133P SUB-CELLULAR LOCALISATION OF ALPHA-1-ADRENOCEPTORS IN LIVE CELLS AND TISSUES

J.C. McGrath, J.F. Mackenzie & C.J. Daly, Institute of Physiology, University of Glasgow, Glasgow G12 8QQ

Fluorescent ligands can permit visualisation and pharmacological analysis of receptor molecules at the subcellular level in isolated tissues. We have established the method of fluorescent ligand binding using the fluorescent α_1-adrenoceptor ligand QAPB (at nanomolar concentrations; McGrath *et al.*, 1996, Daly *et al.*, 1998). Visualisation can be accomplished with high resolution and specific fluorescent-ligand binding sites can be identified on live cells, permitting analysis of their distribution within heterogeneous tissues. Fluorescence can be quantified using confocal microscopy and image analysis can be employed to identify pharmacologically specific sites, for example by image subtraction, analogous to identification of specific binding in conventional radioligand binding (McGrath *et al.*, 1996). This has been applied to the study of receptors in single "living" cells (Mackenzie *et al.*, 1998a).

We initially anticipated that the method would allow observation mainly of receptors on the plasmalemmal membrane due to the ligand binding to the receptor molecules at binding sites on extracellular surface domains. However, since the ligand is lipophilic it can distribute itself throughout cells and, consequently, can bind to receptors wherever they are located.

In initial studies in cells harbouring recombinant α_1-adrenoceptors this technique suggested that the bulk of receptor binding sites were located intracellularly. This might have been unsurprising in such an artificial situation, whereby the cells have been turned into factories for the expression of receptors. However, we then extended the scope of the method to allow localisation and identification of native α_1-adrenoceptors in dissociated smooth muscle cells. This showed remarkable similarities between the intracellular distribution of binding sites in recombinant and native systems (smooth muscle cells from human prostate and rat blood vessels; Mackenzie *et al.*, 1998b). We found intracellular binding sites in smooth muscle cells, particularly around the nucleus, which could represent binding in the Golgi.

The obvious interpretation is that intracellular binding sites represent newly synthesized or recycling stores of receptors not involved in transduction of transmembrane signals. However, we cannot exclude the possibility that intracellular binding sites are involved in signal transduction. Furthermore, accessibility of intracellular binding sites to ligands opens up several lines of thought with respect to receptor theory and the equilibrium between drugs and different receptor states. Although this study has concentrated on alpha-adrenoceptors the principle may be general. Images from this study can be found at the web sites Mackenzie *et al*, 1998b and 1999.

McGrath JC, Arribas SM & Daly CJ (1996). *T.i.P.S;* 17 (11):393-399.

Daly CJ, Milligan CM, Milligan G, Mackenzie JF & McGrath JC (1998). *J. Pharmacol. Exp. Ther.* 286; 984-990.

Mackenzie JF, Daly CJ & McGrath JC (1998a). *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 358; suppl. 2, p6.45.

Mackenzie JF, Daly CJ, Luo D & McGrath JC (1998b). *On-line Proceedings of the 5th Internet World Congress on Biomedical Sciences '98 at McMaster University, Canada* (available from URL: http://www.mcmaster.ca/inabis98/cvdisease/mackenzie0899/index.html

Mackenzie JF, Daly CJ & McGrath JC (1999). Laboratory web site

---

134P AN ANTISENSE APPROACH TO THE PHARMACOLOGY OF THE $\alpha_{2A/D}$-ADRENOCEPTOR IN THE RAT

A.L. Hudson, E.S.J. Robinson, M.D. Lalies & D.J. Nutt
Psychopharmacology Unit, University of Bristol, Bristol BS8 1TD

The α_2-adrenoceptors are known to mediate a diverse range of functions in the CNS by affecting monoamine levels in key brain areas (Nutt *et al.*, 1997). For example, α_2-adrenoceptors play a role in mood, nociception and cognition (French, 1995). Although pharmacological and molecular biological studies have identified four distinct subtypes of α_2-adrenoceptor ($\alpha_{2A/D}$, α_{2B} and α_{2C}), their individual functional roles have still to be fully elucidated (MacKinnon *et al.*, 1994). Currently, there are no highly selective antagonists with which to characterise the functions of the α_2-adrenoceptor subtypes *in vivo*. However, recent developments in techniques to inhibit gene expression, such as antisense oligonucleotides, have enabled the study of such receptor subtypes.

We have characterised an antisense oligonucleotide sequence (Nunes, 1995) to the $\alpha_{2A/D}$-adrenoceptor using quantitative receptor autoradiography and physiological and behavioural parameters. The antisense was administered by unilateral i.c.v. infusion and the distribution determined using a fluorescent uptake study (Robinson *et al.*, 1999, this meeting). α_2-Adrenoceptor density was analysed at the end of the three day infusion and behavioural and physiological parameters were monitored during and after the antisense infusion.

These experiments have shown antisense to $\alpha_{2A/D}$-adrenoceptors specifically reduce binding to α_2-adrenoceptors in discrete brain areas (Table 1). Specific changes in both behavioural and physiological responses were also observed, which included the attenuation of the hypothermic response to UK 14,304 and behavioural activation. The increase in activity was due to increased locomotion and grooming which have previously been shown with an α_2-adrenoceptor antagonist. These data have successfully shown the application of antisense technology to investigating the $\alpha_{2A/D}$-adrenoceptor.

Using the antisense sequence described, the role of the $\alpha_{2A/D}$-adrenoceptor subtype can be further investigated, including determining the contribution of $\alpha_{2A/D}$-adrenoceptors in autoreceptor function. Identification of α_2-adrenoceptor subtype functions may also separate some of the therapeutic effects and side-effects associated with α_2-adrenoceptor occupation.

French (1995) *Pharmac. Ther.*, 68, 175-208
Mackinnon *et al.* (1994) *T.i.P.S.*, 15, 119-123
Nunes (1995) *Eur. J. Pharmacol.*, 278, 183-185
Nutt *et al.* (1997) *J. Psychopharm.*, 11, 163-168

	Left (infusion side)			Right (non-infusion side)		
Brain Region	**Vehicle**	**Antisense**	**Mismatch**	**Vehicle**	**Antisense**	**Mismatch**
Lateral septal nuc.	93.8±10.6	71.2±4.1*††	106.2±12.6	96.6±14.0	78.4±6.7	102.0±13.7
Ant. hypothalamic area	41.2±4.3	28.5±3.8*††	42.2±53.3	44.1±2.8	31.1±3.7*†	46.0±3.9

Table 1: Quantified data for specific [^{3}H]RX821002 binding (1 nM) to rat brain sections following antisense treatment. Results shown as means s.e.means (fmol mg^{-1} wet tissue), 5-6 rats per group, *P<0.05 antisense compared to vehicle, †P<0.05, ††P<0.01 antisense compared to mismatch.

135P PREJUNCTIONAL EFFECTS OF ANGIOTENSIN II AND BRADYKININ IN THE HEART AND BLOOD VESSELS

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

Bradykinin increases noradrenaline release evoked by electrical stimulation of the sympathetic nerves of the heart. It was postulated that bradykinin might act through some endocardium-dependent factor because the facilitation induced by bradykinin is reduced by removal of the endocardium (Vaz-da-Silva *et al.*, 1996).

The hypothesis that angiotensin II mediates the facilitatory effect of bradykinin on noradrenaline release from the rat left ventricle was tested. The influence of angiotensin II antagonists (AT1 selective, losartan; AT2 selective, PD 123319; and non-selective, saralasin) on the effect of angiotensin II and bradykinin was compared in slices of the rat left ventricle preloaded with ^{3}H-noradrenaline. Both angiotensin II and bradykinin concentration-dependently increased tritium overflow evoked by electrical stimulation [EC_{50}=12.0 nM (3.5; 41.1) and EC_{50}=3.5 nM (1.2; 10.2); maximum increases amounting to 205±24% and 208±18% (n=5), respectively]. Neither losartan (3 - 100 nM) nor PD 123319 (30 - 100 nM) changed the facilitatory effect of either angiotensin II or bradykinin, while saralasin (1 - 100 nM) antagonized the effect of both drugs. This indicates that prejunctional effects of bradykinin at the rat ventricle are exerted through angiotensin II.

These results also suggest that the prejunctional receptor for angiotensin II in the rat ventricle is different from the well-established AT1 type. These findings agree with previous results aimed at comparing pre- and post-junctional receptors involved in the responses of blood vessels to angiotensin II (Guimarães *et al.*, 1998).

In the mesenteric artery and pulmonary arteries of the dog, angiotensin II enhances tritium overflow evoked by electrical stimulation (EC30%=5 nM and 1.54 nM, respectively) and causes concentration-dependent contractions (pD2=8.57 and 8.52, respectively). Saralasin antagonizes both pre- and post-junctional effects of angiotensin II, but it is more potent at post- than at pre-junctional level (in the mesenteric artery: pA2 of 9.51 and 8.15, respectively; in the pulmonary artery: pA2 of 9.58 and 8.10), while losartan antagonized exclusively the postjunctional effects of angiotensin II (pA2=8.15 and 7.96, in the mesenteric and pulmonary artery, respectively). PD123319 had no antagonist effect either pre- or post-junctionally. Also *in vivo* (in pithed rats), sub-pressor doses of angiotensin II which shifted to the left the frequency-response curves for increases in blood pressure due to spinal cord stimulation (indicating a facilitatory effect on sympathetic transmitter outflow) were not antagonized by losartan (Ohlstein *et al.*, 1997).

It is concluded that postjunctional receptors in blood vessels belong to AT1 subtype. However, prejunctional receptors that belong to neither AT1 nor AT2 subtype are present in the heart and blood vessels.

Guimarães, S., Paiva, M.Q. & Moura, D. (1998). *Br. J. Pharmacol.* 124, 1207-1212

Ohlstein, E.H., Brooks, D.P., Feuerstein, G.Z. & Ruffolo, R.R. (1997). *Pharmacology* 55, 224-251.

Vaz-da-Silva, M., Magina, S., Domingues-Costa, A. *et al.* (1996). *Br. J. Pharmacol.* 118, 364-368

136P THE ROLE OF CERTAIN AMINO ACIDS FOR THE FUNCTION OF THE DESIPRAMINE-SENSITIVE NORADRENALINE TRANSPORTER

H. Bönisch, F. Runkel, M. Brüss, C. Roubert* & B. Giros* Institute of Pharmacology & Toxicology, University of Bonn, Germany and* INSERM U-288, Neuropsychopharmacology, Paris, France

Released noradrenaline (NA) is rapidly re-moved from the synaptic cleft by reuptake into noradrenergic nerve terminals via the desipramine-sensitive NA transporter (NAT). The human NAT (hNAT) belongs to the family of Na^+- and Cl-dependent neurotransmitter transporters characterized by 12 transmembrane domains (TMs). The hNAT shows high homology to the transporters for g-aminobutyric acid (GAT), dopamine (DAT) and serotonin (SERT). From studies at artificial mutants or chimeras, something is known about the relationship between the primary structure and the function of GAT, DAT and SERT, but little is known in this respect about the NAT.

To define the role of certain amino acids and peptide domains for the function of the hNAT, a series of hNAT variants were produced by site-directed mutagenesis, and their functions were studied in transfected COS7 or HEK293 cells by comparing NA (or dopamine, DA) uptake and affinity for desipramine (DMI) as well as plasma membrane expression with corresponding properties of the wild-type hNAT.

None of the naturally occurring hNAT variants (Stöber *et al.*, 1996) showed a change in the affinity for desipramine and only the G478S variant exhibited a sigificantly reduced NA transport activity due to an approximately 3-fold increase in the Km. A naturally occurring C-terminal splice variant with a shorter and changed amino acid sequence (Pörzgen *et al.*, 1998) was not expressed in the plasma membrane and thus showed no NA transport. Both of two artificial deletion mutants lacking either the C- or the N-terminal region of the protein showed strongly reduced NA transport and DMI binding, which was also due to reduced plasma membrane expression.

All 6 artificial hNAT variants (D75A, W80S, R81H, E113D, S354A, SS354,357AA), in which amino acids were exchanged known to be functionally important as potential Na+, Cl- or monoamine binding site in homologous positions in GAT, SERT or DAT, showed strongly reduced (S354A) or no (all others) NA uptake and reduced DMI binding. From 18 variants in which amino acids between TM5 and TM8 (a region known to be critical for DMI binding) were replaced by their counterparts in the DMI-insensitive hDAT, a double mutation in TM8 (SG397PL) displayed a 3000-fold reduction in DMI binding affinity without a loss of monoamine transport activity, indicating that these two amino acids may be involved in tricyclic antidepressant binding.

Amino acids and peptide domains of the hNAT were identified which are important for plasma membrane expression or are potential binding sites for substrates, co-substrates or inhibitors of the hNAT.

Stöber *et al.*, *Am J Med Genet* 67:523, 1996

Pörzgen *et al.*, Biochim Biophys Acta 1398:365, 1998

137P ARE β1- AND β2-ADRENOCEPTOR-MEDIATED FUNCTIONAL RESPONSES DIFFERENTIALLY DESENSITIZED? A ROLE FOR PHOSPHODIESTERASE?

K.J. BroadleyDivision of Pharmacology, Welsh School of Pharmacy, Cardiff University, Cathays Park, Cardiff CF1 3XF, UK

Prolonged or repeated exposure of humans, animals, cells and tissues to β-adrenoceptor (βAR) agonists results in the well known phenomenon of desensitization, whereby a subsequent agonist challenge produces an attenuated response (Harden, 1983). There is, however, remarkably little agreement whether β_1- and β_2-adrenoceptors undergo desensitization equally. For example, preferential down-regulation of β1AR was reported in patients with dilated cardiomyopathy (Michel *et al.*, 1991). Selective loss of vascular (Cohen & Schenck, 1987) and cardiac (Martin & Broadley, 1994) β_1AR function compared with vascular β_2AR has been reported after chronic isoprenaline infusions. In contrast, there are reports of loss of β_2AR sensitivity and numbers equal to or greater than for β_1AR, following isoprenaline infusions (Molenaar *et al.*, 1990).

In the airways, the situation is also confusing. It has long been believed that tolerance develops to the bronchodilator effects of β-agonists, and abundant studies have shown desensitization of βAR-mediated responses of isolated trachea after prolonged incubation with isoprenaline. The bronchodilator responses to β_2AR agonists are, however, now regarded as being relatively resistant to desensitization (Barnes, 1995; Giembycz, 1996). In contrast, the non-smooth muscle β_2AR responses, including antiinflammatory actions, do appear to undergo desensitization.

Examples of *in vitro* desensitization of β_1AR-mediated functional responses will be illustrated. Selective desensitization of cardiac β_1AR-mediated responses compared with vascular and airways β_2AR-mediated responses after prolonged (4-6h) *in vitro* incubation with isoprenaline (10-6M) will be demonstrated. Factors such as correction from time-matched controls, adequate washout of the isoprenaline and the methods of plotting dose-response curves are taken into account.

Notwithstanding the observed selectivity of β_2AR desensitization and discrepancies in the literature, there is ample evidence that β_2AR are capable of desensitization and uncoupling from G-protein when assessed from adenylyl cyclase activation (Summers *et al.*, 1997).

An early study showed that human β_2AR expressed in CHW cells desensitized after only 10min incubation with isoprenaline (2 μM). The maximum shift of the concentration-response curve for isoprenaline-stimulated adenylyl cyclase was twofold (Hausdorff *et al.*, 1989). Thus, a selective desensitization at the receptor level is unlikely and alternative explanations for the apparent differences in susceptibility to desensitization of βAR-mediated responses is required. Possible reasons include differences in receptor reserve between tissues and variable rates of resensitization during washout of the agonist. Thirdly, upregulation of phosphodiesterase (PDE) during agonist exposure has been suggested to occur during agonist exposure (Giembycz, 1996). The enhanced degradation of second messenger cAMP could reduce sensitivity and if this varied between tissues, could explain different degrees of desensitization.

Results showing that inhibition of PDE by IBMX or rolipram (PDE4-selective) does not interfere with *in vitro* βAR desensitization will be presented.

Barnes PJ (1995) *Am Rev Respir Dis* 152, 838-860.

Cohen ML & Schenck KW (1987) *J Cardiovasc Pharmacol* 10, 365-368.

Giembycz MA (1996) *Trends Pharmacol Sci,* 17, 331-336.

Harden TK (1983) *Pharmacol Rev,* 35, 5-32.

Martin SW & Broadley KJ (1994) Br J Pharmacol, 112, 595-603.

Molenaar P *et al.* (1990) *J Pharmacol Exp Ther*, 255, 393-400.

Hausdorff WP *et al.* (1989) *J Biol Chem,* 264, 12657-12665.

Michel MC, Maisel AS & Brodde O-E (1990) *Br J Clin Pharmacol*, 30, 37S-42S.

Summers RJ, Kompa A & Roberts SJ (1997) *J Auton Pharmacol,* 17, 331-343.

138P ADENOSINE MODULATES PHENOMENA OF SYNAPTIC PLASTICITY IN THE HIPPOCAMPUS OF OLD RATS

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

Adenosine, a neuromodulator with an important role in synaptic transmission and neuronal excitability, acts through adenosine A_1 receptors to modify phenomena of synaptic plasticity, like paired-pulse facilitation (PPF), long-term potentiation (LTP), long-term depression (LTD) and depotentiation (DP) in the hippocampus of young rats (de Mendonça & Ribeiro, 1997). In the present work we investigated whether the neuromodulatory role of adenosine on phenomena of synaptic plasticity would also be present in the hippocampus of old rats.

The experiments were performed *in vitro* on hippocampal slices taken from young adult (5-6 weeks old) and aged (2 years old) male Wistar rats. Two separate sets of the Schaffer pathway were alternately stimulated and the field excitatory postsynaptic potentials (fEPSP) extracellularly recorded in the *stratum radiatum* of CA1 area. To elicit PPF, one set of the Schaffer pathway was stimulated twice with a 50 ms interpulse interval. The synaptic facilitation was quantified as the ratio between the slopes of the second and the first stimulus (S2/S1). To obtain LTD, low frequency stimulation (1 Hz, during 15 min) was applied to the pathway. LTP was induced either by a high frequency stimulation pattern (2 trains of 100 Hz, 100 stimuli, separated by 30 s) or by a θ-burst stimulation pattern (3 trains of 100 Hz, 3 stimuli, separated by 200 ms). To obtain DP, LTP was first induced by high frequency stimulation, and after 1 h DP was elicited by low frequency stimulation. LTD, LTP and DP were quantified as the % change in the average slope of the potentials taken 50-60 min after the induction protocol, in relation to the average slope of the fEPSP considered during the 10 min that preceded the protocol.

The adenosine A_1 selective receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (50 nM), attenuated PPF in young adult rats, ratios S2/S1=1.64±0.05% (n=6) being obtained in the presence of DPCPX (50 nM) and S2/S1=1.76±0.05% (n=6, P<0.05) in the control solution, as well as in old rats, in which ratios S2/S1=1.33±0.05% were observed in the presence of DPCPX (50 nM, n=6) and S2/S1=1.55±0.10% in the control solution (n=6, P<0.05). A larger DP was observed in the presence of DPCPX (50 nM), 27.6±4.4% (n=7), than in the control pathway, 16.8±4.7% (n=7, P<0.05) in young adult rats, as well as in old rats, in which DP was 41.3±5.1% (n=6) in the presence of DPCPX (50 nM) and 16.1±2.7% (n=6, P<0.05) in its absence. High frequency stimulation-induced LTP was not different in the control solution and in DPCPX (50 nM), either in young or old rats. θ-burst stimulation induced a very small LTP (6.4±1.1%, n=5) in control conditions, and a marked LTP 53.9±4.9% (n=5, P<0.05) in the presence of DPCPX (50 nM), in young rats. In contrast, in aged rats θ-burst stimulation was sufficient to elicit a marked LTP in control conditions, 81.8±17.9% (n=7), which was not significantly further enhanced in the presence of DPCPX (50 nM), a value of 98.5±24.2% (n=7) being obtained. Endogenous adenosine thus modulates phenomena of synaptic plasticity in the hippocampus of aged rats.

de Mendonça A. & Ribeiro J.A. (1997) *Life Sci.*, 60, 245-251.