at -20 mV. It was completely blocked by 100 μM $Cd^{2+}$ (4 cells) or by 5 μM nifedipine (5 cells). The current was unaffected by TTX (1 μM). These properties are consistent with this current being Na+ influx through L-type Ca-channels ($ICa_{Na}$). 10nM ET-1 reduced peak $ICa_{Na}$ by 85.5 ± 7.8 % (s.e.m., 5 cells). This inhibition was maintained during persistent exposure to ET-1 (5 min). The effect reversed on washing out. Brief exposure to 10mM caffeine to deplete Ca-stores, prior to ET-1, failed to prevent this inhibition (5 cells). Fura-2 $Ca^{2+}$ microfluorimetry experiments showed that under these latter conditions ET-1 does not induce a rise in $[Ca^{2+}]_{in}$.

These results suggest that ET-1 blocks L-type Ca-channels in MVSMCs of the rabbit choroid, and that this inhibition appears to be independent of $Ca^{2+}$ release from intracellular stores or $Ca^{2+}$ influx from the bathing medium.

This work was supported by the Wellcome Trust

---

10P EFFECT OF CALCIUM CHANNEL BLOCKERS ON BASAL AND TUMOUR NECROSIS FACTOR α-STIMULATED ENDOTHELIN-1 SYNTHESIS IN BOVINE AORTIC ENDOTHELIAL CELLS

R. Corder, N. Khan, E.G. Wood, D.M. Lees & S. Barker, The William Harvey Research Institute, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Charterhouse Square, London, EC1M 6BQ, UK.

There are three main classes of calcium channel blockers: benzothiazepines (e.g. diltiazem), phenylalkylamines (e.g. verapamil), and dihydropyridines (e.g. nicardipine, isradipine). These agents are vasodilators with widespread use in the treatment of angina and hypertension. However, recent studies suggest that some calcium channel blockers have additional actions including inhibition of preproendothelin-1 expression (Feron *et al.*, 1996). In endothelial cells effects may be exerted not only through inhibition of calcium channels but also through actions on chloride channels (Nilius *et al.*, 1997). Here we describe the effects of several different calcium channel blockers on endothelin-1 (ET-1) synthesis in endothelial cells.

Bovine aortic endothelial cells (BAEC) were cultured as previously described (Corder *et al.*, 1995). The effects of diltiazem, verapamil, nicardipine and isradipine were evaluated on both basal and tumour necrosis factor-α (TNFα) stimulated ET-1 synthesis. Confluent cultures (12 x 22 mm well plates) were treated for 6 h in serum free medium with the agents indicated, followed by a 1 h MTT test to ensure the absence of cytotoxic effects. ET-1 secretion was determined by radioimmunoassay (Corder *et al.*, 1995). In each experiment ET-1 secretion was expressed as a % of basal release (mean basal release = 32 ± 1 $fmol.cm^{-2}.6\ h^{-1}$). Results are from at least 4 separate experiments with duplicate or triplicate determinations in each. These are shown as means ± s.e.m., and significant differences were evaluated by ANOVA with Fisher's LSD test.

Table 1. Effect of calcium channel blockers on basal and TNFα-stimulated ET-1 synthesis in BAEC (TNFα values are either alone or with 10 μM $Ca^{2+}$ antagonist, * P <0.05 compared to control, † P <0.05 compared to 10 ng/ml TNFα alone.

| | 1 μM | 10 μM | TNFα |
|---|---|---|---|
| Control | 100 ± 1 | 100 ± 1 | 170 ± 5* |
| Diltiazem | 90 ± 2* | 90 ± 2* | 144 ± 6*† |
| Verapamil | 90 ± 2* | 66 ± 1* | 114 ± 3*† |
| Nicardipine | 88 ± 2* | 72 ± 1* | 119 ± 6*† |
| Isradipine | 93 ± 2* | 78 ± 1* | 138 ± 4*† |

Calcium channel blockers of all classes caused significant reductions in basal and TNFα-stimulated ET-1 synthesis (Table 1). In these studies verapamil tended to be more effective as an inhibitor of both basal and stimulated ET-1 release. Interestingly, verapamil also inhibits TNFα-stimulated adhesion molecule expression in endothelial cells (Yamaguchi *et al.*, 1998). These actions of calcium channel blockers on endothelial function may reduce ET-1-dependent vascular responses, including remodelling, and contribute to the therapeutic benefit of these agents.

Corder, R. *et al.* (1995) *J. Cardiovasc. Pharmacol.* 26(Suppl. 3), S56-S53.
Feron, O. *et al.* (1996) *Br. J. Pharmacol.* 118, 659-664.
Nilius, B. *et al.* (1997) *Br. J. Pharmacol.* 121, 547-555.
Yamaguchi, M. *et al.* (1998) *Transplantation* 15, 756-757.

## 11P COMPARISON OF THE EFFECT OF A PEPTIDOGLYCAN FRAGMENT AND LIPOPOLYSACCHARIDE ON NITRITE PRODUCTION IN J7.DEF3 MACROPHAGE CELLS *IN VITRO*

M. Kengatharan, F. Habens, R. Corder, E.E. Änggård & M.J. Carrier, The William Harvey Research Institute, St Bartholomew's & The Royal London Hospital School of Medicine & Dentistry, Charterhouse Square, London EC1M 6BQ, United Kingdom.

Cellular responses to the Gram-negative cell wall component, lipopolysaccharide (LPS) is dependent on the receptor CD14, but it is unclear whether peptidoglycan (found on Gram-positive cell walls) uses the same receptor even though it binds to CD14 at the same site as LPS (Dziarski, *et al.*, 1998). In this study, we show that, a fragment of peptidoglycan (D-N-acetylglucosamine-β-[1→4]-N-acetylmuramyl-L-ala-D-isoglutamine; NAG-AP) which mediates the effects of this polymer (Kengatharan *et al.*, 1998), is able to facilitate an increase in nitric oxide (NO) formation in J7.DEF3 macrophages which do not express CD14 on their surface (Kirikae *et al.*, 1993).

The murine macrophages J7.DEF3 or the parent cells, J774.1A were cultured in 96-well plates with 200 µl of culture medium (DMEM) containing foetal calf serum (10%) and glutamine (4 mM). At confluence, NAG-AP or LPS was added to the cells either alone or together with interferon-γ (IFNγ; 1 I.U.ml$^{-1}$). In some cases, IFNγ (1 or 10 I.U.ml$^{-1}$) was added to the cells alone. Nitrite accumulation, an indicator of NO formation by inducible NO synthase, was measured 24h later in the conditioned media using the Griess method (Kengatharan *et al.*, 1998). Data are expressed as mean±s.e.mean (n=3-5 independent observations) and significant differences were determined by two-way ANOVA or Student's *t-test*.

LPS produced a concentration-dependent increase in nitrite levels in the supernatants of both macrophage cell lines. But the increase in nitrite was significantly less in J7.DEF3 compared to J774.1A regardless of the presence of IFNγ (see Table 1), confirming that J7.DEF3 do indeed have an impaired response to LPS. In contrast to LPS, nitrite accumulation in response to NAG-AP with IFNγ was comparable in the two macrophage cell lines demonstrating that responses were not affected by the absence of CD14 protein in J7.DEF3. IFNγ alone (10 I.U.ml$^{-1}$) which does not require CD14 for initiating intracellular response induced similar nitrite levels (in µM; 53±13 in J774.1A and 51±11 in J7.DEF3, n=4-5, P>0.05). The increase in nitrite induced by LPS in J7.DEF3 cells is likely to be mediated by the presence of soluble CD14 in the serum.

**Table 1.** Nitrite accumulation in J774.1A and J7.DEF3 cells

| | (µg.ml$^{-1}$) | Nitrite formation at 24h (µM) | | | |
|---|---|---|---|---|---|
| | | + IFNγ (1 I.U.ml$^{-1}$)# | | - IFNγ# | |
| | | J774.1A | J7.DEF3 | J774.1A | J7.DEF3 |
| - | 0 | 4±1 | 2±3 | 0±1 | 1±1 |
| LPS* | 0.01 | 72±4 | 39±12 | 18±3 | 0±0 |
| | 0.1 | 140±6 | 77±16 | 67±8 | 20±8 |
| | 1 | 153±6 | 94±17 | 79±15 | 39±12 |
| NAG-AP | 0.1 | 14±1 | 16±8 | 0±0 | 0±1 |
| | 1 | 19±4 | 26±11 | 1±0 | 0±1 |
| | 10 | 21±7 | 24±12 | 2±2 | 1±1 |

*P<0.05 J774.1A *vs* J7.DEF3, #P<0.05 with IFNγ *vs* without IFNγ

Thus, the NO synthesis induced by NAG-AP in combination with IFNγ in murine macrophages may be mediated by a distinct mechanism independent of the CD14 receptor.

*MK holds a BHF Research Fellowship (FS/98025). This project is supported by the British Heart Foundation.*

Kengatharan, K.M. *et al.*, (1998). *J. Exp. Med.* 188, 305-315.
Dziarski, R., Tapping, R.I. & Tobias, P.S. (1998). *J. Biol. Chem.*, 273, 8680-8690.
Kirikae, T. *et al.*, (1993). *J. Immunol.* 151, 2742-2752.

---

## 12P INHIBITION OF LPS-STIMULATED NEκB SIGNALLING AND iNOS INDUCTION IN RAW 264.7 MACROPHAGHES BY THE TYROSINE PHOSPHATASE INHIBITOR PERVANADATE

**Andrew Paul* Lindsay J. Torrie & Robin Plevin.** Department of Physiology and Pharmacology, University of Strathclyde. SIBS, Glasgow G4 0NR UK.

In RAW 264.7 macrophages, lipopolysacchride(LPS) stimulates the induction of nitric oxide synthase (iNOS) (Paul *et al.*, 1995). This effect is mediated principally through the transcription factor nucleur factor κB (NFκB) which interacts with specific binding sites within the iNOS promoter. Following LPS stimulation NFκB is activated by dissociation from an inhibitory protein, IκB. This process is initated by phosphorylation of IκB by an associated kinase, namely inhibitory κB kinase (IKK) (Regnier *et al.*, 1997). As the intermediates involved in the regulation of IKK have not been fully identified we examined the effect of the tyrosine phosphatase inhibitor pervanadate upon LPS-stimulated NFκB and iNOS induction.

RAW264.7 macrophages were cultured as outlined previously (Paul *et al.*, 1995). LPS-stimulated NFκB-DNA binding activity was assessed by electrophoreticmobility shift assay (EMSA). IKKα and IKKβ activity was assessed by kinase assay *in vitro*. Cellular levels of iNOS and IκB isoforms were assessed by Western blotting. iNOS activity was assayed by measuring the conversion of [$^3$H]arginine to [$^3$H]citrulline in detergent solubilised cell lysates (Paul *et al.*, 1995).

In RAW 264.7 macrophages, LPS stimulated a time-dependent increase in NFκB DNA binding activity and loss in expression of IκBα and IκBβ isoforms. LPS also stimulated a time-dependent increase in IKKα and IKKβ activity (Fold stimulation, IKKα 4.9±0.5; IKKβ 19.1±3.2; mean±s.d, n=3) Pretreament of cells with pervanadate (Per, 30min 100µM), but not component orthovanadate or hydrogen peroxide, abolished LPS-stimulated IKKα and IKKβ activity, prevented LPS-induced degradation of IκBα and β and abolished NFκB-DNA binding activity. LPS also stimulated the expression of iNOS over a 12 hr period which was associated with an increase in enzyme activity. Both iNOS expression and activity were abolished by pervanadate pretreatment (iNOS activity/dpm in [$^3$H]citrulline; control 22471±1896, control±Per 19000±958, LPS 152087± 10791, LPS+Per 17154±988; mean ± s.d, n=3).

These results demonstrate for the first time the possible involvement of a tyrosine phosphatase enzyme in the regulation of IKK isoforms, NFκB activation and iNOS expression in LPS stimulated cells. This may provide the basis for the development of therapies directed against endotoxemia.

Paul, A., Pendrigh, R.H & Plevin, R. (1995) Brit. J. Pharmacol. 114, 482-488.

Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z. & Rothe, M. (1997) Cell 90, 373-383.

This work was supported by The Wellcome Trust.

## 13P EFFECTS OF LIPOCORTIN-1 ON MITOGEN-ACTIVATED PROTEIN KINASES

L.C. Alldridge, R. Plevin & C.E. Bryant
*Department of Clinical Veterinary Medicine, The University of Cambridge, Madingley Road, Cambridge, CB3 OES, UK. *Dept. Physiology and Pharmacolcogy, University of Strathclyde, 204, George St., Glasgow, G1 1XW.*

Lipopolysaccharide (LPS)-activation of cells induces protein phosphorylation including members of the mitogen activated protein (MAP) kinase family: p38, extracellular-signal-regulated kinase (ERK) 1 and 2 and Jun N-terminal kinase (JNK). Lipocortin-1 (LC-1) supresses LPS-induction of inducible nitric oxide synthase (iNOS) in J774.1 macrophages (Wu *et al.*, 1995), suggesting a role for LC-1 in regulating macrophage responses to LPS. In this study we have used stably transfected murine macrophage cell lines (RAW 264) with sense and antisense LC-1 genes (Harris et al., 1998) to study the LPS-activation of ERK, JNK and p38 MAP kinase in these cells.

Sense (F), antisense (R) control (C) and untransfected (RAW) cells were plated at equal density ($1x10^4$/ml) onto 6 well plates for 48h in DMEM supplemented with glutamine (2mM), penicillin (200U/ml), streptomycin (100µl), FCS (10%) and geneticin (G418; 500µg/ml). At 90-100% confluency, cells were treated with LPS (1µg/ml) or vehicle for 0-90 min. After cell lysis the ERK, JNK and p38 activity was assessed using in vitro kinase assays (Belham et al., 1996). Optical densities of phosphorylated substrate bands were quantified using Kodak 1/D software and values compared by ANOVA. Values are reported as fold activity (for n=4).

LPS (1µg/ml) induced a 28-36 fold increase in activation of p38 MAP kinase with similar kinetics of in all cell lines. F, C and RAW cells showed peak activity at 15 minutes whereas the R cells displayed slightly slower kinetics peaking 30 minutes after LPS-stimulation. LPS (1µg/ml) induced a 14-20 fold activation of JNK with similar kinetics in all cell lines, peak activation occuring 15-30 minutes post stimulation. LPS-induced JNK activity was still present at 90 min. LPS (1µg/ml) affected ERK activity in all cell lines. In C and RAW cells ERK became active at 5 minutes after stimulation with LPS and returned to control levels after 30 minutes. R displayed a prolonged activation of ERK/MAP kinase with enzyme activity still present 30 minutes after exposure to LPS. F cells displayed constitutively activated ERK/MAP kinase, which was inhibited after stimulation with LPS. ERK/MAP kinase activity was inhibited by 50% at 5 minutes and by 90% at 30 minutes after exposure to LPS (see figure 1).

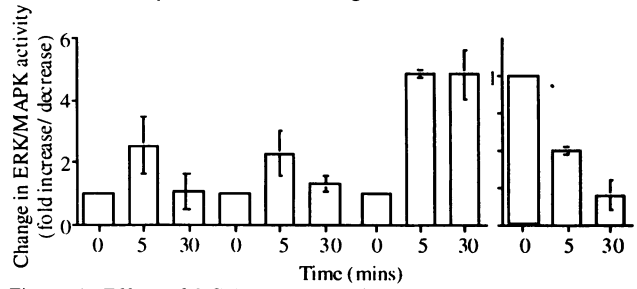



Figure 1: Effect of LC-1 on ERK activity. Data is expressed as fold change in activity (n=4)

Here we show that LC-1 regulates the activity of the ERK/MAP kinase cascade with minimal effects on the JNK or p38 pathways in RAW macrophages. Identification of the site at which LC-1 interacts with the ERK/MAP kinase pathway may help to explain the cellular effects of this protein.

This work was supported by the Wellcome Trust.

Belham, C.M. et al., 1996, *Biochem. J.* **320**, 939-946
Harris, H.J. et al., 1998, *Brit. J. Pharmacol.* **123**, P83
Wu, C.C., et al., 1995, *Proc. Natl. Acad. Sci.* **92**, 3473-3477

---

## 14P DISSOCIATION OF 5-$HT_{1A}$ RECEPTOR-STIMULATED MAP KINASE ACTIVITY AND DNA SYNTHESIS IN TRANSFECTED CHO CELLS

**Elizabeth Fashola*, Andrew Paul, Philip G. Strange⁺ & Robin Plevin,** Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G4 0NR UK; ⁺ School of Animal Microbial Sciences, University of Reading, RG6 6AJ, UK

The 5-$HT_{1A}$ receptor belongs to a large family of seven transmembrane domain-spanning, G-protein coupled receptors and is categorized within the 5-HT1 receptor subfamily. Activation of the $5HT_{1A}$ receptor has been associated with increases in cell proliferation and neuronal differentiation (Singh, *et al.*, 1996). A number of G–protein coupled agonists have been shown to activate homologues of mitogen-activated protein (MAP) kinase, in particular p42/44 but also c-Jun N-terminal kinase (JNK) and p38 MAP kinases (Paul *et al.*, 1997). It is unclear however, as to the role these kinases play in $5HT_{1A}$ receptor-mediated stimulation of cell growth. In this study we have examined activation of MAP kinase activity by 5-$HT_{1A}$ and the correlation between these activities and DNA synthesis.

Cells expressing the $5HT_{1A}$ receptor were established as outlined previously (Newman-Tancredi *et al.*,1992) and rendered quiescent by preincubation for 40 hr in serum free conditions. MAP kinase activation was assessed by analysis of retardation on SDS PAGE or by peptide phosphorylation kinase assay. JNK and p38 MAP kinase were assayed by solid-phase kinase assay. DNA synthesis was assayed by measuring acid insoluble [$^3$H]thymidine uptake over a 24hr period.

In CHO cells, the $5HT_{1A}$ agonist, 8-OH DPAT (100nM) stimulated a transient increase in MAP kinase activity which peaked at 5 min before returning to near basal values by 30min (fold stim: 2min=6.71± 0.14, 15min = 2.03±0.1, 30min = 1.24±0.15, n=4). Activation of MAP kinase was effectively abolished by preincubation with pertussis toxin (PTX) (50ng/ml) and the MAP kinase kinase–1 inhibitor, PD098059 (50µM). 8-OHDPAT also stimulated JNK activity but did not stimulate p38 MAP kinase activity. JNK activity was also abolished by pertussis toxin pretreatment. 8OH-DPAT also stimulated DNA synthesis in CHO cells however, this activation was not effected by preincubation with pertussis toxin and only partially reduced by PD098059. (d.p.m.± s.e.m. Control = 1933±231; 8-OH-DPAT = 5470±2311, PTX =1459±678, 8-OH-DPAT + PTX = 6150 ±250 (ns); PD09805 = 1937 ±231, 8OHDPAT + PD98059= 3955 ± 550 ($p< 0.05$) n=4).

These results show that in transfected CHO cells, stimulation of the $5HT_{1A}$ receptor results in the activation of p42/44 MAP kinase and JNK through a pertussis toxin-sensitive pathway. However, activation of these MAP kinase signalling pathways are clearly dissociated from the effects upon cell growth.

Newman-Tancredi, A. Wootton, R. & Strange, P.G. (1992). Biochem J. 285, 933-938.

Paul, A., Wilson, S., Belham, C. et al., (1997) Cell. Signal. 9, 403-410.

Singh, J.K., Yan, Q., Dawson, G. & Banerjee, P., (1996) Biochem Biophy. Acta. 1310, 201-211.

## 15P INHIBITION OF INHIBITORY KAPPA B KINASE ENHANCES LIPOPOLYSACCHARIDE STIMULATION OF SAP KINASES IN RAT AORTIC SMOOTH MUSCLE CELLS

**Andrew Paul, Susan Wilson & Robin Plevin***, Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G4 0NR UK

In rat aortic smooth muscle cells (RASMCs) lipopolysaccharide (LPS) stimulates the induction of the 130kDa isoform of nitric oxide synthase (iNOS) (Paul *et al.*, 1997) through the activation of a number of key signalling events. LPS activates the stress-activated protein (SAP) kinases, p38 MAP kinase and c-Jun N-terminal kinase (JNK). LPS also activates the transcription factor Nuclear factor kappa B (NFκB) through phosphorylation-dependent degradation of Inhibitory kappa B (IκB). This is regulated by isoforms of a kinase known as inhibitory kappa B kinase (IKK). In this study we examined the effect of well recognised inhibitors of the NFκB signalling pathway, pyrrolidine dithicarbamate (PDTC) (Sherman *et al.*, 1993) and Nα-p-Tosyl-L-lysine chloro-methylketone (TLCK) (Hattorri *et al.*, 1993) upon LPS-stimulated JNK and p38 MAP kinase in RASMCs.

RASMC were prepared and cultured as previously described (Paul *et al.*, 1997). NFκB-DNA binding activity was assayed by electrophoreticmobility shift assay. LPS-stimulated IKK, JNK, p38 MAP kinase and MAPKAP kinase-2 activities were assessed by *in vitro* kinase assays following specific precipitation of the relevant kinase (Paul & Plevin, 1999). Cellular expression of iNOS and IKBα and β isoforms were assessed by Western blotting.

In RAMC pretreatment with PDTC (100μM, 60 min) abolished LPS ($100\mu gml^{-1}$)-induced expression of iNOS and also NFκB-DNA binding activity. However, PTDC also prevented the LPS-induced loss in cellular IκB and inhibited LPS-stimulated IKK activity (fold stim.±s.e.m: IKKα LPS, 7.08±0.27 LPS+PDTC 2.5±0.03; IKKβ LPS 5.64±0.4 LPS+PDTC 1.80±0.04, n=3). Preincubation of RASM with PDTC also enhanced LPS-stimulated JNK activity and prolonged the kinetics of activation (fold stim ± s.e.m.: 90min LPS=2.84± 0.97, LPS+PDTC=21.9±11.0, n=4). A similar phenomenon was observed for p38 MAP kinase and the downstream target MAPKAP kinase-2 (MAPKAP kinase-2 fold stim.±s.e.m.:30min LPS=2.5±0.42, LPS+PDTC=6.95±1.2; 90min LPS=1.61±0.11, LPS+PDTC=8.2±4.0, n=4). Preincubation of RASM with TLCK also inhibited LPS-stimulated NFκB and loss in cellular IκBα and IκBα. However, TLCK also abolished LPS-stimulated IKK activity. TLCK also enhanced LPS-stimulated JNK activity in a manner similar to PDTC.

This study shows that in RASMC the compounds PDTC and TLCK affect the NFκB signalling pathway at a site other than previously proposed, at the level of IKK or above. Secondly, inhibition of IKK signalling results in enhanced activation of the SAP kinase pathways.

This work was sponsored by The Wellcome Trust.

Paul, A., Doherty, K. & Plevin, R. (1997) Brit. J. Pharmacol. 120, 940-946.
Paul, A. & Plevin, R. (1999) Biochem J. (submitted)
Hattori, Y., Nakanishi, N. & Kasai, K. (1997) J. Mol. Cell Cardiol 29, 1585-1492.
Sherman, M.P., Aeberhard, E.E., Wong, V.Z. *et al.*, (1993). Biochem. Biophys. Res. Comm 191, 1301-1308.

---

## 16P CO-ORDINATION OF AGONIST-INDUCED CALCIUM SIGNALLING PATTERNS BY NAADP IN PANCREATIC ACINAR CELLS

Jose Manuel Cancela*, Grant C. Churchill¶and Antony Galione¶
*Department of Physiology, Crown Street, University of Liverpool, L69 3BX, University ¶Department of Pharmacology, Mansfield Road, University of Oxford, OX1 3QT, UK

We have investigated the possible actions and messenger roles in pancreatic acinar cells of nicotinic acid adenine dinucleotide phosphate (NAADP), an endogenous metabolite of β-NADP, and potent $Ca^{2+}$ mobilizing agent in marine invertebrate eggs. The whole-cell configuration of the patch-clamp technique permitted access to the cytosol and monitoring of cytosolic $Ca^{2+}$ concentration changes by measuring $Ca^{2+}$-dependent ionic currents, which have been extensively correlated with different $Ca^{2+}$ signalling patterns.
When NAADP was infused through the intracellular patch-pipette solution it elicited concentration-dependent $Ca^{2+}$ spiking. The intensity of the response to 50 nM NAADP (n=21/27) varied and we classified it in three categories: short-lasting $Ca^{2+}$ spikes (1-2 s, n=4/21); complex patterns of $Ca^{2+}$ spikes which are a mixture of short lasting (1-2 s) and long-lasting (0.2 - 1 min) $Ca^{2+}$ transients (n=7/21); and a sustained $Ca^{2+}$ release (n=10/21). Perifusion of cells with a $Ca^{2+}$ -free solution did not prevent the NAADP-evoked sustained $Ca^{2+}$ response (n=3), indicating that $Ca^{2+}$ is released from intracellular stores. Surprisingly, higher NAADP concentrations (1 to 100 μM) in the patch-pipette solution, failed to evoke any $Ca^{2+}$ release (n=19). One explanation of this is that at high concentrations, NAADP diffusion is very fast and desensitises NAADP receptors rapidly. We also investigated the characteristics of intracellular $Ca^{2+}$ stores mobilised by NAADP. We used 8-$NH_2$-cADPR and heparin as selective inhibitors of cADPR and $InsP_3$-sensitive $Ca^{2+}$ release, respectively. Either 8-$NH_2$-cADPR (18 μM) or heparin (250 μg/ml) in the pipette solution abolished NAADP (50 nM in the pipette) induced $Ca^{2+}$ spikes in pancreatic acinar cells (n=5, n=7, respectively). In contrast, in the absence of inhibitors, cells vigorously responded to 50 nM NAADP (n=9/10). These results indicate that the complex pattern of $Ca^{2+}$ spiking evoked by NAADP also involves activation of both ryanodine and $InsP_3$ receptors. Since high concentrations of NAADP fully inhibited NAADP-evoked $Ca^{2+}$ spikes, we tested the specificity of this effect for NAADP-induced $Ca^{2+}$ release by investigating the ability of cADPR and $InsP_3$ to induce $Ca^{2+}$ spikes in the presence of an inactivating concentration of NAADP (100 μM). In the presence of Ins 2,4,5$P_3$ (10 μM in the patch-pipette solution), cells displayed typical short-lasting $Ca^{2+}$ spikes (1-2 seconds, n=5). Such $Ca^{2+}$ spikes are of a similar pattern to those evoked by 10 μM cADPR (n=5). When NAADP at 100 μM was present in the internal pipette solution, all the cells still responded to both Ins 2,4,5$P_3$ (n=5) and cADPR (n=5). These data indicate that the inhibitory effect of NAADP on NAADP-induced $Ca^{2+}$ spiking is likely due to a desensitisation of the NAADP receptor rather than by inhibiting with $InsP_3$ or ryanodine receptors.
NAADP evoked $Ca^{2+}$ spikes that can be intriguingly similar to those evoked by the secretory hormone cholecystokinin (CCK) (2 and 5 pM). Extracellular applications of CCK at 2 and 5 pM elicited typical $Ca^{2+}$ spikes (n=13/14 and 14/14, respectively). However, the presence of a desensitising NAADP concentration (100 μM) completely abolished the ability of CCK (2 pM) to evoke $Ca^{2+}$ responses (n = 7). In addition, although we increased the concentration of CCK to 5 pM, 57% of the cells did not respond and 43% displayed an attenuated response to 5 pM CCK, indicating that the inhibitory effect of NAADP remains (n=7). This effect of NAADP was highly specific since infusion of cells with its metabolic precursor β-NADP (100 μM) failed to inhibit CCK-induced $Ca^{2+}$ spikes (n=4).
In this study, we report the first description of NAADP-evoked $Ca^{2+}$ mobilization in a mammalian cell. Moreover, the abolition of CCK signalling by perfusing cells with desensitizing concentrations of NAADP, suggests that NAADP is a key intracellular mediator of this hormone. Since NAADP mobilizes $Ca^{2+}$ in pancreatic acinar cells as well as in the oocytes of two diverse species of marine invertebrates, it is likely to be a general $Ca^{2+}$ mobilizing messenger.

## 17P KAINATE RECEPTORS COUPLED TO $G_i/G_o$ PROTEINS IN THE RAT HIPPOCAMPUS

R.A. Cunha[1,2], J.O. Malva[3] & J.A. Ribeiro[1], [1]Lab.Neurosciences, Fac.Medicine, Univ.Lisbon, [2]Dept.Chemistry & Biochemistry, Fac.Sciences, Univ.Lisbon and [3]Center for Neurosciences of Coimbra, University of Coimbra, Portugal

Kainate receptors are a subtype of ionotropic glutamate receptors, permeable to cations and thus expected to have an excitatory depolarising action on neurones. However, kainate receptor activation inhibits GABA release in the hippocampus through activation of protein kinase C in a pertussis toxin-dependent manner, suggesting a coupling of kainate receptors to G proteins (Rodríguez-Moreno and Lerma, 1998). Thus, we now directly investigated the G protein coupling of kainate receptors in the rat hippocampus, using a selective kainate receptor agonist, [$^3$H]-(2S,4R)-4-methylglutamate ([$^3$H]MGA).

Hippocampal membranes were prepared from 6 week old male Wistar rats and pre-treated with G protein modifiers (Cunha *et al.*, 1999) and binding of [$^3$H]MGA was for 90 min at 4°C with 190-370 µg of membrane protein in a final volume of 300 µl in the incubation solution containing 50 mM Tris-HCl and 0.5 mM $MgCl_2$, pH 7.4 (Johansen *et al.*, 1993). Saturation curves were performed with 10 different [$^3$H]MGA concentrations (0.3-120 nM) and competition curves were performed with 25 nM [$^3$H]MGA and 8 different concentrations of competitors (1 nM to 10 µM).

[$^3$H]MGA bound to a single site to hippocampal membranes with a $K_D$ of 32 (95% confidence interval: 29-34) nM and a $B_{max}$ of 1024±39 fmol/mg protein (n=14). Guanylylimidodiphosphate (30 µM), which uncouples all G protein coupled receptors, shifted to the right the saturation curve of [$^3$H]MGA [$K_D$= 133 (67-198) nM, n=4]. This effect was mimicked by pre-treatment of hippocampal membranes with modifiers of $G_i/G_o$ proteins, 30 µM N-ethylmaleimide [$K_D$= 98 (72-124) nM, n=4] or 25 µg/ml pertussis toxin [$K_D$= 95 (88-103) nM, n=3] but not by a modifier of $G_s$ proteins, 50 µg/ml cholera toxin [$K_D$= 32 (13-51) nM, n=3]. Treatment of solubilised hippocampal membranes with pertussis toxin (25 µg/ml) decreased [$^3$H]MGA affinity ($K_D$= 105-113 nM) which was recovered by reconstitution of these pre-treated solubilised hippocampal membranes with $G_i/G_o$ proteins ($K_D$= 41-76 nM) (n=2).

These results indicate that hippocampal kainate receptors are coupled to $G_i/G_o$ proteins.

Cunha, R.A., Constantino, M.D. & Ribeiro, J.A. (1999) *Naunyn Schmiedeberg's Arch.Pharmacol.*, in press
Johansen, T.H. *et al.* (1993) *Eur.J.Pharmacol. Mol.Pharmacol.Sec.* **246**, 195-204
Rodríguez-Moreno, A. & Lerma, J. (1998) *Neuron* **20**, 1211-1218

---

## 18P VISUALIZATION AND PROPERTIES OF TWO N-TERMINAL EPITOPE-TAGGED HUMAN $H_1$ HISTAMINE RECEPTORS

A.C. Scott & S.J. Hill, Institute of Cell Signalling, School of Biomedical Sciences, Medical School, Queen's Medical Centre, Nottingham. NG7 2UH

The addition of an epitope tag to a G-protein coupled receptor enables visualization and isolation of the receptor using techniques such as immunocytochemistry and immunoprecipitation (Smit *et al.*, 1995; Gimpl *et al.*, 1996). This study shows a comparison of binding characteristics, functional responses and visualization (by SDS-PAGE and immunocytochemistry) of two N-terminal epitope tags on the human histamine $H_1$ receptor (Flag: MDYKDDDDK; myc: MEQKLISEEDL).

Chinese Hamster Ovary (CHO) cells were stably transfected with the cDNA for the wild type human $H_1$ receptor ($hH_1$), the human $H_1$ receptor with the Flag epitope tag ($hH_1f$) or the myc epitope tag ($hH_1m$). Clones, named $CHOhH_1$5, $CHOhH_1$f24 and $CHOhH_1$m2, were isolated and used in functional and visualization studies. The functional response was assessed by measuring the accumulation of [$^3$H]-inositol in response to increasing concentrations of histamine (Megson *et al.* 1995). Membrane binding assays using [$^3$H]-mepyramine were carried out to determine receptor number and binding characteristics (Iredale, P.A. *et al.*, 1993). The receptor was visualized by SDS-PAGE followed by immunodetection, or using immunocytochemistry techniques.

Histamine produced a concentration-dependent increase in total [$^3$H]-inositol phosphate accumulation, with $pEC_{50}$ of 5.9±0.1 (mean±s.e.m) in $CHOhH_1$5, 5.8±0.1 in $CHOhH_1$f24 and 5.9±0.1 in $CHOhH_1$m2 (n=6). The addition of mepyramine ($1\times10^{-7}$M) produced a rightward shift of the response to histamine in $CHOhH_1$5, $pK_B$=8.42±0.07 (n=4) and $CHOhH_1$m2, $pK_B$=8.20±0.22 (n=3). The mean $B_{max}$ from [$^3$H]-mepyramine binding for these clones, calculated from membrane binding assays, was 268.8±62.9 fmoles/mg protein in $CHOhH_1$5, 755.2±165.8 fmoles/mg protein $CHOhH_1$f24 (n=6) and 434±114.7 fmoles/mg protein $CHOhH_1$m2 (n=4). Using cell lysates from $CHOhH_1$f24 on SDS-Page gels, no specific band could be measured after immunodetection. However, using cell lysates from myc tagged $hH_1$ receptor a specific band of 60kD was observed (n=3). Results from immunocytochemistry studies showed no specific staining using $CHOhH_1$f24 but specific cell surface staining could be identified using the myc tagged $hH_1$ receptor (n=3).

These results show CHO cells stably express both the native and an epitope tagged $hH_1$ receptor. The isolated clones have similar functional responses and binding characteristics to each other. These results show that neither of these epitope tags has an effect on the functional or binding characteristics of the $hH_1$ receptor. We have shown that a receptor with the myc epitope tag can be detected by both immunoblotting and immunocytochemistry. However, we were not able to visualize the Flag epitope-tagged receptor using either of these techniques. This maybe due either to cleavage of the Flag tag (at the enterokinase site situated between the tag and the receptor) or that the addition of the Flag epitope tag disrupts the folding of the N terminus of the receptor masking the epitope.

ACS holds an MRC studentship

Gimpl, G., Anders, J., Thiele, C., *et al.*, (1996) *Eur. J. Biochem.* 237, 768-777.
Iredale, P.A., Fukui, H. & Hill, S.J. (1993) *Biochem. and Biophys. Res. Commun.* 195, 1294-1300.
Megson A. C., Dickenson, J.M., Townsend-Nicholson, A., *et al.*, *Br. J. Pharmacol.*, (1995); 115, 1415-1424.
Smit, M.J., Timmermen, H., Alewijnse, A.E. *et al.*, (1995) *Biochem. and Biophys. Res. Commun.* 214, 1138-1145.

## 19P CHRONIC L-DOPA ADMINISTRATION INCREASES SECRETOGRANIN II mRNA EXPRESSION IN THE DOPAMINE DEPLETED STRIATUM AND NUCLEUS ACCUMBENS OF 6-OHDA-LESIONED RATS

B-Y. Zeng, B. Dass, A. Owen, S. Rose, C. Cannizzaro, B.C. Tel, P. Jenner, Neurodegenerative Disease Research Centre, Division of Pharmacology & Therapeutics, Guy's, King's and St Thomas' School of Biomedical Sciences, King's College, Manresa Road, London SW3 6LX, UK

Secretogranin II is a member of the protein family of chromogranins involved in modulating endoproteolytic processing, granule formation and calcium binding (Fischer-Colbrie et al., 1995). In brain, proteolytic processing of secretogranin II leads to the formation of the novel neuropeptide, secretonurin (Kirchmair et al., 1993) but the function of secretogranin II in specific brain areas such as basal ganglia is unknown. We now report on the effect of 6-hydroxydopamine (6-OHDA) lesioning of the rat nigro-striatal pathway and subsequent L-DOPA administration on the gene expression of secretogranin II.

Unilateral or sham lesions of the nigro-striatal pathway in male Wistar rats anaesthetised with sodium pentobarbital (50 mg/kg, ip) were produced by stereotaxic injection of 8 µg of 6-OHDA or 0.9% saline into the left medial forebrain bundle (AP-2.2, L+1.5, V-8.0; Paxinos & Watson, 1986). L-DOPA (50 mg/kg/day, ip) plus carbidopa (25 mg/kg/day, ip) was administered to sham- and 6-OHDA-lesioned rats for 3 weeks beginning 4 weeks after the lesion. Subsequently, cryostat sections (20 µm) through the nucleus accumbens (AP 1.7 mm from bregma, the striatum (AP 0.2 mm from bregma) and substantia nigra (AP -5.3 from bregma) were incubated with $^{35}$S-labelled oligodeoxyribonucleotide probes hybridizing with secretogranin II mRNA. Quantitative evaluation of autoradiograms of the hybridization signal was undertaken by computerized densitometry.

Sham lesions had no effect on secretogranin II mRNA labelling. 6-OHDA lesioning significantly decreased secretogranin II mRNA labelling in the lesioned side of nigra but it did not alter secretogranin II mRNA labelling in the striatum or nucleus accumbens (Table 1). L-DOPA administration reversed the decreased secretogranin II mRNA labelling on the lesioned side of substantia nigra and markedly elevated it in lesioned striatum and nucleus accumbens.

The present study shows that although 6-OHDA lesioning does not alter striatal secretogranin II mRNA expression, subsequent L-DOPA administration increases the striatal levels of secretogranin II mRNA. The alterations in striatal neuronal activity following L-DOPA administration may contribute to either the beneficial effect of L-DOPA in treating Parkinson's disease or to the motor complications such as dyskinesia.

Fischer-Colbrie R, Laslop A, Kirchmair R (1995) *Prog in Neurobiol* 46:49-70.
Kirchmair R, Hogue-Angeletti R, Gutierrez J, Fischer-Colbrie R, Winkler H (1993) *Neurosci* 53:359-365.
Paxinos G. & Watson C. (1986) *The rat brain in stereotaxic coordinates*, 2nd ed, Academic Press, London.

Table 1: Secretogranin II mRNA expression (nCi/mg) in the lesioned side of sham- and 6-OHDA-lesioned rats

| | Secretogranin mRNA | | |
|---|---|---|---|
| | Substantia nigra | Striatum | Nucleus accumbens |
| Sham (n=7) | 11.1±0.6 | 2.1±0.1 | 3.7±0.3 |
| Sham+L-DOPA (n=6) | 12.4±0.8 | 2.4±0.2 | 4.2±0.2 |
| 6-OHDA (n=7) | 8.9±0.3* | 2.5±0.2 | 3.6±0.4 |
| 6-OHDA+L-DOPA (n=6) | 11.2±0.6 | 7.7±0.4* | 7.2±0.7* |

Statistics by two-way ANOVA followed by post hoc Newman-Keuls test, *P<0.01 vs Sham-lesioned groups

---

## 20P EFFECT OF BRAIN CATECHOL-*O*-METHYLTRANSFERASE INHIBITION BY TOLCAPONE ON AMPHETAMINE-INDUCED BEHAVIOUR IN THE RAT

A. Parada, N. Borges, M.A.Vieira-Coelho & P. Soares-da-Silva. Dept. Research & Development, BIAL, S. Mamede do Coronado, 4785, Portugal.

The present study was aimed to determine the effect of tolcapone and entacapone, two catechol-*O*-methyltransferase (COMT) inhibitors, on amphetamine-induced behaviour. Behavioural testing was performed in male Wistar rats (8 per group) given orally the vehicle, tolcapone (30 mg kg$^{-1}$) or entacapone (30 mg kg$^{-1}$) 1 or 6 hours prior to behavioural evaluation. The inhibitory effects of both compounds upon brain and liver COMT were also examined, as previously described (Borges et al. 1997). Behavioural testing started 50 min after the s.c. injection of vehicle or amphetamine. Spontaneous locomotion was measured using a San Diego Instruments rodent activity monitor (model Flex Field) with 48 infrared motion sensors. Ten-minute activity recording began immediately after placing the test subject at the centre of the chamber. Stereotypical behaviour (intense sniffing, repetitive head and limb movements and licking and biting) were quantified after being recorded on tape by means of video tracking system (VP200, HVS Image, Ltd). Results are means ± s.e. mean, n=4-8. Statistical analysis were performed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test. Animals were habituated to the test field environment for one hour prior to drug administration. Low doses of amphetamine (0.5 and 2.0 mg kg$^{-1}$, s.c.) were found to produce dose-dependent increases in horizontal activity (from 668±69 to 1862±225 and 2839±332 counts 10 min$^{-1}$, respectively), with no evidence of stereotyped behaviour. A high dose of amphetamine (4.0 mg kg$^{-1}$, s.c.) was found to produce no further increase in locomotion (1317±125 counts 10 min$^{-1}$), but resulted in the appearance of stereotypies. Tolcapone and entacapone (30 mg kg$^{-1}$, p.o.) administered 1 h before amphetamine challenge did not alter amphetamine-induced locomotion; tolcapone increased ($P<0.05$) the duration of stereotypies (from 249±83 to 484±64 s). Tolcapone (30 mg kg$^{-1}$, p.o.) administered 6 h before amphetamine challenge was found to significantly ($P<0.05$) increase locomotion in rats treated with 0.5 and 2.0 mg kg$^{-1}$ amphetamine (from 769±78 to 1729±101 and 3308±253 counts 10 min$^{-1}$, respectively), with no evidence of stereotyped behaviour. By contrast, in rats given 4.0 mg kg$^{-1}$ amphetamine, tolcapone produced a marked ($P<0.05$) decrease in locomotion (from 2216±250 to 1410±120 counts 10 min$^{-1}$) and increased ($P<0.05$) the duration of stereotyped behaviour (from 266±88 to 558±17 s). Rats treated with entacapone (30 mg kg$^{-1}$, p.o.) 1 h or 6 h before amphetamine challenge presented the same pattern of locomotion and stereotyped behaviour as their corresponding controls. The table below shows percent inhibition of COMT activity by entacapone (Enta) and tolcapone (Tolc) in homogenates of rat brain and liver, determined at 0.5, 1, 3 and 6 h after their administration (30 mg kg$^{-1}$).

| **Brain** | 0.5 h | 1 h | 3 h | 6 h |
|---|---|---|---|---|
| Tolc | 98.9±0.1 | 98.7±0.2 | 97.0±0.5 | 85.8±8.2 |
| Enta | 71.7±7.0 | 44.8±7.0 | 30.1±6.4 | 19.9±7.1 |
| **Liver** | 0.5 h | 1 h | 3 h | 6 h |
| Tolc | 100.0±0.0 | 99.9±0.1 | 98.0±0.7 | 94.1±0.3 |
| Enta | 98.2±0.3 | 96.2±1.1 | 85.9±2.2 | 73.6±5.4 |

Results are means ± s.e.mean, n=4.

It is concluded that effective COMT inhibition in brain, as produced by tolcapone, results in potentitation of amphetamine-induced behaviour.

Borges, N., et al. (1997). J. Pharmacol. Exp. Ther., 282, 812-817.

## 21P CHARACTERISATION OF SB-271046, A POTENT AND SELECTIVE 5-HT$_6$ RECEPTOR ANTAGONIST

C. Routledge, G.W. Price, *S.M. Bromidge, *S.F. Moss, H. Newman, G. Riley, T. Gager, A.M. Brown, S. Lightowler, and D.N. Middlemiss. Neuroscience Research and *Department of Medicinal Chemistry, SmithKline Beecham, New Frontiers Science Park, Harlow, Essex, CM19 5AW.

Elucidation of the central function of 5-HT$_6$ receptors has been hampered by the lack of selective ligands. However, recent studies using antisense oligonucleotides directed at 5-HT$_6$ receptor mRNA and the selective 5-HT$_6$ receptor antagonist Ro 04-6790 (Sleight *et al*, 1998) suggest that 5-HT$_6$ receptors may be involved in modulating cholinergic neuronal function (Bourson *et al.*, 1995; Sleight *et al.*, 1998). The present study was undertaken to characterise the profile of the novel, potent and selective 5-HT$_6$ receptor antagonist, 5-Chloro-3-methylbenzo[b]thiophene-2-sulfonic acid (4-methoxy-3-piperazin-1-ylphenyl)amide hydrochloride (SB-271046) (Bromidge *et al.*, 1999).

The affinity of SB-271046 at human and rat 5-HT$_C$ receptors was determined in HeLa and HEK293 cell lines stably expressed with human and rat (RBI) 5-HT The affinity of SB-271046 at human and rat 5-HT$_6$ receptors was determined in HeLa and HEK293 cell lines stably expressed with human and rat (RBI) 5-HT$_6$ receptors respectively. Binding studies were performed using [$^3$H]-LSD as the radioligand, defining non-specific binding with methiothepin. receptors respectively. Binding studies were performed using [$^3$H]-LSD as the radioligand, defining non-specific binding with methiothepin.

In functional studies, the effects of SB-271046 were determined on 5-HT-induced adenylyl cyclase activity in membranes prepared from the HeLa cell line. Adenylyl cyclase activity was measured as the formation of [$^{33}$P]-cAMP from [$\alpha$-$^{33}$P]-ATP. [$^{33}$P]-cAMP was separated from [$\alpha$-$^{33}$P]-ATP by Dowex-Alumina chromatography using a method adapted from Salomon (1979).

In studies carried out to determine *in vivo* efficacy, the effects of SB-271046 on physostigmine-induced yawning in rats were evaluated. SB-271046 (1, 3, 10 mg/kg p.o.) or vehicle (1% methylcellulose) was administered to Sprague Dawley rats (200-250 g) followed 2 h later by physostigmine (0.3 mg/kg i.p.) or vehicle. Yawning was observed over a 20 min period commencing 20 min following physostigmine administration. Data were analysed using a pairwise statistical analysis and presented as mean ± s.e.mean.

SB-271046 showed high affinity for the human and rat 5-HT$_6$ receptors with pKi values of 8.9 ± 0.1 and 9.3 ± 0.1 respectively. The compound was greater than 200 fold selective over 54 other receptors and ion channels.

5-HT elicited a concentration-dependent increase in adenylyl cyclase activity in the HeLa cell membranes. SB-271046 inhibited the effect of 5-HT in a competitive manner, displaying a concentration-dependent rightward shift in the 5-HT concentration effect curve with no depression of the maximum. Linear regression analysis of the Schild plot data revealed a correlation coefficient of unity, a slope of 1.04 and a pA$_2$ of 8.7.

SB-271046 (1, 3 and 10 mg/kg p.o.) had no effect on yawning *per se*. Physostigmine (0.3 mg/kg i.p.) increased yawning in rats to 0.4 ± 0.1 yawns per 20 min. SB-217046 enhanced the physostigmine effects at all doses with significance being achieved at the 10 mg/kg dose (from 0.4 ± 0.1 to 1.7 ± 0.5, $p<0.05$).

These data demonstrate that SB-271046 is a potent, selective and orally active 5-HT$_6$ receptor antagonist which modulates central cholinergic function.

Bourson A. *et al.* (1995) *JPET.* 274: 173-180.
Bromidge S.M. *et al.* (1999) *J. Med. Chem* 42: 202-205.
Sleight A.J. *et al.* (1998) *Br. J. Pharmacol.* 124:556-562.
Solomon Y. (1979) *Adv. Cyclic. Nucleotide Res.* 10: 35-55.

---

## 22P COGNITIVE ENHANCEMENT EFFECTS OF THE SELECTIVE 5-HT$_6$ ANTAGONIST SB-271046

D.C. Rogers, T.L. Robinson, C.A. Quilter, A.J. Hunter, C. Routledge and J.J. Hagan. Neuroscience Research, SmithKline Beecham, New Frontiers Science Park, Harlow, Essex, CM19 5AW.

Several lines of evidence indicate that 5-ht$_6$ receptors are involved in the modulation of cholinergic neuronal function. Antisense oligonucleotides studies have suggested that 5-ht$_6$ receptors mediate an inhibitory tone on cholinergic neurones (Bourson *et al.* 1995) and more recently, a 5ht$_6$ receptor antagonist has been shown to reverse scopolamine-induced rotation in 6-OHDA lesioned rats (Bourson *et al.* 1998). The aim of this study was to determine the effects of SB-271046, a selective 5-ht$_6$ antagonist (Bromidge *et al.* 1999), on cognitive function in two rat models of learning and memory.

Forty-eight male Hooded-Lister rats (225-260g) received either 1% aqueous methylcellulose vehicle, 3 mg/kg or 10 mg/kg SB-271046 p.o., 2 h prior to test in a dose-volume of 2 ml/kg (n=16). Each animal received water maze training over five days to acquire the position of a hidden platform. At the end of training, each rat received a probe trial (transfer test) to assess the degree of learning. Retention of the position of the platform was assessed by transfer tests carried out both 4 and 7 days after completion of training. In a separate study, 10 aged (26-30 month) Wistar rats which had been trained previously in an operant delayed-alternation procedure, received either 0, 1, 3 or 10 mg/kg SB-271046, as described above, on each of 4 test days in a balanced cross-over design. Each rat received 36 paired alternation trials at either 5, 10 or 20s delay interval in a randomised design.

There was no significant effect of treatment on acquisition of the water maze as quantified by latency or pathlength to find the platform and by percentage time spent in the platform quadrant during the probe trial at the end of 5 days training. Repeated measures ANOVA indicated a significant effect of treatment on the percentage time spent in the platform quadrant during the transfer tests [$F2,45=3.69$, $P=0.033$]. Multicomparisons revealed that there was a significant difference between vehicle (30.3±2.3%) and 10 mg/kg (40.5±2.8%) treatment groups at Day 7 ($P<0.01$). There was a significant effect of treatment on performance of the delayed alternation task at the 5 sec delay interval [$F3,36=2.93$, $P=0.047$] and multicomparisons revealed a significant difference between vehicle (80.0±4.0%) and 1 mg/kg (93.3±2.1%) treatment groups ($P=0.015$).

This study has demonstrated evidence of cognitive enhancement properties of 5-ht$_6$ receptor antagonism in two different models of learning and memory in the rat. SB-271046 improved retention of a previously learned platform position in the water maze test of spatial learning and memory. This compound also produced significant improvement in the performance of an operant delayed alternation task by aged rats. These data suggest that 5-ht$_6$ receptor antagonism is involved in cognitive function and that SB-271046 may be effective in the clinical treatment of cognitive dysfunction.

Bourson, A., Borroni, E., Austin, R.H. *et al.* (1995) *J. Pharmacol. Exp. Ther.* 274: 173-180.
Bourson, A., Boess, F.G., Bos, M. *et al.* (1998) *Br. J. Pharmacol.* 125: 1562-1566.
Bromidge S.M., Brown, A.M., Clarke, S.E. *et al.* (1999) *J. Med. Chem.* in press.

## 23P [$^{125}$I]SB-258585 - A SELECTIVE ANTAGONIST RADIOLIGAND FOR 5-HT$_6$ RECEPTORS

W.D. Hirst, J.A.L. Minton, S.M. Bromidge*, C. Routledge, D.N. Middlemiss and G.W. Price.
Department of Neuroscience Research and *Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex, CM19 5AW.

Prior to the recent introduction of [$^3$H]Ro 63-0563 (Boess *et al.* 1998), there have been no selective radioligands for the 5-HT$_6$ receptor. However, non-specific binding with this ligand is high, particularly in native tissues. We now report on an analogue of SB-271046 (Bromidge *et al.*, 1999), [$^{125}$I]SB-258585 (4-Iodo-N-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]benzene-sulfonamide), a novel, selective 5-HT$_6$ receptor antagonist, which labels 5-HT$_6$ receptors with high specific binding.

In the present study we have characterised the binding of [$^{125}$I]SB-258585 to recombinant human 5-HT$_6$ receptors. We also undertook similar experiments using [$^3$H]LSD, a ligand which has previously been used to characterise this receptor subtype (Kohen *et al.*, 1996). Studies were performed using membranes of HeLa cells stably transfected with the human 5-HT$_6$ receptor. Binding of both radioligands was carried out in 20mM HEPES, 3mM $MgCl_2$ and 2mM ascorbate (pH 7.4) for 45 min. at 37°C (except for the kinetic studies). [$^{125}$I]SB-258585 was used at a concentration of 0.1nM in saturation, association, dissociation and competition studies. Non-specific binding was measured in the presence of 10μM methiothepin. The experiments were terminated by rapid filtration through Whatman GF/B filters, pre-treated with 0.3% polyethyleneimine, and washed with 6 ml of ice cold buffer.

All measurements were performed in at least 3 independent experiments.

SB-258585 has high affinity for 5-HT$_6$ receptors and displays over 100-fold selectivity over all other 5-HT receptors. Specific binding of [$^{125}$I]SB-258585, which was >95% of total binding, reached a maximum within 45 minutes of incubation. Non-transfected cells displayed only non-specific binding levels. Specific binding was reversible, with a dissociation rate constant ($k_{-1}$) of 0.06 ± 0.004 min$^{-1}$. In saturation binding studies, both [$^{125}$I]SB-258585 and [$^3$H]LSD labelled a single class of high affinity sites, with a $K_D$ of 0.8 ± 0.05 nM for [$^{125}$I]SB-258585 and a $K_D$ of 2.1 ± 0.56 nM for [$^3$H]LSD, with a $B_{max}$ of 6.1 ± 0.95 and 3.8 ± 0.7 pmol mg protein$^{-1}$ respectively. The pharmacological profile of [$^{125}$I]SB-258585 binding to recombinant human 5-HT$_6$ receptors was similar to that of [$^3$H]LSD. The pKi rank order of SB-271046 >SB-258585 >methiothepin >clozapine >mianserin >ritanserin >5-HT >5-CT >mesulergine, differed by <0.2 pKi between the 2 ligands and correlated with previously published data (Kohen *et al.*, 1996).

In conclusion, [$^{125}$I]SB-258585 is a highly selective 5-HT$_6$ receptor antagonist, which labels these receptors with high affinity and displays high levels of specific binding. In addition, the high specific activity (2000Ci/mmol) may enable binding studies to be performed on tissues with a low density of 5-HT$_6$ receptors and may allow for short-exposure autoradiographic studies.

Boess F.G. *et al.*, (1998) *Mol. Pharmacol.* **54**: 577-583
Bromidge S.M. *et al.*, (1999) *J. Med. Chem.* **42:**1 202:205
Kohen R. *et al.*, (1996) *J.Neurochem.* **66**: 47-56

---

## 24P THE CENTRAL DOPA DECARBOXYLASE INHIBITOR, NSD-1015, DOES NOT PREVENT L-DOPA-INDUCED CIRCLING BEHAVIOUR IN 6OHDA-LESIONED RATS

S.A. Treseder, S. Rose, P. Jenner, Neurodegenerative Diseases Research Centre, Division of Pharmacology and Therapeutics, Guy's, King's and St Thomas' School of Biomedical Sciences, Manresa Rd, London UK.

The central dopa decarboxylase (DDC) inhibitor, NSD-1015 (3-hydroxybenzyl hydrazine), is widely used to study the role of L-DOPA itself in brain. However, its effect on L-DOPA-induced locomotor activity in rats is unclear as both increases and decreases have been reported (Melamed *et al*, 1984, Nakazato & Akiyama, 1988). For this reason, we have investigated the effects of NSD-1015 on L-DOPA-induced contralateral rotations and efflux of L-DOPA and dopamine (DA) in 6OHDA lesioned rats.

Initially, striatal DDC activity was assessed 40min after NSD-1015 (50-200mg/kg ip; n=5-6) or vehicle (n=5) administration to male Wistar rats (180-200g) as previously described (Nagatsu *et al*, 1979). In a second group of rats (200-380g) with 6OHDA or sham lesions of the medial forbrain bundle (MFB) (n=5), L-DOPA methyl ester (L-DOPA, 25mg/kg free base ip) was administered twice daily for 3 consecutive days to carbidopa (CD; 12.5mg/kg ip) pre-treated rats. On day 4, animals were pre-treated with NSD-1015 (100mg/kg ip) + CD 40 min prior to L-DOPA. Circling behaviour was assessed for 6.5h after the first dose of NSD-1015 on days 3 and 4. In a third group of rats (200-380g) with established 6OHDA lesions to the left and sham lesions to the right MFB, microdialysis probes were bilaterally implanted into the striatum under chloral hydrate anaesthesia. Rats were pre-treated with CD (12.5mg/kg i.p.), and either NSD-1015 (100mg/kg ip; n=8) or vehicle (phosphate buffered saline, n=6) 40min prior to the administration of L-DOPA (25mg/kg ip). Samples (20min) were collected for 5h and levels of L-DOPA, DA, and DOPAC were measured by HPLC. Data, expressed as mean ± s.e.m., were analysed by ANOVA followed by Neuman-Keuls post hoc test.

NSD-1015 (50, 100, and 150mg/kg) reduced striatal DDC activity by 83.5, 90, and 97.8% respectively (control levels: 4.5±0.3nmole DA formed /mg tissue/20min). NSD-1015 (100mg/kg) was used for all further studies. L-DOPA-induced circling behaviour was not observed in sham-lesioned rats (p>0.05, ANOVA). NSD-1015 did not alter the total number of L-DOPA-induced contralateral turns (vehicle + CD 1405±155; NSD-1015 + CD, 1865±390, p>0.05), however, it delayed the onset of circling by 45min (onset: vehicle + CD, 45min, p<0.05; NSD-1015 + CD, 90min, p<0.05) and prolonged the duration of circling (duration: vehicle + CD, 160min, p<0.05; NSD-1015 + CD, 275min, p<0.05). The maximum rate of L-DOPA-induced circling was not affected by NSD-1015 (maximum turns/min: vehicle + CD, 10.2±1.1; NSD-1015 + CD, 10.4±2.3, p>0.05).

| | Time (min) | vehicle | | NSD-1015 | |
|---|---|---|---|---|---|
| | | 6OHDA | sham | 6OHDA | sham |
| L-DOPA | 0 | 18 ± 2 | 16 ± 4 | 16 ± 4 | 21 ± 3 |
| | 240 | 143 ± 18 | 98 ± 23 | 738 ± 194* | 2111 ± 231* |
| DA | 0 | 0 ± 0+ | 31 ± 3 | 0 ± 0+ | 34 ± 4 |
| | 240 | 21 ± 5 | 30 ± 4 | 20 ± 6 | 31 ± 6 |
| DOPAC | 0 | 21 ± 8 | 5202 ± 489 | 35 ± 25+ | 8624 ± 1047* |
| | 240 | 1551 ± 169+ | 8100 ± 540 | 50 ± 20+* | 1985 ± 217* |

Table: Dialysate levels of L-DOPA, DA and DOPAC at 0 and 240min after NSD-1015 + CD or vehicle + CD. L-DOPA was administered at 40min. *p<0.05 vs. vehicle, + p<0.05 vs. sham.

The 6OHDA lesion reduced dialysate levels of DA and DOPAC but had no effect on L-DOPA (Table). Following L-DOPA administration, NSD-1015 increased dialysate L-DOPA levels to a greater extent in sham than 6OHDA lesioned striata, had no effect on the L-DOPA-induced increase in DA levels in the lesioned striata, but prevented the L-DOPA-induced increase in DOPAC levels. Indeed, DOPAC levels were reduced by NSD-1015 in the sham-lesioned striatum.

These data show that NSD-1015 increased extracellular fluid levels of L-DOPA, but did not produce a concomitant decrease in DA, although DOPAC levels were dramatically reduced. This suggests that NSD-1015, as well as inhibiting DDC activity, may also affect DA release and/or metabolism by monoamine oxidase, thus explaining the alteration in time course of L-DOPA–induced circling. This indicates that NSD-1015 is not a good tool for the study of L-DOPA itself in rat brain *in vivo*.

Melamed, E., Hefti, F., Bitton, V. *et al.* (1984) Neurology 34, 1566-1570.
Nagatsu, T., Yamamoto, T. & Kato, T. (1979) Anal. Biochem. 100, 160-165.
Nakazato, T. & Akiyama, A. (1988) Brain Res. 490,.332-338.

## 25P EVIDENCE THAT $P2Y_1$ AND $P2Y_2$ RECEPTORS REGULATE CALCIUM OSCILLATIONS IN SINGLE HEPATOCYTES

C.J. Dixon, N.M. Woods & A.K. Green (introduced by M.R. Boarder), Dept. of Human Anatomy and Cell Biology, The University of Liverpool, New Medical School, Ashton St., Liverpool L69 3GE U.K.

Single rat hepatocytes micro-injected with the photoprotein aequorin display oscillations in intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) when stimulated with $Ca^{2+}$-mobilizing agonists, including ADP and ATP. The duration of the $[Ca^{2+}]_i$ oscillations is dependent on the receptor species being activated; oscillations of very different duration can be induced in the same individual hepatocyte by different agonists. Activation of a given receptor induces oscillations of consistent duration both within an individual hepatocyte and also between cells. ADP and ATP induce very different patterns of oscillations, indicating activation of distinct receptors by these agonists (Dixon et al., 1990). The receptors involved have not been identified. We have studied the effects of UDP and UTP on single rat hepatocytes. In addition the effect of the antagonist, suramin, on the oscillations induced by ADP, ATP and UTP has been investigated.

Methods are as described in Dixon et al. (1995). Hepatocytes were prepared by collagenase digestion of livers from male Wistar rats (200-250g) after cannulation of the hepatic portal vein. Single cells were isolated and micro-injected with aequorin. Individual injected cells were placed under a photomultiplier and continuously superfused with William's Medium E to which agonists or antagonists were added. Photon counts were sampled every 50ms by computer and the fractional rate of consumption of aequorin was plotted as $[Ca^{2+}]_i$ using in vitro calibration data as described in Cobbold & Rink (1987).

The application of extracellular UTP (0.4-8μM) to 19 aequorin-injected hepatocytes resulted in $[Ca^{2+}]_i$ oscillations. In 17 of these19 cells the oscillations evoked were indistinguishable from those induced in the same cell by ATP. Co-application of the cell permeant cAMP analogue, dibutyryl cAMP, resulted in conversion of the UTP-induced oscillations to a sustained rise in $[Ca^{2+}]_i$ in 3/3 cells. This modulatory effect has previously been observed for oscillations induced by ATP but not ADP (Green et al., 1994). The effect of hexokinase-treated UDP was investigated; 100μM UDP had no effect on $[Ca^{2+}]_i$ in 5 cells.

Hepatocytes producing oscillations in response to ADP, ATP and UTP were co-applied with suramin. ADP-induced oscillations were inhibited in 6/8 cells by the application of 50μM suramin. The remaining 2/8 cells displayed a decrease in the frequency of oscillations. In contrast, ATP-induced oscillations were inhibited in only 2/8 cells; oscillations continued in the remaining 6 cells although at a reduced frequency in 5. $[Ca^{2+}]_i$ oscillations induced by ATP and UTP in an individual cell were inhibited by the same concentration of suramin (n=4).

These results are consistent with the effects of UTP and ATP on hepatocytes being mediated through $P2Y_{2/4}$ receptors and of ADP being through $P2Y_1$ receptors. The lack of effect of UDP suggests hepatocytes do not express $P2Y_6$ receptors.

Cobbold, P.H. & Rink, T.J. (1987) *Biochem. J.* 248, 313-328.
Dixon, C.J., Woods, N.M., Cuthbertson, K.S.R. & Cobbold, P.H. (1990) *Biochem. J.* 269, 499-502.
Dixon, C.J., Cobbold, P.H. & Green, A.K. (1995) *Br. J. Pharmacol.* 116, 1979-1984.
Green, A.K., Cobbold, P.H. & Dixon, C.J. (1994) *Biochem. J.* 302, 949-955.

---

## 26P REDUCED EXPRESSION OF GRK2 IN NG108-15 CELLS LEADS TO A SPECIFIC REDUCTION IN ADENOSINE RECEPTOR DESENSITIZATION

J.M.Willets, *J-L.Parent, *J.L.Benovic and E.Kelly, Department of Pharmacology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD and *Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia 19107, U.S.A.

We have previously shown that in NG108-15 mouse neuroblastoma x rat glioma cells stable expression of a dominant negative mutant G-protein receptor kinase 2 (GRK) construct suppresses the desensitization of adenosine $A_2$ receptors (Mundell *et al.*1997). Furthermore, overexpression of rat wild-type GRK2 in NG108-15 cells enhances the desensitization of adenosine $A_{2a}$ receptors (Mundell *et al.*1998). To further explore the role of GRK2 in adenosine $A_2$ receptor desensitization we attempted to reduce GRK2 levels in intact NG108-15 cells through the introduction of a full length antisense cDNA to the rat wild-type GRK2 in pcDNA3. Stably transfected NG108-15 cells were expanded into cell lines of which two were selected for further study (AS5 and AS8) which expressed approximately 60% less GRK2 than plasmid controls, when determined by Western blot analysis.

Antisense GRK2 or plasmid transfected control cells were grown in DMEM containing 6% foetal calf serum supplemented with geneticin (300 μg/ml) in 24 well plates. When confluent media were exchanged for 0.5 mL of fresh media 1 hour prior to experimentation. With the exception of basal, cells were challenged with either 5'-(N-ethylcarboxamido)-adenosine (NECA 10 μM, adenosine agonist), iloprost (1 μM, IP-prostanoid agonist), secretin (100 nM) or forskolin (10 μM) for up to 2 hours at 37°C in the presence of the phosphodiesterase inhibitor Ro201724 (250 μM). At appropriate time points trichloroacetic acid was added to terminate the reaction and cAMP concentrations determined by a protein binding assay (Mundell *et al.*1997). Data are expressed as pmol cAMP / mg protein.

NECA stimulation produced significantly increased cAMP accumulation in antisense cells when compared to plasmid controls at all time points (Table 1, similar data were obtained with both P2 and AS8 cells). In contrast, cAMP accumulation stimulated via either the IP-prostanoid, or secretin receptors, or directly through forskolin, was not significantly different between antisense or plasmid control cells.

Table 1. NECA (10 μM) stimulated cAMP accumulation in plasmid control (P1) and antisense GRK2 cells (AS5).

| Time (min) | P1 (n =4) | AS5 (n =4) |
|---|---|---|
| Basal | 44 ±6.5 | 83.0 ±5.6 |
| 10 | 352 ± 21 | 669 ±43 |
| 20 | 592 ± 26 | 1130 ± 49 |
| 30 | 734 ± 12 | 1512 ± 54 |
| 60 | 942 ± 21 | 1971 ± 98 |
| 120 | 939 ± 37 | 1727 ± 165 |

cAMP accumulation was significantly ($P<0.01$) increased in AS5 cells when compared to P1 cells via two way analysis of variance.

These data show for the first time a direct correlation between expression of GRK2 and desensitization of adenosine $A_2$ receptors in NG108-15 cells, which in turn, suggests that GRK2 plays a leading role in the regulation of adenosine $A_2$ receptors.

Mundell,S.J. *et al.* (1997) Mol. Pharmacol., 51,991-998.
Mundell,S.J. *et al.* (1998) Brit. J. Pharm., 125, 347-356.

## 27P 2-CHLOROADENOSINE INHIBITS NMDA CURRENTS IN RAT CULTURED BIPOLAR RETINA CELLS

A.R. Costenla, A de Mendonça, A.M. Sebastião & J.A. Ribeiro. Laboratory of Neurosciences, Faculty of .Medicine, University of Lisbon, Portugal.

Neurotoxicity associated with excitatory amino acids is known to be largely mediated through the activation of a specific subtype of ionotropic glutamate receptors, the *N*-methyl-D-aspartate (NMDA) receptors (Simon *et al.*, 1984). In the retina, ischemic damage was also found to be dependent upon the activation of NMDA receptors (Yoon *et al.*, 1989). Endogenous adenosine has neuroprotective effects against hypoxic or ischemic damage in brain (von Lubitz *et al.*, 1995). Under hypoxia or ischemia, a pronounced increase in the release of adenosine can be detected in retina-choroid preparations (Roth *et al.*, 1997). It has been previously observed that NMDA receptor-mediated currents in hippocampal neurons are decreased by adenosine $A_1$ receptor activation (de Mendonça *et al.*, 1995). We therefore investigated whether NMDA receptor-mediated responses in rat cultured bipolar retina cells are inhibited by an adenosine analogue.

Cells from one-day-old male Wistar rat retina were isolated and cultured as described (Leinders-Zufall *et al.*, 1994), with modifications. After being deeply anaesthetised with halothane, the rat was killed by decapitation. The eyes were gently enucleated, immersed in a previous cooled and gassed (100% $O_2$) Hank's balanced salt solution (HBSS). For enzymatic incubation (10 min, 37°C), papain (0.25% w/v) and EDTA (0.04%) were used in a $Ca^{2+}$- and $Mg^{2+}$-free HBSS solution. Retinas were then mechanically dispersed by trituration through a glass pipette and cells were plated on poli-L-lysine-coated glass coverslips. The culture medium consisted in (g/L): Eagle's minimum essential medium 9.6; HEPES 2.38; glucose 3.954; $NaHCO_3$ 2.2; 10% (v/v) fetal calf serum and penicillin and streptomycin (1 ml/100 ml medium). Cells were studied from 6 to 17 days in culture, bipolar cells being identified by morphological inspection. NMDA currents were induced by iontophoretic application of NMDA (75 mM) every 60 s and were recorded with the patch-clamp technique in the whole-cell configuration (de Mendonça *et al.*, 1995).

Iontophoretic application of NMDA to bipolar cells clamped at -50 mV induced an inward current of 140±25 pA peak amplitude, which was virtually completed blocked by the NMDA receptor antagonist DL-2-amino-5-phosphonopentanoate (AP5, 200 μM). Perfusion of the stable adenosine analogue, 2-chloroadenosine (CADO, 100 nM), caused a decrease in the amplitude of NMDA receptor-mediated currents, an action which was reversible upon removal of the CADO from the bath. The decrease of the maximal peak amplitude of NMDA current induced by CADO (100 nM) was 62.5 ± 6.1 % (n=5).

These results show that 2-chloroadenosine inhibits NMDA currents in bipolar cells suggesting that adenosine could protect bipolar cells against NMDA-induced toxicity.

de Mendonça, A. *et al.* (1995) *NeuroReport*, **6**, 1097-100.
Leinders-Zufal, T. *et al.* (1994) *J. Neurophysiol.*, **72**, 2503-6.
Roth, S. *et al.* (1997) *Exp. Eye Res.*, **65**, 771-9.
Simon, R.P. *et al.* (1984) *Science*, **226**, 850-2.
von Lubitz, D. *et al.* (1995) *Eur.J. Pharmacol.* **283**, 185-92.
Yoon, Y.H. *et al.* (1989) *Arch. Ophtalmol.*, **107**, 409-11.

---

## 28P INCREASE IN THE NUMBER, G-PROTEIN COUPLING AND EFFICIENCY OF ADENOSINE $A_{2A}$ RECEPTORS IN THE CORTEX OF AGED RATS

R.A. Cunha[1,2], & J.A. Ribeiro[1], [1]Lab.Neurosciences, Fac.Medicine, Univ.Lisbon, [2]Dept.Chemistry & Biochemistry, Fac.Sciences, Univ.Lisbon, Portugal

Adenosine $A_{2A}$ receptors are facilitatory receptors coupled to $G_s$ proteins and activating the adenylate cyclase/cAMP transducing system (Cunha, 1997). The density of $A_{2A}$ receptors is changed in the cerebral cortex of aged rats (2 years old) when compared with young adult rats (6 weeks old) (Cunha *et al.*, 1995). Since a variation in the density of $A_{2A}$ receptors may be due to an increase of spare receptors or of uncoupled receptors (Luthin *et al.*, 1995), we now re-evaluated whether the G protein coupling and efficiency of $A_{2A}$ receptors are also modified in aged rats.

Binding of adenosine $A_{2A}$ ligands to cortical membranes was performed as described (Cunha et al., 1999).

The $B_{max}$ of the $A_{2A}$ agonist [$^3$H]2-[4-(2-*p*-carboxyethyl) phenylamino]-5'-N-ethylcarboxamido adenosine ([$^3$H]CGS21680) was higher in cortical membranes of aged (413±22 fmol/mg protein, n=4) than of young adult rats (193±18 fmol/mg protein, n=4), with no significant (P>0.05) change in $K_D$ (84.2 nM in young *versus* 89.1 nM in aged rats). The $B_{max}$ of the $A_{2A}$ antagonist [$^3$H]4-(2-[7-amino-2-(2-furyl {1,2,4} -triazolo{2,3-a{1,3,5} triazin-5-yl-aminoethyl)phenol ([$^3$H]ZM241385) was also higher in cortical membranes of aged (435±34 fmol/mg protein, n=4) than of young adult rats (248±18 fmol/mg protein, n=4), with no significant (P>0.05) change in $K_D$ (0.29 nM in young *versus* 0.17 nM in aged rats). The difference in the density of binding of the $A_{2A}$ agonist and antagonist lead us to test the G protein coupling of $A_{2A}$ receptors by testing the effect of guanylylimidodiphosphate ($Gpp_{NH}p$, 100 μM) on the displacement by CGS21680 (1nM-1μM) of [$^3$H]ZM241385 (1nM) binding. In cortical membranes, the $K_i$ of CGS21680 was 102 nM (95% CI: 79-125 nM, n=4) in the absence and 216 nM (127-304 nM, n=4) in the presence of $Gpp_{NH}p$ (100μM) in young adult rats and 75 nM (69-80 nM, n=4) in the absence and 316 nM (282-350 nM, n=4) in the presence of $Gpp_{NH}p$ (100μM) in aged rats. In cortical slices, CGS21680 (30-1000 nM) was virtually devoid of effect on cAMP accumulation in young adults but increased cAMP accumulation with an $EC_{50}$ of 152.6 nM (72-233 nM, n=4) in aged rats, an effect prevented by ZM241385 (20 nM, n=4).

These results show that the number, G protein coupling and efficiency of adenosine $A_{2A}$ receptors are increased in the limbic cortex of aged rats.

Cunha, R.A. (1997) in *The Role of Adenosine in the Nervous System*. ed. Okada, Y. pp 135-142. Elsevier.
Cunha, R.A., Constantino, M.D. & Ribeiro, J.A. (1995) *NeuroReport* **6**, 1583-1588
Cunha, R.A., Constantino, M.D. & Ribeiro, J.A. (1999) *Naunyn Schmiedeberg's Arch.Pharmacol.*, in press
Luthin, D.R. *et al.* (1995) *Mol.Pharmacol.* **47**, 307-313

## 29P CROSS-TALK BETWEEN $A_1/A_{2A}$ ADENOSINE RECEPTORS IN THE RAT HIPPOCAMPUS AND CORTEX

L.V. Lopes[1], R.A. Cunha[1,2] & J.A.Ribeiro[1], [1]Lab.Neurosciences, Fac.Medicine and [2]Dept. Chemistry & Biochemistry, Fac.Sciences, Univ.Lisbon, Portugal.

Adenosine $A_1$ and $A_{2A}$ receptors are co-localised and modulate neuronal excitability of CA1 hippocampal area, and the effect of endogenous adenosine depends on a balance between inhibitory and excitatory responses (Cunha *et al*, 1994a). The observation that $A_{2A}$ receptor activation attenuates $A_1$ receptor responses (Cunha *et al*, 1994b) lead us to investigate the mechanism of these interactions.

Through extracellular electrophysiological recordings from the CA1 area of 6 weeks old male Wistar rat hippocampal slices (Cunha *et al.*, 1994b), we studied the effect of the selective $A_{2A}$ adenosine receptor agonist, 2-[4-(2-carboxyethyl) phenethylamino]-5'-N-ethyl carboxamido adenosine (CGS21680) on population spike (PS) depression caused by application of the selective $A_1$ adenosine receptor agonist, $N^6$-cyclopentyladenosine (CPA). CPA (10 nM) caused a 66.1± 8.4% (n=5) inhibition of PS amplitude. However, in the presence of CGS21680 (10 nM), CPA (10 nM) only reduced by 40.5 ± 13.1% the PS amplitude. This effect of CGS21680 (10 nM) was observed in four of seven experiments. CGS21680 (10 nM) by itself elicited a moderate facilitatory effect of 10.3 ± 4.6% (n=4) on PS amplitude. To investigate whether this interaction occurred at the pre-synaptic level we performed displacement binding curves of the selective $A_1$ receptor antagonist, [$^3$H]8-cyclopentyl-1,3-dipropylxanthine ([$^3$H]DPCPX, 2 nM) by the selective $A_1$ agonist CPA (0.1 nM to 1 μM) in rat hippocampal synaptosomes (Cunha *et al.*, 1996). We obtained a Ki of 2.3 (95% confidence interval: 1.3-3.3) nM (n=4) in control conditions and a shift to the right of the displacement binding curve with a Ki of 7.8 (7.5-8.2) nM (n=4) in presence of CGS21680 (30 nM). This desensitisation-like effect of CGS21680 (30 nM) on $A_1$ receptors was prevented by the selective $A_{2A}$ adenosine receptor antagonist (4-(2-[7-amino-2-(2 - furyl {1,2,4} - triazolo {2,3-a{1,3,5} triazin - 5 - yl - aminoethyl)phenol) (ZM241385, 20 nM, n=2). We observed a similar effect of CGS21680 (30 nM) on $A_1$ receptors in rat cortical synaptosomes (n=4). Chelerytrine (6 μM, n=3) - a protein kinase C inhibitor - prevented the effect of CGS21680 (30 nM) on $A_1$ receptor binding. In contrast, HA-1004 (10 μM, n=2) - a protein kinase A inhibitor - did not modify the effect of CGS21680 (30 nM) on $A_1$ receptor binding.

Therefore, we conclude that activation of $A_{2A}$ adenosine receptors attenuates the response of $A_1$ adenosine receptors in limbic cortex, possibly in a PKC-dependent, PKA-independent manner.

*(Supported by FCT Project 2/2.1/BIA/137/94)*

Cunha, R.A. *et al.*,(1994a) *J. Neurochem.*, **63**, 207-214
Cunha, R.A. *et al.*,(1994b) *Brain Research*, **649**, 208-216
Cunha, R.A. *et al.* (1996) *Na...yn Schmiedeberg's Arch.Pharmacol.* **353**, 261-271.

---

## 30P ADENOSINE MODULATES THE NEUROMODULATORY ACTION OF VIP ON [$^3$H]-GABA RELEASE FROM RAT HIPPOCAMPAL SYNAPTOSOMES

D. Cunha Reis, A. M. Sebastião and J. A. Ribeiro, Laboratory of Neurosciences, Faculty of Medicine, University of Lisbon Av. Prof. Egas Moniz, 1600 Lisboa

Vasoactive intestinal peptide (VIP) is a 28 a.a. peptide which, in the rat hippocampus, has been essentially associated to the GABAergic interneurones (Acsády *et al*, 1996). In the rat hippocampus, VIP also increases cAMP formation and this effect is dependent on endogenous adenosine (Wright and Schoepp, 1996). In the present work we investigated the action of VIP at GABAergic nerve terminals and whether the effect of this neuropeptide was modulated by tonic $A_{2A}$ and $A_1$ receptor activation by endogenous adenosine.

The presynaptic action of VIP was tested on the $K^+$-evoked [$^3$H]-GABA release from hippocampal synaptosomes of the rat (male, Wistar, 5-7 weeks old), according to a method previously described (Cunha *et al.*, 1997). Synaptosomes were pre-incubated with [$^3$H]-GABA (1.25μCi/ml for 20min) and the release of [$^3$H]-GABA was evoked by stimulating the synaptosomes twice ($S_1$ at 4 and $S_2$ at 22min after starting sample collection) with 28mM $K^+$. The effect of VIP on evoked release of [$^3$H]-GABA was expressed as alterations of the $S_2/S_1$ ratio (i.e., the ratio between the evoked release of [$^3$H]-GABA induced by $S_2$ and by $S_1$) compared to control. The involvement of adenosine in the action of VIP was acessed by testing the effect of VIP in the presence of adenosine deaminase (ADA, 1 U/ml). The influence of $A_{2A}$ or $A_1$ receptor activation on the effect of VIP was evaluated by testing the effect of VIP in the presence of the $A_{2A}$ receptor antagonist, ZM 241385 (20nM) or the $A_1$ receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 10nM), respectively. Significance of the effects of ,VIP was calculated by the Student's *t* test.

VIP (0.3-100 nM ) caused a biphasic effect on the evoked release of [$^3$H]-GABA. At concentrations up to 30 nM VIP induced an increase in [$^3$H]-GABA release, the maximum excitatory effect being observed with 1 nM VIP (55,8±4,5%, n=9); at a higher concentration (100nM) VIP had an inhibitory effect (-17,5±1,8%, P<0,05, n=6) on the evoked release of [$^3$H]-GABA. The basal release of tritium was virtually not modified by VIP (0.3-100 nM). In the presence of ADA (1U/ml) the excitatory effect of VIP (1 nM) on the evoked release of [$^3$H]GABA was abolished (% modification of [$^3$H]-GABA release caused by VIP was -12.8±5.1, P>0.05, n=6). ZM 241385 (20nM) attenuated the excitatory effect of 1nM VIP, which under these conditions increased the evoked [$^3$H]-GABA release by 23,5±4,6% (P<0,05, n=6). DPCPX (10 nM) prevented the excitatory effect of 1nM VIP (% modification of [$^3$H]-GABA release caused by VIP: -13.5±3.9 %, P<0,05, n=6).

It is concluded that at low nanomolar concentrations VIP facilitates the release of GABA, and that this effect is strongly dependent on activation of both $A_1$ and $A_{2A}$ receptors by endogenous adenosine.

Supported by FCT (Project grant Praxis/2/2.1/BIA/137/94)

Acsády, L. *et al* (1996) *Neuroscience*, 73, 299
Cunha, R. *et al.* (1997) *Eur. J. Pharmacol.*, 323, 167
Wright, R. A. and Schoepp, D. D. (1996) *Eur. J. Pharmacol.*, 297, 275-282

## 31P AGE-RELATED CHANGES IN THE HISTAMINE $H_3$-RECEPTOR OF THE RAT BRAIN

A. Alves-Rodrigues[1,3], R .Leurs[1], P. Panula[2], and H.Timmerman[1]; (J.A. Ribeiro[3]) 1-Dept. Pharmacochemistry, Vrije Universiteit Amsterdam, The Netherlands; 2-Dept. Biology, University of Turku, Finland; 3-Present address: Lab. Neurosciences, Fac. Medicine, Univ. Lisbon, Portugal.

The histamine H3 receptor is a presynaptic receptor that regulates the synthesis and release of histamine as well as several other neurotransmitters including noradrenaline, serotonin, and dopamine (Leurs *et al.*, 1998). Recent developments in the field of the H3 receptor indicate an important role of the histaminergic neuronal system in learning and memory (Leurs *et al.*, 1998). Since ageing is commonly associated with cognitive impairments, in this study we assessed changes in the histamine H3-receptor in the rat cerebral cortex, striatum, and hippocampus of young (2-4 months) *vs* old (27-29 months) rats.

Following the methods described by Alves-Rodrigues *et al.* (1995) we performed saturation binding assays of the radiolabelled H3-receptor antagonist [125I]-iodophenpropit to membranes prepared from male FBNF1 rat brains. As a functional assay the H3-receptor mediated inhibition of the electrically evoked release of [3H]-noradrenaline was measured from brain slices of the same rats. Data are expressed as mean±SEM, and Student *t*-tests applied to detect statistical significance.

A significant ($P<0.01$) increase in the density of H3-receptors was observed in the hippocampus of the aged (Bmax= 241±16 fmol/mg protein, n=4) *vs* the young (Bmax=123±18 fmol/mg protein, n=4) rats. In contrast, a decrease occurred in the cerebral cortex of the old rats (Bmax=109±64 fmol/mg protein, n=4) in relation to the young controls (Bmax=173±18 fmol/mg protein, n=4), whereas virtually no age-related changes in the H3-density were detected in the striatum. In the three brain regions examined, no significant differences were obtained for the Kd values of the radiolabelled H3-antagonist [125I]-iodophenpropit with age. The H3-receptor mediated inhibition of the electrically induced release of [3H]-noradrenaline was then measured in the cerebral cortex and hippocampus of the rat brain. Age-associated changes were neither observed in the basal release nor in the total content of [3H]-noradrenaline released from the slices. However, the level of maximum inhibition, achieved with 1μM of the selective H3 receptor agonist (R)-α-methylhistamine, decreased with age both in the cerebral cortex (49±0.7% *vs* 37±2.6%; n=6, $P<0.01$) and in the hippocampus (47±1.1% *vs* 39±2.0%, n=7, $P<0.01$). Furthermore, the pD2 value for (R)-α-methylhistamine was significantly higher in the hippocampus of young rats (7.7±0.02, n=7) when compared to their old counterparts (7.2±0.2, n=6). This study reveals an age-related increase in the density of H3 receptors in the hippocampus along with a loss in H3 receptor function both in the rat cerebral cortex and hippocampus. (Supported by PRAXIS XXI)

Alves Rodrigues A., *et al.* (1995) *Br. J. Pharmacol.*, 114, 1523-1524.

Leurs R., *et al.* (1998). *Trends Pharmacol. Sci.*, 19, 177-83.

---

## 32P CONTRIBUTION OF MUSCARINIC AND $\alpha_2$-ADRENERGIC RECEPTORS TO THE DEPRESSION OF SYNAPTIC TRANSMISSION INDUCED BY HYPOXIA

J. E. Coelho, A. de Mendonça & J. A. Ribeiro
Laboratory of Neurosciences, Faculty of Medicine, University of Lisbon, Portugal.

Ischemia and hypoxia are known to markedly increase extracellular adenosine levels (Schubert *et al.*, 1994). The depression of synaptic transmission induced in such conditions is largely due to activation of inhibitory adenosine $A_1$ receptors (Fowler, 1989). Under normoxic conditions, activation of pre-synaptic muscarinic or $\alpha_2$-adrenergic receptors inhibits synaptic transmisssion. Since increased extracellular levels of acetylcholine (Ueno *et al.*, 1991) and noradrenaline (Globus *et al.*, 1989) occur during ischemia/hypoxia, we studied whether acetylcholine muscarinic receptors and $\alpha_2$-adrenergic receptors might also be involved in the hypoxia-induced depression of synaptic transmission.

Extracellular electrophysiological recordings were performed in the CA1 area of hippocampal slices taken from young (5-6 weeks old) male Wistar rats. Hypoxia was induced during 14 min by changing from a superfusion medium gassed with a $95\%O_2/5\%CO_2$ mixture to a medium saturated with a $95\%N_2/5\%CO_2$ mixture. Bath temperature was maintained at 32°C (de Mendonça & Ribeiro, 1997).

The muscarinic receptor antagonist, atropine (10 μM) did not by itself modify the depression of synaptic transmission induced by hypoxia (100.0±0.0% in the presence of atropine (10 μM), and 99.7±0.3% in control conditions; *n*=4). The selective adenosine $A_1$ receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine, DPCPX (50 nM), attenuated the depression of synaptic transmisssion to a value of 75.6±11.0% (*n*=4). Atropine (10 μM), the muscarinic receptor antagonist, in the presence of DPCPX (50 nM), further reduced the hypoxia-induced depression of synaptic transmission to 63.3±11.1% (*n*=4, $P<0.05$)

The selective $\alpha_2$-adrenergic receptor antagonist, rauwolscine (10 μM), did not by itself modify the depression of synaptic transmission (100.0±0.0% decrease both in the presence and in the absence of rauwolscine; *n*=4). Rauwolscine (10 μM) also did not modify the hypoxia-induced depression of synaptic transmission in the presence of DPCPX (50 nM): 79.5±13.5% decrease in the presence of DPCPX (50 nM, *n*=4) alone, and 86.4±5.3% depression in the presence of DPCPX (50 nM) plus rauwolscine (10 μM; *n*=4).

These results indicate a possible role for muscarinic receptors activation in the depression of synaptic transmission induced by hypoxia, namely in situations where adenosine $A_1$ receptors are blocked. *(Supported by FCT)*

de Mendonça A. & Ribeiro J. A.(1997) *NeuroReport*, 8, 3667-3671.

Fowler J.(1989) *Brain Res.*, 490, 378-384.

Globus M. Y., Busto R., Dietrich W. D. *et al.* (1989) *J. Cereb. Blood Flow Metab.*, 9, 892-896.

Schubert P., Rudolphi K. A., Fredholm B. B. *et al.* (1994) *Int. J. Biochem.*, 26, 1227-1236.

Ueno M., Itakura T., Yokote H. *et al.* (1991) *No To Shinkei*, 43, 467-472.

## 33P EFFECTS OF sCR1 ON TROPONIN T RELEASE AND ON THE INFARCT SIZE FOLLOWING REGIONAL MYOCARDIAL ISCHAEMIA AND REPERFUSION IN THE ANAESTHETISED RAT

Kai Zacharowski, Gerd Hafner[1], Henry C Marsh[2] & Christoph Thiemermann. The William Harvey Res. Inst., St. Bartholomew's and the Royal London School of Medicine, Charterhouse Square, London, UK; [1]Clinical Chemistry, University of Mainz, Germany; [2]AVANT Immunotherap., Needham, USA.

There is evidence that non-immunological activation of the complement system in response to myocardial ischaemia may initiate a number of pathophysiologic responses that contribute to the progression of myocardial ischaemia and reperfusion injury (Schafer *et al.*, 1986). Auto-immunological activation of the complement system causes tissue injury, e.g. glomerulonephritis (Couser *et al.*, 1985). Complement-mediated tissue injury involves two pathobiological mechanisms: (i) a direct effect, mediated by C5b-C9, and (ii) an indirect mechanism of injury mediated by the fragments of activated C3 and C5 (Frank, 1987). This study sought to examine whether inhibition of the complement system with soluble complement receptor type 1 (sCR1) reduces injury after myocardial ischaemia and reperfusion in the rat *in vivo*.

Thirty-two male Wistar rats (210-310 g) were anaesthetised (thiopentone sodium, 120 mg·kg$^{-1}$ i.p.), tracheotomised and ventilated (tidal volume: 10 ml·kg$^{-1}$, 70 strokes·min$^{-1}$, inspiratory oxygen-conc.: 30%, positive end-exspiratory pressure: 1-2 mmHg) as previously described (Zacharowki *et al.*, 1999). The jugular vein and carotid artery were cannulated to administer drugs and measure mean arterial blood pressure (MAP) respectively. After a left-sided thoracotomy, a needle was placed around the left anterior descending coronary artery (LAD). 30 min after recovery, the LAD was occluded for 30 min and then reperfused for 2 h, followed by collection of blood samples (1 ml) to obtain plasma. The LAD was then re-occluded, and 1 ml of Evans Blue dye (2% w/v) was injected i.v. to determine the perfused and the non-perfused (area at risk, AR) myocardium. Infarct size (IS) was measured using p-nitro-blue tetrazolium (0.5 mg·ml$^{-1}$). The plasma levels of cardiac Troponin T (TnT) were determined by the short-turn-around-time assay (Boehringer Mannheim) using an Elecixs® System 2010. The plasma levels of sCR1 were determined by an immunoassay (Nickells *et al.*, 1998).

The following groups were studied: LAD-occlusion and 5 min prior to reperfusion injection of (1) vehicle (3.4 ml·kg$^{-1}$ of PBS, n=10); (2) sCR1 (1 mg·kg$^{-1}$, n=5); (3) sCR1 (5 mg·kg$^{-1}$, n=6); (4) sCR1 (15 mg·kg$^{-1}$, n=5) or no LAD-occlusion (sham operation) plus injection of (5) vehicle (3.4 ml·kg$^{-1}$ of PBS, n=3) or (6) sCR1 (15 mg·kg$^{-1}$, n=3).

The AR (mean) were similar in all groups studied (data not shown). When compared to vehicle, injection of sCR1 (5 or 15 mg·kg$^{-1}$ i.v.) caused a significant reduction in IS of approximately 50 % (Table 1). The high dose of sCR1 also caused a significant inhibition of the release of TnT (Table 1). Administration of sCR1 (1, 5 or 15 mg·kg$^{-1}$) causes dose dependent plasma concentrations of sCR1 *in vivo* (Table 1). None of the drugs used had any haemodynamic effects (data not shown). ND=not determined.

**Table 1:** Effects of sCR1 on TnT release and on myocardial injury. Data are expressed as mean±s.e.mean. Statistic: ANOVA and Bonferoni's test. * $P<0.05$, when compared to control.

| group | IS (%) | TnT (ng·ml$^{-1}$) | plasma levels of sCR1 (µg·ml$^{-1}$) |
|---|---|---|---|
| 1 | 61±2 | 80±20 | undetectable |
| 2 | 46±8 | ND | 15±1 |
| 3 | 21±10* | ND | 83±6 |
| 4 | 31±7* | 13±7* | 224±17 |
| 5 | < 3* | < 0.05* | undetectable |
| 6 | < 3* | ND | 219±16 |

Thus, sCR1 causes a substantial reduction in the release of cardiac TnT and myocardial infarct size. The mechanism of the cardioprotective effects of this agent may involve the inhibition of complement-activation.

Couser, W.G., Baker, P.J. & Adler, S. (1985) *Kidney Int.*, **28,** 879-890
Frank, M.M. (1987) *N. Engl. J. Med.*, **316,** 1525-30
Nickells, M., Hauhart, R., Krynch, M., *et al.* (1998) *Clin. Exp. Immunol.* **112,** 27-33
Schafer, H., Mathey, D., Hugo, F. *et al.* (1986) *J. Immunol.*, **137,** 1945-1949
Zacharowski, K., Olbrich, A., Otto, M. *et al.* (1999) *Br. J. Pharmacol.*, **126,** 849-858

## 34P PRETREATMENT WITH ENDOTOXIN REDUCES MYOCARDIAL INFARCT SIZE IN THE ANAESTHETISED RAT

Kai Zacharowski & Christoph Thiemermann. The William Harvey Research Inst., St. Bartholomew's and the Royal London School of Medicine and Dentistry, Charterhouse Square, London, UK.

Myocardial protection associated with "classic" ischaemic preconditioning (IPC) is known to last approximately 1-2 h in many species (e.g. Murry *et al.*, 1991). It has recently been reported, that a delayed phase of protection appears 24 h after IPC (Marber *et al.*, 1993). This phenomenon has been termed the "second window of protection" (SWOP) (Yellon *et al.*, 1995). Sublethal doses of Gram-negative bacterial endotoxins (lipopolysaccarides, LPS) can increase myocardial tolerance to a subsequent challenge with ischaemia and reperfusion (Brown *et al.*, 1989). This study was designed to investigate the time course of LPS-induced cardioprotection by extending the interval between administration of LPS and coronary artery ligation beyond 2 h.

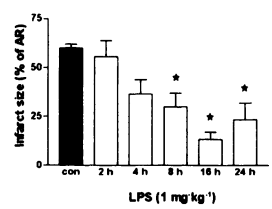
Thirty-nine male Wistar rats (240-300 g) were pretreated with i.p. boluses of either LPS 1 mg·kg$^{-1}$(*E. coli* serotype .127:B8, Sigma) or saline. At 2, 4, 8, 16 or 24 h after LPS, rats were anaesthetised (thiopentone sodium, 120 mg·kg$^{-1}$ i.p.), tracheotomised and ventilated (tidal volume: 10 ml·kg$^{-1}$, 70 strokes·min$^{-1}$, inspiratory oxygen-concentration: 30%, positive end-exspiratory pressure: 1-2 mmHg). The carotid artery was cannulated to measure mean arterial blood pressure (MAP) and the jugular vein was cannulated for the administration of drugs. The chest was opened by a left-sided thoracotomy, the pericardium incised and a needle was placed around the left anterior descending coronary artery (LAD). The animals were allowed to recover for 30 min and subsequently the LAD was occluded for 25 min and then reperfused for 2 h. At the end of the experiment, the LAD was re-occluded and 1 ml of Evans Blue dye (2% w/v) was injected into the jugular vein to determine the perfused and the non-perfused (area at risk, AR) myocardium. Infarct size (IS) was determined by incubation of the slices of the heart with p-nitro-blue tetrazolium (NBT, 0.5 mg·ml$^{-1}$).

The following groups were studied: LAD-occlusion and reperfusion of **(1)** vehicle (n=8); **(2)** 2 h LPS (n=5); **(3)** 4 h LPS (n=5); **(4)** 8 h LPS (n=8); **(5)** 16 h LPS (n=6) or **(6)** 24 h LPS (n=7).

The mean AR were similar in all groups studied (data not shown). When compared to vehicle, pretreatment of rats with LPS for 8 h caused a significant reduction in IS of approximately 40 % (Figure 1). The reduction in IS afforded by LPS was maximal at 16 h (Figure 1). There were no haemodynamic differences between any of the groups studied (data not shown).

**Figure 1:**

LPS induces a time-dependent SWOP.
Data are expressed as mean ±s.e.mean. Statistic: ANOVA and Bonferoni's test. * $P<0.05$, when compared to control.




Thus, pretreatment of rats with LPS causes a substantial protection against a subsequent period of myocardial ischaemia and reperfusion.

Brown, J.M., Grosso, M.A., Terada, L.S., *et al.* (1989). *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 2516-2520.
Marber, M.S., Walker, J.M., Latchman, D.S., *et al.* (1993). *Circulation*, **88,** 1264-1272.
Murry, C.E., Jennings, R.B., & Reimer, K.A. (1991). *Circulation*, **74,** 1124-1136.
Yellon, D.M. & Baxter, G.F. (1995). *J. Mol. Cell. Cardiol.*, **27,** 1023-1034.

## 35P THE STABLE NITROXIDE RADICAL TEMPOL ATTENUATES THE MULTIPLE ORGAN DYSFUNCTION CAUSED BY HAEMORRHAGIC SHOCK IN THE RAT

Helder Mota-Filipe, Michelle McDonald & Christoph Thiemermann. The William Harvey Research Institute, St. Bartholomew's & the Royal London School of Medicine and Dentistry, Charterhouse Square, London, EC1M 6BQ,UK.

Reactive oxygen species (ROS) generation contribute to the pathophysiology haemorrhagic shock (Niessen *et al.*, 1998). Tempol is a stable piperidine nitroxide (stable free radical) of low molecular weight, which permeates biological membranes and scavenges superoxide anions *in vitro* (Laight *et al.*, 1997) and protects against cellular injury caused by hydrogen peroxide in cultures of human endothelial cells (Mota-Filipe *et al.*, 1999) and cardiomyocytes (McDonald *et al.*, 1998). Furthermore tempol reduces the infarct size caused by regional myocardial ischaemia and reperfusion in the isolated perfused heart of the rat (McDonald *et al.*, 1998) and attenuates the renal and liver injury caused by endotoxin in the rat (Leach *et al.*, 1998). In this study, we investigated the effect of tempol on the circulatory failure and hepatic and renal injury/dysfunction caused by haemorrhagic shock in the rat.

Male Wistar rats (250-320g) were anaesthetised with thiopentone sodium (120 $mg \cdot kg^{-1}$, i.p.). The trachea was cannulated to facilitate respiration, and rectal temperature maintained at 37°C. The left carotid and jugular vein were cannulated for the measurement of mean arterial blood pressure (MAP) and drug administration, respectively. The bladder was cannulated to facilitate urine flow. The left femoral artery was also cannulated for blood withdrawal. Rats were than bled to and maintained at a mean arterial pressure of 40 mmHg for 90 minutes. After this period of time, the rats were resuscitated with the shed blood. At the beginning of resuscitation the animals received tempol (30 $mg \cdot kg^{-1}$, i.v. bolus followed by a continuous infusion of 30 $mg \cdot kg^{-1}h^{-1}$, n=9) or saline (1 $ml \cdot kg^{-1}$, followed by 1.5 $ml \cdot kg^{-1}h^{-1}$, infusion, n=10). Sham-operated animals (no shock) received either tempol (n=9) or saline (n=9). Four hours after the resuscitation, blood was collected and the serum levels of urea, creatinine, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured. Statistical differences between groups were analysed by ANOVA followed by a Dunnett's test ($p<0.05$).

Haemorrhage (90 min) and resuscitation (4 h) resulted in significant rises in the serum levels of urea (an indicator of impaired excretory function of the kidney and/or increased catabolism), creatinine (an indicator of renal failure), AST (a non-specific marker for hepatic injury) and ALT (a specific marker for hepatic parenchyma injury) (Table 1). Haemorrhage and resuscitation cause a progressive decline in MAP that was not affected by tempol (Table 1).

Table 1: Effect of tempol on renal and hepatic injury and on MAP caused by haemorrhage (90 min) and resuscitation (4 h) (HS). Mean ± s.e.m. (*$p<0.05$ vs. sham).

| | Urea ($mmol \cdot l^{-1}$) | creatinine ($\mu mol \cdot l^{-1}$) | AST ($iu \cdot l^{-1}$) | ALT ($iu \cdot l^{-1}$) | MAP (mmHg) |
|---|---|---|---|---|---|
| Sham | 7 ± 0.8 | 42 ± 3 | 234 ± 38 | 138 ± 23 | 114 ± 7 |
| Sham+tempol | 9 ± 0.8 | 56 ± 4 | 258 ± 23 | 142 ± 12 | 108 ± 5 |
| HS | 17 ± 0.8* | 104 ± 9* | 824 ± 225* | 342 ± 68* | 63 ± 5* |
| HS+tempol | 11 ± 1 | 49 ± 5 | 357 ± 51 | 203 ± 22 | 83 ± 7* |

Thus, the treatment of rats with tempol at the onset of the resuscitation attenuates the renal and liver dysfunction and/or injury caused by haemorrhage and resuscitation in the rat.

*H.M.F. is the recipient of a post-doctoral grant from the FCT (Praxis XXI/BPD/16333/98). C.T. is a Senior Fellow of the BHF (FS 96/018).*

Laight, D.W., Andrews, T.J., Haj-Yehia, A.I. *et al.* (1997) *Environ. Toxicol. Pharmacol.* **3**, 65-68.

Leach, M., Frank, S., Olbrich, A. *et al.* (1998) *Br. J. Pharmacol.* **125**, 817-825.

McDonald, M.C., Bowes, J., Thiemermann, C. (1998) *Br. J. Pharmacol. (in press).*

Mota-Filipe, H., Bowes, J., Thiemermann, C.(1999) *Br. J. Pharmacol. (in press).*

Niessen , F., Isselhard, W., Minor, T. (1999) *Adv. Exp. Med. Biol.* **442**, 193-200.

---

## 36P INVOLVEMENT OF MAST CELL DEGENERATION IN LPS-INDUCED PLASMA LEAKAGE IN RAT SKIN

Teresa Iuvone, F. D'Acquisto, R. Carnuccio and [1]A.G. Herman
Department of Experimental Pharmacology, University of Naples "Federico II", via D. Montesano 49,80131 Naples, Italy, [1]Division of Pharmacology, Faculty of Medicine, University of Antwerpen UIA, B2610 Wilrijk, Belgium

Mast cells have been traditionally associated with immediate hypersensitivity resulting from the release of histamine following IgE-mediated activation. Recently the direct involvement of mast cells in the inflammatory and immune process has been demonstrated since mast cells are potential source of cytokines, such as tumour necrosis factor-α (TNFα) (Gordon and Galli, 1991). Both histamine and TNFα release by mast cells is not only a response to IgE-dependent activation, but it may occur in response to a variety of stimuli including LPS (Leal-Berumen et al., 1994). We have previously demonstrated that LPS administration in rat skin causes an increased plasma exudation which reached a peak at 2 hours after LPS administration (Iuvone et al., 1998). The aim of this study was to investigate whether mast cell degranulation and release of both histamine and TNFα occurred in this model of inflammation.

Plasma leakage in the skin was measured in male Wistar rats (250-300 g) anaesthesized with Nembutal (60 mg/kg i.p.). The back of rats was shaved and $^{125}$I-labelled human serum albumin (HSA) (2μCi/kg of body weight in 1% Evans blue solution) was injected i.v. After 2 hours blood samples were taken by intracardiac puncture and animals were killed. The injection sites were punched out, samples were counted in an automatic gamma-counter and edema at each site was expressed as μl of plasma. LPS, ketotifen, chlorpheniramine, anti-TNFα were injected intradermally in duplicate immediately before $^{125}$I-HSA administration.

LPS (10 μg/site), produced an increase of plasma leakage (50.1± 2.3 μl/site, $P<0.01$) as compared to saline (13.0 ± 3.2 μl/site). Histological analysis of rat tissue showed that LPS induced a remarkable mast cell degranulation (59.8±2.1%, $P<0.05$) as compared to saline (13.5 ± 2.2%). Ketotifen ($3.9x10^{-9}$ - $3.9x10^{-7}$ mol/site), an $H_1$ receptor antagonist mainly used as mast cell-membrane stabiliser, caused a dose-related inhibition of LPS-induced plasma leakage by 36±3.5%, 47±4.0% ($P<0.05$), 60±3.3% ($P<0.01$) respectively. In addition, ketotifen ($3.9x10^{-8}$ mol/site) inhibited mast cell degranulation by 59.2±2.7% ($P<0.05$). Chlorpheniramine ($10^{-9}$ -$10^{-7}$ mol/site), an $H_1$ histamine receptor antagonist, only partially inhibited LPS-induced plasma leakage in rat skin by 38±1.1% ($P<0.05$) at the highest dose. Moreover, chlorpheniramine did not protect mast cell from degranulation, as shown by histological analysis. In contrast, a polyclonal antibody against TNFα (1:500 to1:50 v/v dilution) decreased LPS-induced plasma leakage in the skin by 15±2.0%, 24±2.1% ($P<0.05$) and 50±3.0% ($P<0.01$) respectively.

Our results suggest that LPS-induced plasma leakage in rat skin is mediated, at least in part, by mast cell degranulation since ketotifen strongly inhibited plasma leakage. The increased plasma exudation seems to be sustained mainly by TNFα rather than by histamine released from mast cells.

Gordon, J.R. & Galli, S.J. (1991) J.Exp. Med. 174, 103-107.
Iuvone, T., et al., (1998) Br. J. Pharmacol. 123, 1325-1330.
Leal-Berumen et al., (1994) J. Immunol. 152, 5468-5476.

## 37P PARASYMPATHETIC NERVES FACILITATE INSULIN ACTION IN THE POST-PRANDIAL STATE

M. P. Macedo*, S. Takayama[1], D.J. Legare[1], W.W. Lautt[1]; *Institute of Health Sciences, Monte da Caparica, 2825 Portugal and [1]Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, Winnipeg, Canada, R3E OW3

The disposal of glucose in response to insulin has been proposed to depend upon a hepatic parasympathetic nerve (HPN) reflex release of a hormone that sensitises skeletal muscle to insulin (Xie and Lautt, 1996a). Hepatic denervation or atropine results in insulin resistance. Insulin action consists of an HPN independent and a variable HPN-dependent component (Xie and Lautt, 1996b). We test the hypothesis that rats in the postprandial period have a high HPN-dependent component of insulin action and that fasting decreases this component and results in reduced insulin action. Insulin sensitivity was quantitated by a Rapid Insulin Sensitivity Test (RIST) with the RIST Index being the amount of glucose infused (mg/kg) over 30 minutes following the administration of 50 mU/kg insulin using a euglycemic clamp (Xie et al, 1996; Lautt et al, 1998). Two series of experiments were performed. In the first series, rats were fasted during 8 hours, fed for one hour and then divided in three groups, the first group was used immediately (group a, n=6), the second group was used 6 hours later (group b, n=6) and the third group 18 hours later (group c, n=3). This series of experiments consisted in a control RIST and in a RIST performed after atropine administration (3 mg/kg). The control RIST was significantly higher for group a (212 ± 4.5 mg/kg glucose) than for group b (149 ± 7.2 mg/kg glucose) or group c (110.1 ± 15.2 mg/kg glucose). RISTs after atropine were similar in the three groups (97.4 ± 15.8; 74.7 ± 9.5; 73.5 ± 16.1 mg/kg glucose for group a, b and c, respectively). In the second series of experiments, a control RIST was performed after a fasting period of 12 hours with a second RIST performed after the administration of food directly into the stomach (1 g/100 g of body weight in 5 ml of saline). Control RIST (125.0 ± 5.0 mg/kg of glucose) was significantly lower than the second RIST (190.0 ± 5.0 mg/kg glucose; n=5, $p<0.05$). In conclusion, increased insulin sensitivity in the post-prandial state is mediated by an activation of the parasympathetic system . On the other hand, hepatic parasympathetic nerves are inhibited during fasting causing insulin resistance.

Lautt, W.W.; Wang, X.; Sadri, P.; Legare D.; Macedo, M.P. , (1998); Can. J. Physiol. Pharmacol, 76: 1080-1086.
Xie, H. and Lautt, W.W., (1996a) Am J Physiol, 270: E858-863.
Xie, H. and Lautt, W.W., (1996b); Am J Physiol 271: E587-592.
Xie H., Zhu L., Zhang Y.L., Legare D.J. and Lautt W.W., (1996); J Pharmacol Toxicol Methods 35:77-82.

Suported by A.P.D.P., Portugal.

---

## 38P AN IMIDAZOLINE COMPOUND (S 22954) PRODUCES HYPERGLYCAEMIA IN CBA/Ca MICE

S. Slough & P. V. Taberner, Department of Pharmacology, University of Bristol, University Walk, Bristol BS8 1TD.

Imidazoline compounds have been shown to stimulate insulin secretion from pancreatic β-cells (Chan *et al,* 1993), and it has been proposed that this effect is mediated by a novel imidazoline binding site, possibly via an interaction with $K_{ATP}$ channels (Rustenbeck *et al,* 1997). Since previous work has suggested that the imidazoline 4-methyl-2-(4,5-dihydro-1H-imidazol-2-yl) piperazine (S 22954) might enhance $K_{ATP}$ channel activation in rat isolated aortic rings (Chambers *et al,* 1998),. we have studied the effects of this compound on blood glucose levels (BGL) in mice.

All experiments used adult male CBA mice (28-30 g), and were begun between 09:00h and 10:00h. BGLs were measured in fed mice given i.p. S 22954 (0.125-25 mg/kg, i.p.), diazoxide (25-100 mg/kg, i.p.) or saline (10 ml/kg); BGL was measured immediately before treatment, then at 30 min intervals. Blood was obtained by tail venesection. BGLs were determined using a Glucochek II and BM-Test strips. Differences between groups (shown as means ± s.e. mean of n) were analysed by Students unpaired t-test.

S 22954 25 mg/kg in fed mice produced a rapid and sustained increase in BGL compared with saline treated mice; 30 min after administration BGL had reached 17.2 ± 0.4 mM compared with 7.7 ± 0.2 mM in saline treated mice ($p<0.0001$, n=6). This level was sustained for 2 h; glucose levels were still elevated compared to those in saline treated mice 4 h after administration (14.6 ± 0.7mM compared with 9.5 ± 0.5 mM, $p<0.01$, n=6). The minimum effective dose of S 22954 was 0.25 mg/kg; which produced a peak BGL of 10.3 ± 0.6 mM recorded at 30 min after administration ($p<0.001$, n=6). In fasted mice, 25 mg/kg S 22954 still elevated blood glucose significantly compared with saline treated mice (6.6 ± 0.6 mM and 2.7 ± 0.4 mM respectively at 30 min); however, the glucose concentration was not increased as markedly in fasted mice, and the increase was considerably slower in onset. Diazoxide was a less potent hyperglycaemic: at 25 mg/kg there was a less significant increase in BGL; 100 mg/kg diazoxide produced a similar peak BGL (19.7 mM) to 25 mg/kg S 22954, but this peak was not reached until 150 min compared with 30 min for S 22954.

We conclude that S 22954 is a rapid and potent hyperglycaemic agent in CBA/Ca mice. Since it was active in fasted mice, where plasma insulin levels would be low, we suggest that its hyperglycaemic action is not entirely a consequence of inhibition of insulin secretion. This is consistent with the different time course seen with diazoxide, which is known to decrease insulin secretion.

We are grateful to I. R. I. S. for their support, and for supplying S 22954.

Chambers, C.J. *et al.* (1998) *Br. J. Pharmacol.* **125,** 105P.
Chan, S.L.F. *et al.* (1993) *Eur. J. Pharmacol.* **230,** 375-378.
Rustenbeck, I. *et al.* (1997) *Naunyn Schmiedeberg's Arch. Pharmacol.* **356,** 410-417.

## 39P COPPER AUGMENTS THE INHIBITORY EFFECT OF HOMOCYSTEINE ON RELAXATION OF THE RABBIT CORPUS CAVERNOSUM: NEW RISK FACTORS FOR ERECTILE DYSFUNCTION

M. Khan, C.S. Thompson, A.M. Emsley[1], F. Mumtaz, G.D. Angelini[1], D.P. Mikhailidis, R.J. Morgan, J.Y. Jeremy[1]. Department of Chemical Pathology, Royal Free Hospital, London, UK & Bristol Heart Institute[1], Bristol, UK.

Homocysteinaemia is an independent risk factor for atherosclerosis (AS), an effect augmented by copper ($Cu^{2+}$) (Emsley *et al.*, 1998; 1999). In turn, AS predisposes to erectile dysfunction (Jeremy & Mikhailidis, 1998). The angiopathic impact of homocysteine (HCy) and $Cu^{2+}$ is thought to be a result of the generation of superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) and impairment of nitric oxide (NO) formation (Emsley *et al.*, 1999). NO also plays a key role in mediating erection (Jeremy *et al.*, 1997). It is not known, however, whether HCy and $Cu^{2+}$ are risk factors for angiopathic erectile dysfunction (ED). In order to explore this possibility, the interactive effects of HCy and $Cu^{2+}$ on the relaxation of corpus cavernosum (CC) was investigated.

New Zealand White rabbits were killed by cervical dislocation, CC excised and mounted in organ baths for recording of isometric tension. Tissues were pre-contracted with phenyl - ephrine and relaxation responses to carbachol assessed in the presence of combinations of HCy and $Cu^{2+}$ as well as with superoxide dismutase (SOD; removes $O_2^-$) and catalase (CAT; removes $H_2O_2$).

HCy (100 μM) alone inhibited carbachol (10 μM)-stimulated relaxation (NO-dependent) of the CC (figure 1), an effect augmented by $Cu^{2+}$ (20 μM) (figure 1). SOD (100 U / ml) and CAT (100 U / ml) reversed this effect (data not shown). The same combination of Hcy and $Cu^{2+}$ had no effect on sodium nitroprusside-stimulated relaxation (NO release independent: data not shown ).

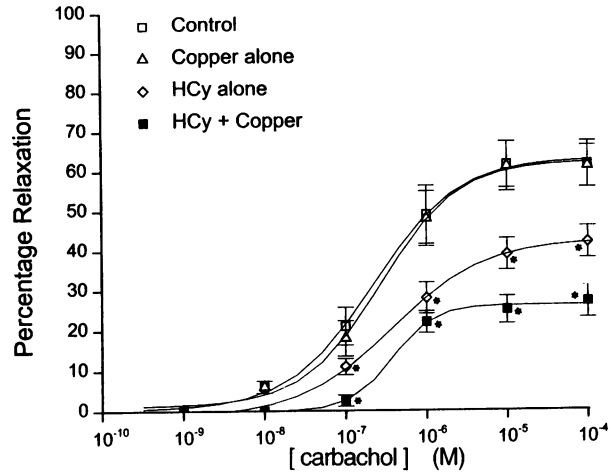



Figure 1. Effect of HCy and $Cu^{2+}$ on maximal mean relaxation (± s.e.m., n = 4) of rabbit CC. * $p < 0.05$, (ANOVA) compared to untreated values.

The reversal of the inhibition of relaxation of the CC by HCy and $Cu^{2+}$ by SOD and catalase suggests that $H_2O_2$ and $O_2^-$ mediate this effect, possibly through the reaction of $O_2^-$ with NO to form peroxynitrite. These data suggest that HCy and its interaction with copper constitute new risk factors for ED.

Emsley, A.M., *et al.*, (1999) *Br. J. Pharmacol.*, **126**: 1034-1040.
Emsley, A.M., *et al.*, (1998)*Vasc. Dis.*, **1**: 3 -10
Jeremy, J.Y. & Mikhailidis, D.P. (1998) *J. Roy. Soc. Health*, **118**: 151-155.
Jeremy, J.Y., *et al.*, (1997) *Br. J. Urol.*, **79**: 958-963

## 40P SEMI-QUANTITATIVE RT-PCR ANALYSIS OF EXCITATORY PROSTANOID RECEPTOR mRNA EXPRESSION IN HUMAN NONPREGNANT MYOMETRIUM

Michelle Senchyna[1], Nicole Stallwood[2] & D. J. Crankshaw[2], [1]School of Optometry, University of Waterloo, Waterloo, Ontario, Canada, [2]Dept of Obstetrics & Gynecology, McMaster University, Hamilton, Ontario, Canada.

Altered expression of prostanoid receptors in human nonpregnant myometrium (HNPM) may underlie pathological disorders such as dysmenorrhoea. To accurately evaluate this hypothesis, we have attempted to develop semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) assays for DP, $EP_1$, $EP_2$, $EP_3$, $EP_4$, FP, IP, TP and alternatively spliced TP (TP-β) receptor mRNAs, and to compare the distribution of these mRNAs in four different human tissues.

Total RNA from HNPM was extracted by a modified method of Chomczynski & Sacchi (1987) and RNA integrity was verified by visualization of ribosomal RNA and $A_{260}/A_{280} \geq 1.6$. 0.5 μg of total RNA was converted to cDNA, which was then amplified by PCR using oligonucleotide primers designed against portions of each of the reported human prostanoid receptor sequences (Senchyna & Crankshaw, 1995). The range of exponential amplification for each sequence was determined from time course analysis. PCR products were analysed by gel electrophoresis. The quantity of each PCR product was determined by calculating the integrated density (ID) of each band from a positive photographic image using Scion Image for Windows (freeware, Scion Corporation, USA). Data were expressed relative to the housekeeping gene human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Total RNA from human kidney (HK), lung (HL), and small intestine (HSI) was purchased from Clontech Laboratories.

Table 1 Distribution of excitatory prostanoid receptor mRNAs among different human tissues.

| Tissue | Prostanoid Receptor mRNA | | | |
|---|---|---|---|---|
| | $EP_1$ | $EP_3$ | FP | TP |
| HNPM | 0.9 ± 0.4 | 5 ± 2 | 1.4 ± 0.4 | 1.4 ± 1.1 |
| HSI | 0.5 ± 0.2 | 1.0 ± 0.3 | 1.4 ± 0.4 | 0.6 ± 0.4 |
| HL | 1.3 ± 0.3 | 1.0 ± 0.1 | 2 ± 1 | 0.9 ± 0.3 |
| HK | 2.0 ± 0.3 | 4 ± 2 | 1.0 ± 0.1 | 0.4 ± 0.2 |

(Values represent receptor amplification product ID/ GAPDH amplification product ID and are means ± s.d.mean, n=3 in all cases)

Each prostanoid receptor mRNA, with the exception of $EP_4$, was expressed in each of the four tissues studied. The presence of $EP_4$ receptor mRNA was inconclusive due to the failure of a negative control. Table 1 shows the results of a semi-quantitative analysis of excitatory ($EP_1$, $EP_3$, FP and TP) prostanoid receptor mRNA expression. There were large inter-experiment fluctuations (up to 7 fold) in PCR product yield. In no tissue was the level of expression of any excitatory prostanoid receptor mRNA significantly different from that in HNPM ($p < 0.05$, Kruskal-Wallis test).

We conclude that the method of semi-quantification of excitatory prostanoid receptor mRNA that we have described here is insufficiently accurate to determine small changes in expression that might contribute to myometrial pathology. Further refinements to reduce inter-experimental variability are required.

Supported by the Medical Research Council of Canada

Chomczynski, P., and Sacchi, N. (1987). *Analyt. Biochem.* (162): 156-159.
Senchyna, M and Crankshaw, D. J. (1995) *Br. J. Pharmacol.* (116): 280P.

## 41P PRELIMINARY RESULTS ON THE URINARY EXCRETION OF 2C-B (4-BROMO-2,5-DIMETHOXYPHENETHYLAMINE) AND ITS METABOLITES IN HUMANS

D. de Boer[¶,§], L.J.A.L.dos Reys[¶], , N. Pylon[§], M. Gijzels[§], I.J. Bosman[§] and R.A.A. Maes[§] [¶] Laboratório de Análises de Dopagem e Bioquímica, Lisboa, Portugal [§] Department of Human Toxicology, Utrecht Institute of Pharmaceutical Sciences, Utrecht, The Netherlands

4-Bromo-2,5-dimethoxyphenethylamine (2C-B) is a phen-*ethyl*amine, which is closely related to mescaline (3,4,5-trimethoxyphen*ethyl*amine). It displays high affinity and selectivity for central 5-$HT_2$ receptors. As 2C-B also binds significantly to 5-HT1a, 5-$HT_{1b}$ and 5-$HT_{1c}$ receptors, it is less selective than the very potent hallucinogenic compounds DOB (4-bromo-2,5-dimethoxy*isopropyl*amine) or DOM (4-methyl-2,5-dimethoxyphen*isopropyl*amine) (Glennon *et al.* 1988). It is a potential drug-of-abuse and since recently it is available on the European drug market (Giroud *et al.* 1998). We analysed some urine samples collected from a 2C-B abuser in order to obtain some preliminary data regarding its excretion in urine.

Urine samples were collected on spontaneous voiding from a male subject abusing 2C-B. The basic and acidic organic fractions were isolated after applying a solid phase extraction at pH 9 and a liquid/liquid extraction at pH 3, respectively. The solvent was evaporated and the respective residues were dried in vacuum over di$P_2O_5$/KOH. The residues of the basic extraction were derivatised in a two step reaction with MSTFA followed by MBTFA resulting in *N*-TFA-*O*-TMS derivatives. Those of the acidic extraction were derivatised with of a mixture of MSTFA/$NH_4I$/ethanethiol resulting in *O*-TMS derivatives. The samples were analysed by Electron Ionisation GC/MS.

2C-B was found only in those urine samples collected during the first 3 hours after administration. Urinary concentrations of 2C-B were in the range of 0.3 to 0.7 µg/ml. The presence of some metabolites could also be observed, but the amounts were too small in order to be able to quantify the concentrations with the analytical methods applied. The metabolite identified unambiguously was 4-bromo-2,5-dimethoxyphenylacetic acid. Furthermore the formation of 4-bromo-2,5-dimethoxybenzoic acid and of 4-bromo-5-hydroxy-2-methoxyphenethylamine was indicated by GC/MS.

These results indicate that the metabolism of 2C-B in humans probably follows similar pathways compared to that of mescaline, i.e. oxidative deamination leading to phenylacetic and benzoic acid-like metabolites and *O*-demethylation of the aromatic methoxy groups (Clarke 1986). As with mescaline the parent compound is excreted in urine as the main compound. The observed urinary concentrations of 2C-B are consistent with the low dose tablets available on the drug market (Giroud *et al.* 1998).

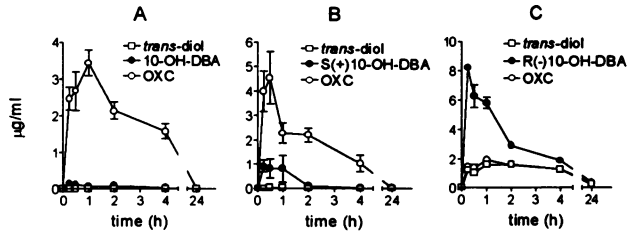
Glennon R.A., Titeler M. and Lyon R.A. (1988) *Pharmacol. Biochem. Behav.* 30, 597-601.
Giroud C., Augsburger M., Rivier L. *et al.* (1998) *J. Anal. Toxicol.* 22, 345-354.
Clarke's Isolation and Identification of Drugs (1986) 2nd Ed. The Pharmaceutical Press, London. pp. 737-738.

---

## 42P ENANTIOSELECTIVE OXIDATION OF 10,11-DIHYDRO-10-HYDROXY-DIBENZ/b,f/AZEPINE-5-CARBOXAMIDE, AN ACTIVE METABOLITE OF OXCARBAZEPINE, IN THE RAT

D. Hainzl, A. Parada & P. Soares-da-Silva. Dept. Research & Development, BIAL, S. Mamede do Coronado, 4785, Portugal.

Oxcarbazepine (OXC) is an analogue of carbamazepine in which a keto group has been added to the 10 position of the azepine ring. Both compounds are comparable in efficacy of treating most forms of epilepsy. In humans and non-human primates, enzymatic reduction of the keto group of the azepine ring of OXC yields a monohydroxy derivative (10,11-dihydro-10-hydroxy-dibenz/b,f/azepine-5-carboxamide; 10-OH-DBA) as an active metabolite, which is then inactivated by glucoronidation or undergoes conversion to 10,11-dihydro-*trans*-10,11-dihydroxy-dibenz/b,f/azepine-5-carboxamide (*trans*-diol derivative). Of all the species examined, only in rat and dog OXC apparently escapes reduction to 10-OH-DBA (Feldmann et al., 1978). In the present study, the *in vivo* metabolism of OXC and 10-OH-DBA in the rat was further examined by considering the racemic nature of 10-OH-DBA and it was found that S(+)10-OH-DBA, but not the R(-) enantiomer, is easily converted back to OXC. Male Wistar rats were treated orally with 20 mg $kg^{-1}$ OXC, S(+)- or R(-)10-OH-DBA in carboxymethylcellulose (0.5% w/v), and blood, livers and brains were taken from pentobarbital anaesthetised animals (60 mg $kg^{-1}$) after 0.25, 0.5, 1, 2, 4 and 24 hours. The blood samples were centrifuged (3000*g*, 15 min) and then subjected to solid phase extraction. HPLC-MSD (Series 1100, Hewlett-Packard) with selected ion monitoring was used for detection and quantification of the parent compound and metabolites. A good chromatographic separation was achieved by using an EcoCART 125-3 column (LiChrospher 100 RP-18, 5µm, Merck) and water/methanol/acetonitrile (62:25:13) as mobile phase (flow 0.8 ml $min^{-1}$). Results are means ± s.e. mean, n=4. As shown in the figure, OXC in rats is not metabolized to a great extent to 10-OH-DBA or the *trans*-diol derivative. In agreement with this, S(+)10-OH-DBA is very rapidly oxidized to OXC (10-OH-DBA/OXC-ratio ~0.25 after only 15 min), indicating that in rats the equilibrium of this redox reaction is extremely shifted to the OXC side. In contrast, R(-) 10-OH-DBA is oxidized only very slowly (10-OH-DBA/OXC-ratio is still ~2 after 2 h) and it is the only compound being metabolised in considerable amounts to the *trans*-diol derivative.




Plasma levels in rats after administration of 20 mg/kg OXC (A), S(+)10-OH-DBA (B) and R(-)10-OH-DBA (C).

Tissue levels of OXC, S(+)10-OH-DBA and R(-)10-OH-DBA, and their metabolites in brain and liver closely followed the pattern observed in plasma. It is concluded that in rats the involved enzymes preferably oxidize S(+)10-OH-DBA to OXC, whereas R(-)10-OH-DBA is only very slowly converted to OXC and the *trans*-diol derivative.

Feldmann, K.F., et al. (1978). Advances in Epileptology. Amsterdam Lisse, 290-294.

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

There is evidence suggesting that uptake of L-DOPA, the precursor of natriuretic hormone dopamine, in renal epithelial cells is dependent on apical-to-basal flux of $Na^+$ and the $Na^+/H^+$ exchanger (Gomes & Soares-da-Silva, 1998). Reducing apical $Na^+$ (from 137 to 0 mM) without changing basal $Na^+$ (137 mM) produced a 37% reduction in apical uptake of L-DOPA. Amiloride (100 µM) reduced the apical-to-basal flux and the basal extrusion of L-DOPA by 41 to 49%. A similar effect was observed when pH at the apical cell border was lowered from 7.4 to 6.9; a further reduction in apical pH to 6.4 markedly reduced the apical-to-basal flux and the basal extrusion of L-DOPA by 40 to 60%. The present study examined the possibility that L-DOPA-$Na^+$ coupled uptake is accompanied by changes in intracellular pH. OK cells (ATCC 1840-CRL) were grown at 37° C in a humidified atmosphere (5% $CO_2$) on 2 $cm^2$ plastic culture clusters (Costar, 3524) in Minimum Essential Medium supplemented with 10% foetal bovine serum and 100 U $ml^{-1}$ penicillin G, 0.25 µg $ml^{-1}$ amphotericin B and 100 µg $ml^{-1}$ streptomycin. After 6 days, the cells formed a monolayer and each 2 $cm^2$ culture well contained about 100 µg of cell protein; 24 h before the experiments the cell culture medium was changed to a serum free medium. OK cells were loaded with BCECF (10 µM) and pH changes were measured by spectrofluorometry for 1400 s. Results are arithmetic means with s.e.mean or geometric means with 95% confidence limits, n=4-5. Statistical differences between experimental groups were determined by ANOVA followed by the Newman-Keuls test. The effect of $Na^+$ removal, amiloride (100 µM) and amphotericin B (2.5 µg $ml^{-1}$) (from t=600 s to t=1400 s) was a significant (P>0.05) reduction in intracellular pH (from 7.38±0.05 to 6.62±0.05, 6.82±0.08 and 6.96±0.02, respectively). The addition of L-DOPA (5, 25 and 100 µM) from the apical cell border also resulted in a concentration dependent decrease in intracellular pH (from 7.38±0.05 to 7.16±0.05, 7.03±0.08 and 6.97±0.07, respectively). It is suggested that L-DOPA uptake at the apical cell side is coupled to $Na^+$. Changes in intracellular pH may have to do with inhibition of $Na^+/H^+$ exchange resulting from increases in L-DOPA-$Na^+$ co-transport.

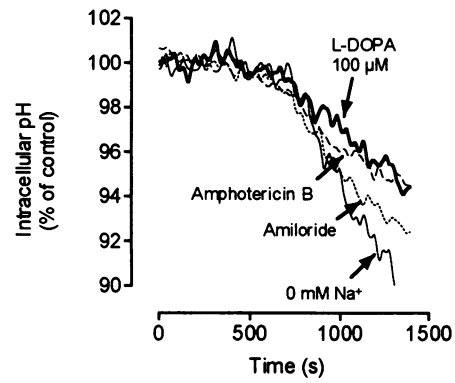



**Figure 1**. Effect of $Na^+$ removal, amiloride, amphotericin B and L-DOPA, on intracellular pH in OK cells.

Gomes, P. & Soares-da-Silva, P. (1998). FASEB J., 12, A1013.

Supported by grant PRAXIS/2/2.1/SAU/1836/95

---

44P EFFECT OF P-GLYCOPROTEIN SUBSTRATES AND INHIBITORS ON THE APICAL OUTWARD TRANSFER OF L-DOPA IN LLC-$PK_1$ AND LLC-GA5 Col300 RENAL CELLS

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

L-DOPA, the precursor of natriuretic hormone dopamine, has been suggested to be extruded at the apical membrane of renal tubular epithelial cells by P-glycoprotein (P-gp) (Soares-da-Silva et al., 1998). The present study examined the effects of several compounds that either inhibit P-gp or are substrates for P-gp, on the apical and basolateral extrusion of L-DOPA in LLC-$PK_1$ and LLC-GA5 Col300 cells, a renal cell line expressing the human P-glycoprotein in the apical membrane (Saeki et al., 1993). LLC-$PK_1$ cells (ATCC CRL 1392; passages 210-215) were grown in Medium 199 supplemented with 100 U $ml^{-1}$ penicillin G, 0.25 µg $ml^{-1}$ amphotericin B, 100 µg $ml^{-1}$ streptomycin, 3% foetal bovine serum and 25 mM HEPES. The culture medium used to grow LLC-GA5 Col300 cells (passages 16-25) was similar to that described above with the exception that it contained 300 ng $ml^{-1}$ colchicine. For transport studies, the cells were seeded onto collagen treated 0.2 µm polycarbonate filter supports (internal diameter 12 mm Transwell, Costar) at a density 13,000 cells per well (2.0 x $10^4$ cells $cm^2$). L-DOPA was applied from either the basolateral or the apical cell side at a non-saturating concentration (2.5 µM) for 6 min. For 24 hours prior to each experiment, the cell medium was free of foetal bovine serum. Results are arithmetic means with s.e.mean, n=4. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons. A P value less than 0.05 was assumed to denote a significant difference. The accumulation of L-DOPA applied from the basolateral cell border in LLC-$PK_1$ cells (378±45 pmol mg $protein^{-1}$) was similar to that observed in LLC-GA5 Col300 cells (366±10 pmol mg $protein^{-1}$). The basal-to-apical flux of L-DOPA in LLC-GA5 Col300 cells (2080±136 pmol mg $protein^{-1}$) was 15-fold that in LLC-$PK_1$ cells (135±25 pmol mg $protein^{-1}$). As shown in the table, 20 min exposure to verapamil, vinblastine, rhodamine 123, quinidine and daunomicine markedly reduced L-DOPA apical extrusion in both cell lines. By contrast, neither rhodamine 123 nor daunomicine affect the uptake and apical-to-basal flux of L-DOPA applied from the apical cell border.

**Table 1**. Apical fractional outflow of L-DOPA.

| | LLC-$PK_1$ | LLC-GA5 Col300 |
|---|---|---|
| Control | 100.0±10.6 | 100.0±10.2 |
| Verapamil (25 µM) | 54.4±2.0 | 67.2±3.9 |
| Vinblastine (10 µM) | 51.0±1.9 | 69.9±14.2 |
| Rhodamine (10 µM) | 19.1±3.2 | 42.6±5.6 |
| Quinidine (50 µM) | 16.9±1.7 | 26.5±2.8 |
| Daunomicine (10 µM) | 34.1±4.4 | 43.8±5.0 |

Values are percent of control (mean±s.e.mean., n=4)

It is concluded, that apical but not basolateral extrusion of L-DOPA, in LLC-GA5 Col300 and LLC-$PK_1$ cells, is promoted through P-glycoprotein and, that native P-glycoprotein in LLC-$PK_1$ cells is more sensitive to inhibition by P-gp inhibitors than cells expressing the human P-glycoprotein.

Saeki, T., et al. (1993). J. Biol. Chem., 268, 6077-6080.
Soares-da-Silva, P., et al. (1998) Br. J. Pharmacol., 123, 13-22.

Supported by grant SAU 123/96.

## 45P COMPARISON OF ANOXIC AND ISCHAEMIC DAMAGE IN A NOVEL ORGANOTYPIC *IN VITRO* SPHEROID MODEL OF STROKE: ASSESSMENT WITH LACTATE DEHYDROGENASE (LDH) AND THE CYTOKINE TNFα

K.J. Presland[1], K.M. Doyle[1], D.J. Heal[2], W.M. Purcell[3], C.K. Atterwill[4].
[1]University of Hertfordshire, Hatfield, Hertfordshire, AL10 9AB;
[2]Knoll Pharmaceuticals, Pennyfoot St. Nottingham, NG1 1GF;
[3]University of the West of England, Coldharbour Lane, Bristol, BS16 1QY;
[4]Roche Discovery, Welwyn Garden City, Hertfordshire, AL7 3AY.

Stroke is the third highest cause of death in Western Countries and the leading cause of disablement. Excitotoxicity, free radicals and cytokines have been implicated in the pathogenesis of ischaemic strokes, though precise mechanisms are still poorly understood. Ischaemia is not synonymous with anoxia as lack of blood supply to tissues results in glucose deprivation as well as oxygen deprivation and a build up of toxic metabolites such as lactic acid. Organotypic *in vitro* models, such as the whole brain spheroid culture which consists of differentiated neurones and glia (Atterwill, 1987) may allow analysis of more discrete cellular events associated with anoxic and ischaemic cell damage.

Cultures of foetal Wistar rat whole brain spheroids were grown in Dulbecco's Modified Eagle's Medium and 10% foetal calf serum at 37°C, 9% $CO_2$ for 12 days. One or two hour periods of anoxia or ischaemia were achieved by suspending the spheroid culture in glucose or glucose-free Tris-Krebs medium respectively. Subsequently, oxygen was removed from cultures by $N_2$ gassing in sealable tissue culture chambers. Following treatments, the spheroids were immediately harvested, homogenised and assayed for protein content, the cytokine tumor necrosis factor alpha (TNFα) and the general cytotoxicity marker lactate dehydrogenase (LDH).

Table 1: Effects of 1 and 2 h anoxia/ischaemia on LDH and TNFα

| | LDH (media/homogenate) | | TNFα (pg/mg protein) | |
|---|---|---|---|---|
| | mean | % control | mean | % control |
| 1 h Control | 0.05+/-0.01 | | 65.1+/-17 | |
| 1 h Anoxia | 0.04+/-0.08 | 90+/-24 | 167.1+/-45 | 257+/-79 ** |
| 1 h Ischaemia | 0.08+/-0.02 | 171+/-45* | 434.3+/-115 | 667+/-203*** |
| 2 h Control | 0.04+/-0.01 | | 37.4+/-10 | |
| 2 h Anoxia | 0.10+/-0.02 | 233+/-61 ** | 281.2+/-74 | 752+/-229*** |
| 2 h Ischaemia | 0.19+/-0.04 | 446 +/-119*** | 983.4+/-260 | 2629+/-799*** |

*=P<0.05 **=P<0.01 ***=P<0.001 multiple t test (n=6)

Over 500% increases in TNFα levels were found for both 1 h & 2 h ischaemia, with bigger increases occurring at 2 h (Table 1). This reflects previous findings of TNFα level increases in experimental ischaemia (Hopkins & Rothwell, 1995). Additionally, significant LDH release was observed with 1 and 2 h ischaemic insults. Significant TNFα elevation was also detected for 1 & 2 h anoxia, though of less magnitude than the ischaemic changes, whilst significant LDH release was only detected at 2 h anoxia. These data indicated early cytokine involvement in anoxia and ischaemia associated with cytotoxicity. Ischaemic damage was more pronounced than anoxia, and of greater detriment with prolonged insult which was in accordance with other *in vitro* studies (Huang et al., 1993). The anoxic/ ischaemic damage and cytokine involvement demonstrated in spheroids suggests they would be a suitable model to develop further as a pharmacological screen.

K.P is supported by the BBSRC and Knoll Pharmaceuticals

Atterwill, C.K. (1987) In *In Vitro Methods Of Toxicology* ed. Atterwill, C.K. & Steele, C.E. 132-161. Cambridge University Press.
Hopkins, S.J. & Rothwell, N.J. (1995) Trends Neurosci. **18**, 83-88.
Huang, R., Shuaib, A. & Hertz, L. (1993) Brain Res. **618**, 346-351.

---

## 46P INHIBITION OF L-TYPE $Ca^{2+}$ CHANNELS BY CARBAMAZEPINE IN RAT CULTURED HIPPOCAMPAL NEURONS STIMULATED WITH IONOTROPIC GLUTAMATE RECEPTOR AGONISTS

[1]António F. Ambrósio, [1]Ana P. Silva, [1]João O. Malva, [2]Patrício Soares-da-Silva, [1]Caetana M. Carvalho and [1]Arsélio P. Carvalho
[1]Center for Neuroscience of Coimbra, Dept. of Zoology, University of Coimbra, Coimbra, Portugal, and [2]BIAL Laboratories, Portugal

Carbamazepine (CBZ) is an antiepileptic drug that has been extensively used in the treatment of epilepsies. It is established that CBZ inhibits voltage-gated $Na^+$ channels (Rush and Elliot, 1997). However, the mechanisms underlying the effects of CBZ have not been completely identified, and are still a matter of debate. It has been also shown that CBZ inhibits $Ca^{2+}$ channels (Schumacher *et al.*, 1998) and N-methyl-D-aspartate (NMDA)-evoked $Ca^{2+}$ influx (Hough *et al.*, 1996). In order to better understand the mechanism(s) of action of CBZ, we studied its effects on $[Ca^{2+}]_i$ and $[Na^+]_i$ increases stimulated by ionotropic glutamate receptor agonists, in cultured rat hippocampal neurons. The changes in the $[Ca^{2+}]_i$ and $[Na^+]_i$ were followed by the Indo-1 or SBFI fluorescence, respectively.

Carbamazepine inhibited the increase in the $[Ca^{2+}]_i$ stimulated either by glutamate, kainate, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA), or NMDA in a concentration-dependent manner. The calculated $EC_{97}$'s values were 223.0 μM for glutamate, 185.4 μM for kainate, 201.4μM for AMPA and 217.0 μM for NMDA. In order to discriminate the effects of CBZ on the activation of glutamate receptors from possible effects on $Ca^{2+}$ channels, we determined the inhibitory effects of $Ca^{2+}$ channel blockers on $[Ca^{2+}]_i$ changes stimulated by kainate, in the absence or in the presence of CBZ. The presence of 1 μM nitrendipine, 0.5 μM ω-conotoxin GVIA (ω-CgTx GVIA), or of both blockers inhibited the increase in $[Ca^{2+}]_i$ stimulated with 185.4 μM kainate by 51.6±2.8% (S.E.M.; n=7), 32.9±1.3% (n=5) or 68.7±2.9% (n=5), respectively. Carbamazepine (100 μM) inhibited the $[Ca^{2+}]_i$ increase due to kainate by 41.0±4.2% (n=8). In the presence of both 100 μM CBZ and nitrendipine, the inhibition, 54.1±6.5% (n=5), was similar to that obtained with nitrendipine alone, but in the presence of both CBZ and ω-CgTx GVIA, the inhibition was greater, 54.0±4.5% (n=7), than that caused by ω-CgTx GVIA alone. The presence of MK-801 (10 μM) or tetrodotoxin (1 μM) did not inhibit the changes in $[Ca^{2+}]_i$, indicating that NMDA receptors or tetrodotoxin-sensitive $Na^+$ channels are not involved in the changes of $[Ca^{2+}]_i$. Moreover, CBZ did not inhibit the increase in the $[Na^+]_i$ stimulated by the ionotropic glutamate receptor agonists, but inhibited the increase in the $[Na^+]_i$ due to veratridine by 53.4±4.7% (n=5). Tetrodotoxin, or MK-801, did not inhibit the influx of $Na^+$ stimulated by kainate, indicating that $Na^+$ influx occurs mainly through the non-NMDA ionotropic glutamate receptors. The $[Na^+]_i$ response to kainate or AMPA was inhibited by LY 303070, a specific AMPA receptor antagonist, by 67.6±2.9% (n=6) or 79.1±3.3% (n=5), respectively, suggesting that AMPA receptors are mainly involved.

Taken together, the results suggest that CBZ inhibits L-type $Ca^{2+}$ channels and $Na^+$ channels, but does not inhibit ionotropic glutamate receptors activation.

(Supported by PRAXIS XXI Program, Portugal; and BIAL Laboratories, Portugal; LY 303070 was a kind gift of Eli Lilly)

References

Hough, C.J., Irwin, R.P., Gao, X.-M., *et al.*, 1996, J. Pharmacol. Exp. Ther., 276, 143-149.
Rush, A.M. and Elliot, J.R., 1997, Neurosci. Lett. 226, 95-98.
Schumacher, T.B., Beck, H., Steinhäuser, C., *et al.*, 1998, Epilepsia, 39, 355-363.

## 47P BINDING OF [$^3$H]GABA AND OF [$^3$H]CGP 62349 TO THE $GABA_B$ RECEPTOR ARE DIFFERENTIALLY AFFECTED IN ALZHEIMER'S DISEASE PREFRONTAL CORTEX

P. Meoni, M. Uroda, B.J. Bowery and N.G. Bowery, Dept of Pharmacology, University of Birmingham, B15 2TT, UK

Electrophysiological and behavioural studies have suggested a role for $GABA_B$ receptors in memory processes in experimental animals (see Bowery, 1993). In line with these findings, binding studies conducted on human post-mortem brain samples with [$^3$H]GABA have shown that binding to $GABA_B$ receptors is severely reduced in Alzheimer's disease (AD) brain (Chu *et al.*, 1987), suggesting an involvement of this receptor in the memory impairment characteristic of this disease. However, preliminary studies in our laboratory suggest that binding to the antagonist site of the $GABA_B$ receptor might be preserved in samples of human AD cortex.

The aim of the present study was to examine relative changes in agonist and antagonist binding to the prefrontal cortex of control and AD brain. The role of G-protein coupling to the receptor was also examined to highlight any possible difference in $GABA_B$ signal transduction in AD.

Levels of binding of [$^3$H]CGP 62349, a high affinity antagonist of the $GABA_B$ receptor (Bittiger *et al.* 1996) and of [$^3$H]GABA, in conditions allowing specific binding to the $GABA_B$ receptor (Bowery *et al*, 1983) were measured in control and AD prefrontal cortex.

Levels of agonist and antagonist binding to control and age and pH-matched samples from AD brains were measured with Biospace (Paris, France), Beta-Imager 2000 avalanche β-ray detector and levels of radioactivity normalised with plastic microscales (Amersham UK). Sections (12μm) of human control (n=5) and AD (n=6) prefrontal cortex (MRC Brain Bank, Institute of Psychiatry) were incubated with 6 concentrations of [$^3$H]CGP 62349 ranging from 0.125 to 8nM, and the resulting saturation curve was fitted to a one-site binding hyperbola (Prism, Graph Pad software Inc., USA).

Saturation studies revealed a Kd ~ 1.1 nM in control tissue, with no significant difference in $K_d$ or $B_{max}$ between the control and AD group. Binding of [$^3$H]GABA (50 nM) to the $GABA_B$ receptor on consecutive sections of the same tissue showed a significant decrease (50-55%) in AD prefrontal cortex compared to control as reported by Chu *et al* (1987). [$^3$H]GABA binding to $GABA_B$ sites in control sections was reduced by 72% in the presence of GTP (30-100 μM). In Alzheimer brain sections the low level of [$^3$H]-GABA binding was further reduced by 80-85% in the presence of GTP suggesting that receptors coupled to G-protein were still present. Binding of [3H]CGP 62349, not surprisingly, was unaffected by GTP in any tissue section.

It has recently been reported that $GABA_B$ receptors may exist as heterodimers (Jones *et al*, 1998; White *et al* 1998; Kaupmann *et al*, 1998) providing the possibility of differential binding properties. . An alteration in the expression of these subunits might provide the basis for the difference in agonist and antagonist binding in prefrontal cortex of AD human brain. Studies to determine the detailed kinetics of [$^3$H]GABA binding are in progress.

The present data show that antagonist, in contrast to agonist binding to the $GABA_B$ receptor is not affected in AD, suggesting that the receptor might be differentially affected in this pathology.

We thank Novartis for [$^3$H]CGP 62349.

Bittiger H. *et al* (1996) Pharm Rev & Comm. **8**: 97-98
Bowery N.G. (1993) Ann. Rev. Pharmacol. Toxicol. **33**: 109-147
Bowery N.G. *et al* (1983) Br. J. Pharmacol, **78**: 191-206
Chu *et al* (1987) Neurol. **37**: 1454-1459
Jones K.A. *et al* (1998) Nature, **396**: 674-679
Kaupmann K. *et al* (1998) Nature, **396**: 683-687
White J. H. *et al* (1998) **396**, 679-682

---

## 48P $NK_2$-RECEPTOR ACTIVATION INCREASES SPONTANEOUS GABA-RELEASE IN THE RAT NUCLEUS TRACTATUS SOLITARIUS *IN VITRO*

C.P. Bailey & R.S.G. Jones. Department of Physiology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD

Previous studies in rat brain slices have shown that $NK_1$-receptor activation caused a postsynaptic depolarization of neurones in the nucleus tractus solitarii (NTS) (Maubach & Jones, 1997). In addition, $NK_1$-receptor agonists increased spontaneous synaptic synaptic activity, but this effect was shown to be due to excitation in local circuits rather than an effect on presynaptic terminals (Maubach *et al.*, 1995). In contrast, a specific $NK_2$-receptor agonist, GR64349, had no postsynaptic depolarizing effect (Maubach & Jones, 1997), but did increase spontaneous synaptic activity (Maubach and Jones, unpublished). Subsequent whole-cell voltage-clamp recordings in the NTS (Bailey & Jones, 1998) showed that $NK_2$-receptor activation increased the frequency of both miniature and spontaneous excitatory postsynaptic currents (EPSCs), indicating a presynaptic location of $NK_2$-receptors, activation of which could increase glutamate release. We have now examined whether $NK_2$-receptors can also modify GABA rlease in the NTS by studying the effects of GR64349 on spontaneous inhibitory postsynaptic currents (IPSCs).

Experiments were conducted in brainstem slices prepared from young adult male Wistar rats. Whole-cell voltage-clamp recordings were made from neurones in the NTS voltage clamped at −80 mV. Recording electrodes were filled with a solution containing (in mM): 130 KCl, 5 Hepes, 1 $MgCl_2$, 1 NaCl, 0.34 $CaCl_2$ and 5 EGTA (pH 7.3, 285 mOsmol). Such recordings showed continuous spontaneous synaptic events, manifested as inward currents. Pooled data from 4 neurones (minimum of 150 events per neurone gave a mean (±s.e.mean) frequencies of 14.8±3.3 Hz, and amplitudes of 14.9±2.4 pA. In order to isolate spontaneous IPSCs (sIPSCs), D,L-AP5 (50μM) and NBQX (5 μM) were bath applied. This resulted in a decrease in frequency of 87.2±2.2%, and a decrease in amplitude of 30.0±9.8%. A non-parametric analysis of cumulative probability distributions (Kolmogorov-Smirnov (KS) test) showed these effects to be highly significant ($P$<0.0001). Subsequent addition of GR64349 (500 nM) significantly increased both the frequency and amplitude of the isolated sIPSCs by 108.2±53.8% and 50.3±18.5%, respectively (n=4). Comparison of cumulative probability distributions of sIPSCs in the presence and absence of GR64349 showed its effects on both frequency and amplitude to be highly statistically significant ($P$<0.0001, KS test). In three neurones addition of GR64349 increased the frequency of sIPSCs from 2.4±0.5 Hz to 5.0±0.8 Hz. Subsequent addition of bicuculline (5 μM) blocked most of the sIPSCs, indicating that GR64349 is effective in increasing inhibitory transmission predominantly via an increase $GABA_A$-receptor mediated events. However, a few small events remained (frequency 0.5±0.4 Hz). It is possible that these events may be mediated by glycine release (Feldman and Felder, 1991) and we are currently looking at this possibility.

Thus, in addition to increasing spontaneous glutamate release (Bailey & Jones, 1998) activation of $NK_2$-receptors also increases both the frequency and amplitude of spontaneous $GABA_A$-receptor mediated sIPSCs in the rat NTS. Whether this reflects a direct effect on GABA-terminals, or reflects a local network effect remains to be determined.

We thank the Wellcome Trust for Financial support

Bailey, C.P. & Jones, R.S.G. (1998) *J.Physiol.* **513**, 17P
Feldman, P.D. & Felder, R.B. (1991) *Neuropharm.* **30**: 225-231
Maubach, K. A. & Jones, R. S. G. (1997). *Br. J. Pharmac.*, **122**, 1151-1159
Maubach, K. A. *et al* (1995) *J. Physiol.* **489**: 18P

## 49P PHENYTOIN ENHANCES SPONTANEOUS GABA-MEDIATED INHIBITION IN THE RAT ENTORHINAL CORTEX

M.O. Cunningham, S.J. Wood & R.S.G. Jones. Department of Physiology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD

The overall level of excitability of cortical networks may depend on the balance between spontaneous synaptic excitation, mediated by glutamate, and inhibition mediated by GABA (Otis *et al.*, 1991). We have recently shown that phenytoin, decreases the spontaneous release of glutamate at synapses in rat entorhinal cortex (EC) via a presynaptic action (Cunningham and Jones, 1998). In the present study we have looked at the effect of phenytoin on inhibitory synaptic transmission by studying it's effects on spontaneous and miniature IPSCs mediated by $GABA_A$-receptors.

Experiments were conducted in brain slices containing EC from adult rats prepared and maintained *in vitro* by conventional means. Whole-cell patch-clamp recordings were made blind from neurones in layer II. Recording electrodes were filled with a solution containing (in mM): 135 CsCl, 10 Hepes, 2 $MgCl_2$, 5 QX-314, 0.5 $CaCl_2$ and 5 EGTA (pH 7.3, 290 mOsmol). At a holding potential of -60 mV, in the presence of D,L-AP5 (100μM) and NBQX (5 μM) neurones displayed fast, high frequency inward currents. Addition of bicuculline (5μM) abolished these events, showing that they were IPSCs mediated via activation of $GABA_A$ receptors.

In pooled data from 5 layer II neurones (minimum of 100 events per neurone), IPSCs had a mean (±S.E.M) amplitude of 17.94±0.26 pA and an inter-event interval of 0.29±0.01 sec. Bath application of phenytoin (50 μM) resulted in an increase in mean amplitude to 20.73±0.38 pA, accompanied by a decrease in inter-event interval (i.e. increase in frequency) to 0.13±0.004 s. Comparison of cumulative probability distributions using the non-parametric, Kolmogorov-Smirnov test showed that the increase in both amplitude and frequency was highly significant ($P<0.0001$). In a further 5 layer II neurones, TTX (1μM) was perfused (in addition to NBQX and D,L-AP5) to block voltage-gated $Na^+$ channels, allowing us to record activity-independent, miniature IPSCs (mIPSCs). Before application of phenytoin, these had a mean amplitude of 40.7±1.03 pA and an inter-event interval of 0.16±0.009 sec. In the presence of phenytoin, mIPSCs had an inter-event interval of 0.1±0.004 s and an amplitude of 42.16±0.96 pA. Again, comparison of cumulative probability distributions using the Kolmogorov-Smirnov test confirmed that both frequency and amplitude of mIPSCs were significantly increased by phenytoin. We also have preliminary data from two layer V neurones showing similar effects of phenytoin on IPSC frequency (mean control interval, 0.25s *v* 0.20s in phenytoin) and amplitude (32.6 *v* 38.6 pA).

The increase in frequency of IPSCs is indicative of a presynaptic action of phenytoin to increase inhibition. However, the increase in amplitude may suggest that this is combined with a postsynaptic action of the drug. Whatever the basis of these effects, the current data, combined with our previous results on glutamate release suggest that the phenytoin can exert a dual influence on spontaneous transmitter release, tipping the balance towards inhibition. This may well underlie the anticonvulsant actions of the drug.

M.O.C. is an MRC student. We thank the Wellcome Trust for financial support.

Cunningham, M.O & Jones, R.S.G. (1998) *J. Physiol.* **511**, 126P
Otis, T.S., *et al.* (1991) *Brain Res.* **545**, 142-150

---

## 50P IFENPRODIL BLOCKS NMDA AUTORECEPTORS IN THE RAT ENTORHINAL CORTEX *IN VITRO*

G.L. Woodhall & R.S.G. Jones. Department of Physiology, School of Medical Sciences, University Walk, University of Bristol, Bristol BS8 1TD.

Recent work in this laboratory has demonstrated that glutamate terminals in the entorhinal cortex (EC) possess NMDA autoreceptors which play a role in control of glutamate release (Berretta and Jones, 1996). Tonic activation of these receptors by glutamate facilitates subsequent release of the excitatory transmitter. However, as yet we do not know the subtype(s) of NMDA-receptor present on the glutamate terminals. The present study has examined the effect of ifenprodil on spontaneous excitatory post-synaptic currents EPSCs in rat EC neurones. The effect of this antagonist suggests that the presynaptic autoreceptor may contain the NR2B sub-unit of the NMDA receptor.

Whole-cell patch-clamp recordings were made from layer II and layer V neurones in EC slices prepared from male Wistar rats (70-110g) and maintained *in vitro*. Neurones were visually identified using a microscope fitted with an infra red camera and DIC optics. Recording electrodes were filled with a solution containing (in mM): 130 Cs-methanesulphonate, 10 HEPES, 5 QX-314, 0.5 EGTA, 1 NaCl, 0.34 $CaCl_2$, 1 $MgCl_2$, 1 MK-801, (290 mOsmol, adjusted to pH 7.3 with CsOH). Neurones were voltage-clamped at –60 mV, and repeatedly step depolarized to –20 mV during an initial five minute recording period. This facilitated open channel blockade of NMDA receptors by the internally dialysed MK-801. This approach allowed us to record postsynaptic AMPA receptor mediated EPSCs in isolation, uncontaminated by any action of spontaneously released glutamate at postsynaptic NMDA receptors. TTX (1 μM) was included in the perfusate to isolate activity-independent miniature EPSCs (mEPSCs). Ifenprodil (50 μM) was applied by bath perfusion.

Pooled data from six layer II neurones (at least 100 events from per neurone) gave a mean (±SEM) mEPSC frequency and amplitude of 2.42±0.14 Hz and 8.91±0.24 pA respectively. Application of ifenprodil decreased mEPSC frequency to 1.04±0.07 Hz with no change in amplitude (8.61±0.17 pA). A comparison of cumulative probability distributions with the Kolmogorov-Smirnov (KS) test showed that the decrease in frequency was highly significant ($P<0.0001$), whereas there was no significant change in amplitude. A two-way ANOVA comparing inter-event intervals confirmed the significance of the reduction in mEPSC frequency by ifenprodil. Similar experiments on layer V neurons (n=6), revealed a reduction in mEPSC frequency from 4.18±0.22 Hz to 1.32±0.07 Hz with ifenprodil, again without a change in amplitude (9.06±0.3 pA *v* 8.53±0.20 pA). KS tests confirmed that the decrease in frequency was statistically significant, whilst the amplitude changes were not ($P<0.0001$ and 0.0015, respectively). Again these effects were confirmed using the two-way ANOVA ($P<$ 0.005 and 0.07, respectively).

These data suggest that the presynaptic NMDA autoreceptor in rat EC is ifenprodil-sensitive, and therefore probably contains the NR2B sub-unit. Confirmation of this will require further experimentation, and we are currently looking at the effects of an NR2B specific agonist. The ability to control glutamate release via sub-unit specific activation or blockade of presynaptic receptors presents a potentially important therapeutic strategy in pathological conditions such as temporal lobe epilepsy.

We thank the Wellcome Trust for financial support

Berretta, N. & Jones, R.S.G. (1996) *Neurosci.* **75**, 339-344

## 51P THE $A_{2A}$-ADENOSINE AGONIST, CGS 21680C, FACILITATES ACETYLCHOLINE RELEASE BY ACTIVATING L-TYPE CALCIUM CURRENTS DURING PROLONGED DEPOLARISATION OF THE RAT MOTOR NERVE TERMINALS

P. Correia-de-Sá, M.A. Timóteo & J.A. Ribeiro* Lab. Farmacologia, ICBAS - Univ. do Porto, Porto and *Lab. Neurociências, Fac. Medicina - Univ. Lisboa, Portugal.

The rat motor nerve terminals possess both $A_1$-inhibitory and $A_{2A}$-excitatory adenosine receptors modulating acetylcholine release (Correia-de-Sá *et al.*, 1991). The $A_1/A_{2A}$-receptor activation balance is highly dependent on the stimulation pattern, *e.g.* tonic $A_{2A}$-receptor-mediated facilitation predominates as the stimulus pulse duration increased to the milisecond range (Correia-de-Sá *et al.*, 1997). Dudel (1989) showed that changes in the duration of depolarization significantly modify neuronal calcium influx into nerve cells. This prompted us to evaluate the influence of several type-specific voltage sensitive calcium channel (VSCC) blockers on $A_{2A}$-adenosine receptor facilitation of [$^3$H]-acetylcholine ([$^3$H]-ACh) release evoked by pulses of variable duration.

The experiments were carried out on phrenic nerve-hemidiaphragm preparations taken from Wistar rats of either sexes weighting about 200 g. The phrenic motor nerve terminals were loaded with 1 μM [$^3$H]-choline during 40 min under electrical stimulation (1 Hz frequency). After a 60 min washout period, [$^3$H]-ACh release was evoked by supramaximal stimulation of the phrenic nerve using 750 electrical pulses applied with a frequency of 5 Hz ($S_1$ and $S_2$). The duration of each pulse ranged from 40 μs and 1 ms. Drugs were added 15 min before $S_2$. Data were expressed as mean±s.e.m., and Student's *t*-test applied to detect statistical significance; Values were considered significant if $P<0.05$.

Electrical stimulation of the phrenic nerve increased the release of [$^3$H]-ACh in a pulse duration-dependent manner. During short stimulation pulses (40 μs), the P-type VSCC blocker, ω-agatoxin IVA (100 nM), decreased evoked [$^3$H]-ACh release by 21±4% ($n$=4). Upon increasing the stimulation pulse duration to 1 ms, ω-agatoxin IVA (100 nM) failed to modify evoked [$^3$H]-ACh release, while the inhibitory effect of the L-type VSCC blocker, nifedipine (1 μM), became significant (23±5%, $n$=4). Neither the N-type, ω-conotoxin GVIA (1 μM), nor the P/Q-type, ω-conotoxin MVIIC (150 nM), VSCC blockers significantly ($P>0.05$) modified evoked [$^3$H]-ACh release. The magnitude of the $A_{2A}$-receptor agonist, CGS 21680C (3 nM), facilitation on [$^3$H]-ACh release was not modified by changes in stimulation pulse duration. However, with pulses of 40 μs duration, nifedipine (1 μM) significantly attenuated the CGS 21680C (3 nM) facilitatory effect from 63±2% ($n$=4) to 21±4% ($n$=5). In contrast, with 1 ms pulses, where calcium influx through L-type channels is dominant, CGS 21680C (3 nM) facilitation (55±8%, $n$=5) was blocked by pretreatment with the P-type VDCC blocker, ω-agatoxin IVA (100 nM)(5±9%, $n$=4).

The results suggest that $A_{2A}$-adenosine receptor activation facilitates acetylcholine release from motor nerve endings by alternatively mobilizing calcium influx through either P- or L-type VSCC depending on the stimulus paradigm.

Correia-de-Sá, Sebastião & Ribeiro (1991). *Br. J. Pharmacol.* **103**: 1614-20.
Correia-de-Sá, Timóteo & Ribeiro (1997). *J. Neurophysiol.* **76**: 3910-19.
Dudel (1989). In: *Neuromuscular Junction*. ed. Sellin, L.C., Libelius, R. & Thesleff, S. pp.149-159. Amsterdam: Elsevier.

This work was supported by PRAXIS XXI and UMIB grants.

---

## 52P COMPARATIVE EFFECTS OF MORPHINE, COCAINE AND MORPHINE PLUS COCAINE ON PLASMA AND BRAIN CATECHOLAMINES IN RATS

T.R.A. Macedo, C.M. Pinto, S. Arede, M.T. Morgadinho, M. F. Pacheco, A.M. Macedo, C.A. Fontes Ribeiro. Inst. Pharm. Exp. Ther.,Fac. of Medicine, Univ. of Coimbra, Portugal.

In the present study we compare the effects of acute administration of morphine (Mph), cocaine (Coc) and morphine plus cocaine (Mph + Coc) on the concentrations of catecholamines in plasma and distinct areas of brain in rats.

Male Wistar rats (200-250 g) were given Mph (50 or 100 mg/Kg, s.c.), Coc (10 or 50 mg/Kg, i.p.) or 50 mg/Kg Mph plus 10 mg/Kg Coc. The control (C) animals received s.c. or i.p. physiological saline. Two hours (Mph) or 30 min (Coc) later blood samples from pentobarbital (35 mg/Kg) anesthetised rats were collected *via* cardiac puncture into ice-cooled tubes containing 0.7 mM Na2EDTA and immediately centrifuged to separate plasma and platelets. Brains were rapidly removed and seven regions were carefully isolated and dissected - cerebellum (CB), hypothalamus (HT), hippocampus (HC), striatum (ST), prefrontal cortex (PC), rest of cortex (RC) and pons (PO) - and immersed in 0.1 M perchloric acid to determine noradrenaline (NA), dopamine (DA) and DA major metabolites contents after 24 h at 4°C, using HPLC-ED. The wet weight of all the tissues was registered. Data were recorded as ng amine/mL plasma or per gram of brain tissue. With Coc the metabolite/neurotransmitter ratios were converted to percent change of drug values over control values to facilitate evaluation. Analysis of variance (ANOVA) was used.

Plasma NA levels were significantly increased by the two Mph doses used, whereas the DA level was increased only by 100 mg/Kg Mph. With 10 mg/Kg Coc the plasma NA level was not affected but DA was enhanced. The simultaneous use of Mph plus Coc produced a significant increase of NA (similar to Mph alone) and DA in plasma.

Table 1. Effects of acute morphine, cocaine and morphine plus cocaine on NA and DA plasma levels (pg/mL). Results are means ± s.e.m. for n rats. *P<0.05 compared to control (one way ANOVA).

| Drug | NA | DA | n |
|---|---|---|---|
| Morphine (50 mg/Kg) | 633±132* | 96±32 | 9 |
| Control | 284±48 | 92±27 | 9 |
| Morphine (100 mg/Kg) | 2848±900* | 966±238* | 11 |
| Control | 614±141 | 134±44 | 11 |
| Cocaine (10 mg/Kg) | 657±131 | 439±81* | 10 |
| Control | 454±48 | 92±27 | 10 |
| Cocaine (50 mg/Kg) | 1820±264* | 362±121* | 10 |
| Control | 635±94 | 77±13 | 10 |
| Morphine (50 mg/Kg) + Cocaine (10 mg/Kg) | 2055±648* | 133±28* | 10 |
| Control | 305±80 | 92±18 | 9 |

In brain the most profound changes with Mph were seen in ST and PC. In PC the values of 3,4-dihidroxyphenylacetic acid (DOPAC) (Mph-50mg/Kg=533±79* ng/g; C=227±52 ng/g; n=9) and homovanillic acid (HVA) (Mph=180±27* ng/g; C=49±12ng/g; n=9) were significantly increased. With 10 mg/Kg Coc, in the same area the DOPAC/DA ratio (21±4*%; C=36±3%; n=9) and HVA/DA ratio decreased (Coc=5.0±0.4*%; C=10±0.9%; n=10). Thus, the DOPAC and HVA routes of DA degradation were both utilized by the prefrontal cortex. When Mph plus Coc was used only the DA level was significantly reduced (Coc+Mph=879±84*ng/g; C=1220±91 ng/g; n=5) and the DOPAC/DA ratio (Coc+Mph=41±4%*; C=23±2%; n=5) and the HVA/DA ratio (Coc+Mph=13±3%; C=5±1%; n=5) were significantly higher (*P<0.05).
These results in the plasma and in the brain indicate that both drugs change the DA turnover.

[Supported by PRAXIS/2/2.1/SAU/1401/95]

## 53P EFFECTS OF NICOTINE ON INTRACELLULAR CALCIUM DYNAMICS IN SYMPATHETIC NERVE TERMINAL VARICOSITIES

Trout, S.J., Brain, K.L, Dass, N. and Cunnane, T.C.
Department of Pharmacology, University of Oxford, Mansfield Road, Oxford, OX1 3QT. U.K.

It is well known that many presynaptic receptors modulate neurotransmitter release by affecting the intracellular calcium concentration (Augustine *et al.*, 1987). It is more unusual, however, to have a ligand-gated ion channel modulate transmitter release. Nicotinic acetylcholine receptors have been found on presynaptic sympathetic nerve terminals, which increase the efflux of neurotransmitter upon nerve stimulation (Trout & Cunnane, 1997). Fluorescence laser scanning confocal microscopy was used to image sympathetic nerve terminal varicosities, preloaded with the calcium indicator Oregon Green 488 BAPTA-1, as described by Brain and Bennett (1997).

Data are expressed as mean ± s.e.mean. Statistical comparisons were made with the Mann-Whitney U test: values of $P < 0.05$ were considered to be significant. Field stimulation (pulse width, 0.06 ms; voltage, 10-60 V) was used to elicit calcium transients within the varicosities. These transient changes in intracellular calcium concentration ($\Delta[Ca^{2+}]_i$) were abolished by 300 nM tetrodotoxin (TTX; amplitude of the $\Delta[Ca^{2+}]_i$ transient in TTX was 0.4 ± 0.3% of the control amplitude; $n$ = 32 varicosities in 5 *vasa deferentia*), suggesting that they occurred subsequent to an action potential. Nicotine (30 μM) had no significant effect on the amplitude of the evoked $\Delta[Ca^{2+}]_i$ transient (-2.8 ± 3.0 % change, $n$ = 13 varicosities in 3 separate preparations; $P > 0.05$), but nicotine did increase the resting calcium concentration (by 17 ± 3 nM; $n$ = 48 varicosities) and elicit spontaneous $\Delta[Ca^{2+}]_i$ transients (calcium spikes). Results obtained in 27 varicosities in 5 *vasa deferentia* showed the calcium spike frequency was 0.085 ± 0.010 Hz above the control spike count. These nicotine-induced calcium spikes also occurred in the presence of TTX (frequency 0.105 ± 0.020 Hz; $n$ = 15 varicosities), showing that they were not induced by action potentials. These results shed new light on the mechanism of action of nicotine in presynaptic nerve terminals, including its ability to facilitate evoked transmitter release. One possibility is that calcium-induced calcium release is involved in the observed effects of nicotine.

References

Augustine, G.J., Charlton, M.P. & Smith, S.J. (1987), *Annu. Rev. Neurosci.* **10**, 633-693.
Brain, K.L. & Bennett, M.R. (1997), *J. Physiol.* **502**, 521-536.
Trout, S.J. and Cunnane, T.C. (1997), *Pharmacologist* **30**, 357.

---

## 54P ACUTE HISTAMINE EXPOSURE INDUCED THE PHOSPHORYLATION OF A 58 kDa PROTEIN IN MOUSE HYPOTHALAMIC GT1-7 NEURONAL CELLS

Andrea H. Leeson, M. Reza Zamani & David R. Bristow
Division of Neuroscience, School of Biological Sciences, University of Manchester, 1.124 Stopford Building, Manchester, M13 9PT, U.K.

The mechanism underlying homologous histamine $H_1$ receptor desensitisation has been studied in a number of systems and the results infer an important role for protein phosphorylation (Smit *et al.*, 1992; Zamani & Bristow, 1996). However, it has not been determined whether the site of phosphorylation occurs at the receptor or at some other cellular protein. This study investigated whether an acute histamine exposure sufficient to induce $H_1$-receptor desensitisation caused changes in phosphoprotein expression in GT1-7 neuronal cells.

GT1-7 cells were cultured and harvested as described elsewhere (Zamani & Bristow, 1996). Cells (approx. 1.5 x $10^6$ /treatment) for 2D-phosphoprotein studies were incubated with 100 μCi [$^{32}$P]orthophosphate for 1.5 hrs at 37 °C and then treated with vehicle (HEPES-Krebs phosphate buffer, in mM: NaCl, - 120, KCl - 4.7, HEPES - 25, $NaHCO_3$ - 5, CaCl - 1.8, $MgCl_2$ - 1.2, and glucose 0.45%, w/v) (control), histamine (30 μM) or histamine (30 μM) and KN-62 (10 μM) for 30 min (37 °C). The incubation was terminated by centrifugation (13,000 x *g*, 1 min), the supernatant removed, and the cell pellet was solubilised in denaturing buffer (urea 9.5 M, NP40 5%, β-mercaptoethanol 5%, Ampholyte 5/7 1.6%, Ampholyte 3/10 0.4%, all v/v). The samples were subjected to 2D-gel electrophoresis. First dimension was separation by isoelectric point using rod gels (750 V, 3.5 hrs) and the second dimension was separation by molecular weight (100 V, 1.5 hrs). Molecular weight markers were included on each gel. The gels were exposed to X-ray film overnight. Autoradiographs were analysed by computer-assisted 2D-gel analysis (Bio-image Software). The experiments were repeated on four different cultures, and each drug condition was performed in duplicate. All data are given as mean ± s.e.mean.

Analysis of the 2D-phosphoprotein patterns identified 187 phosphoproteins present on both duplicate gels from all experiments. Quantitative measurements of [$^{32}$P] signal density (integrated intensity) were carried out and the level of each phosphoprotein spot was compared between control and histamine-treated, and histamine-treated and histamine-treated plus KN-62. Two-tailed Student's t-test was used to determine significant changes in individual phosphoprotein levels between experimental groups. However, the 2D-maps did not always overlay exactly, because of slight gel distortion within experiments, or incomplete separation of protein signals, or the [$^{32}$P] signal had saturated the X-ray film. Thus statistical testing of differences in protein levels was only carried out on a proportion of the phosphoproteins detected when n ≥ 3. One phosphoprotein with a molecular weight of 58 ± 1.9 KDa (n=4) was present in histamine treated cells (mean density 28 ± 6 arbitrary units (AU), n=4) but completely absent in control cells (n=4), and significantly reduced ($P < 0.04$) in cells exposed to histamine in the presence of KN-62 (6.8 ± 6.8 AU, n=4, signal absent in 3 of 4 experiments).

The results from this study demonstrate that a 58 KDa protein is phosphorylated in GT1-7 cells following acute histamine stimulation, which is almost completely abolished in the presence of KN-62, a selective inhibitor of calcium/calmodulin-dependent protein kinase II (CaMKII). These results are consistent with a role for CaMKII phosphorylation in histamine $H_1$ receptor desensitisation. The histamine-induced phosphoprotein corresponds with the estimated size of the histamine $H_1$ receptor (Yamashita *et al.*, 1991), implying that the site of phosphorylation may be the $H_1$ receptor protein.

Smit M.J. *et al.*, (1992) *Br. J. Pharmacol.*, **107**, 448-455
Yamashita M. *et al.*, (1991) *Proc. Natl. Acad. Sci.*, **88**, 11515-11519
Zamani M.R. & Bristow D.R. (1996) *Br. J. Pharmacol.*, **109**, 353-359