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Comparison of the interaction of agmatine and crude methanolic extracts of bovine lung and brain with α_2-adrenoceptor binding sites

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1 In the present study we have evaluated whether α_2-adrenoceptor binding sites on bovine cerebral cortex membranes labelled by [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 can distinguish between known agonists and antagonists. This model has then been used to compare the binding profiles of the putative non-catecholamine, clonidine-displacing substance (CDS), agmatine and crude methanolic extracts of bovine lung and brain.

2 Saturation studies carried out in the presence and absence of noradrenaline, 10 μmol l^{-1}, revealed that the maximum number of binding sites on bovine cerebral cortex membranes for [^{3}H]-idazoxan and [^{3}H]-RX-821002 were approximately 60-80% greater than those for [^{3}H]-clonidine (62.6 fmol mg^{-1} protein). Rauwolscine, the selective α_2-adrenoceptor antagonist, was approximately 100 fold more potent against each of the ligands than the selective α_1-adrenoceptor diastereoisomer, corynanthine. Also, the pK_i value for the selective α_1-adrenoceptor prazosin against each ligand was less than 6.

3 Adrenaline, UK-14034, rauwolscine, corynanthine, RX-811059 and prazosin produced concentration-dependent inhibition of binding of all three ^{3}H-ligands. The agonists, adrenaline and UK-14304, were approximately 5 and 10 fold less potent against [^{3}H]-idazoxan and [^{3}H]-RX-821002, respectively, than against [^{3}H]-clonidine. In marked contrast, the antagonists, rauwolscine, corynanthine, RX-811059 and prazosin exhibited a different profile, being approximately 2-3 fold more potent against sites labelled by [^{3}H]-RX-821002 and [^{3}H]-idazoxan compared to sites labelled by [^{3}H]-clonidine.

4 Agmatine and histamine produced a concentration-dependent displacement of [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 binding to bovine cerebral cortex membranes. The pK_i values for agmatine and histamine were independent of the ^{3}H-ligand employed, approximately 4.8 and 4.5, respectively.

5 Crude methanolic extracts of bovine brain and lung produced a concentration-dependent inhibition of [^{3}H]-clonidine binding to bovine cerebral cortex membranes (>90%). Based on the volume of the extract that caused 50% inhibition of [^{3}H]-clonidine binding, bovine lung contains 3 fold more CDS than bovine brain. Both extracts were at least 5 fold more potent against α_2-adrenoceptor sites labelled by [^{3}H]-clonidine than those labelled by [^{3}H]-idazoxan and [^{3}H]-RX-821002.

6 All three ^{3}H-ligands label the same population of α_2-adrenoceptor binding sites on bovine cerebral cortex membranes, but [^{3}H]-clonidine appears to label selectively the 'agonist' state of the sites: for which known agonists, adrenaline and UK-14304, exhibit a higher affinity. Our results indicate that neither agmatine nor histamine can account for the CDS activity present in crude extracts of bovine brain and lung. Moreover, these extracts appear to possess a binding profile similar to that of adrenaline and UK-14304, suggesting that they may possess agonist activity.

Keywords: α_2-Adrenoceptors; agmatine; clonidine-displacing substances; [^{3}H]-clonidine; [^{3}H]-idazoxan

Introduction

Atlas & Burstein (1984a,b) coined the term 'clonidine-displacing substance' (CDS) to describe the ability of a non-catecholamine substance(s), contained in a partially purified extract of rat and bovine brain, to displace [^{3}H]-clonidine from α_2-adrenoceptor binding sites on bovine cerebral cortex membranes. In subsequent experiment these workers reported that the extract was able to recognize non-adrenoceptor, imidazoline binding sites labelled by [^{3}H]-idazoxan, and activate α_2-adrenoceptors in human platelets and the rat vas deferens to produce a pro-aggregatory response and to inhibit neurogenic contractions, respectively (Diamant & Atlas, 1986; Diamant *et al.*, 1987; Parini *et al.*, 1989). The ability of a similarly prepared, crude brain extract to recognize α_2-adrenoceptor and non-adrenoceptor, imidazoline binding sites labelled by [^{3}H]-*para*-aminoclonidine has also been described by Ernsberger *et al.* (1988), although no data were presented to show that this extract could activate α_2-adrenoceptors. We have recently described the presence of a non-catecholamine activity in crude extracts of bovine brain and lung, capable of selective interaction with both α_2-adrenoceptor and non-adrenoceptor, imidazoline binding sites (Singh *et al.*, 1995). However, it was not possible to examine whether these extracts were able to activate α_2-adrenoceptors because of the presence of many impurities (eg., catecholamines, histamine and monovalent cations).

Li *et al.* (1994) recently proposed that agmatine, which could be extracted from bovine brain and which is able to displace [^{3}H]-clonidine from α_2-adrenoceptor binding sites, may represent a CDS. We have confirmed that agmatine can recognize α_2-adrenoceptor binding sites, but in functional studies it failed to activate or inhibit prejunctional α_2-adrenoceptors in the rat vas deferens (Pinthong *et al.*, 1995). Thus, agmatine cannot account for the CDS activity originally described by Atlas & Burnstein (1984a). Furthermore, since agmatine also failed to exhibit biological activity, even in preparations in which it recognized the associated binding site (in the rat cerebral cortex), this raises serious questions about the appropriateness of radioligand binding assay as the principal detection system for 'CDS'.

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Whilst the ability of an agent to activate a receptor cannot be gleaned from radioligand binding studies involving a single radioligand, the potency of agonists and antagonists at α_2-adrenoceptor binding sites are known to be differentially affected by the affinity state of the site. For example, α_2-adrenoceptor agonists possess a higher affinity for $\alpha_{2A/D}$-sites labelled by ^{3}H-agonists (e.g., [^{3}H]-adrenaline or [^{3}H]-UK-14304) compared to the same sites labelled by ^{3}H-antagonists (e.g., [^{3}H]-rauwolscine). Conversely, antagonists appear to possess a higher affinity for α_2-adrenoceptors labelled by ^{3}H-antagonists compared to the corresponding site labelled by an ^{3}H-agonist (Gleason & Hieble, 1991; MacKinnon *et al.*, 1993; see also Wilson *et al.*, 1991). Examples of preparations possessing α_2-adrenoceptors that exhibit this type of behaviour include human platelets (Garcia-Seville & Fuster, 1986; MacKinnon *et al.*, 1993). HT-29 cells (Turner *et al.*, 1985; Bylund *et al.*, 1988; Gleason & Hieble, 1991) and rat cerebral cortex membranes (Wallace *et al.*, 1994): preparations that belong to the $\alpha_{2A/D}$ subtypes.

The aims of the present study were two fold. First, to assess whether α_2-adrenoceptor binding sites on the bovine cerebral cortex membranes, when labelled by [^{3}H]-clonidine (a selective agonist U'Prichard & Synder, 1980), [^{3}H]-RX-821002 and [^{3}H]-idazoxan (selective antagonists; Brown *et al.*, 1990; Mallard *et al.*, 1992), can distinguish between known agonists and antagonists. Secondly, to determine whether the putative, non-catecholamine endogenous ligands for α_2-adrenoceptors, agmatine (Li *et al.*, 1994) and bovine brain and lung 'CDS' (Singh *et al.*, 1995), exhibit 'agonist-like' or 'antagonist-like' characteristics in this system.

Methods

Preparation of bovine brain and lung methanolic CDS

Bovine brain and lung were obtained from a local abattoir immediately after slaughter of the animal; 100 g wet weight of brain (minus cerebellum) and 80–100 g wet weight of lung were finely chopped and placed in 10 volumes (w/v) of boiling distilled water. Both tissues were homogenized in an OMNI-GEN sealed homogenizer (Setting 5 for 3 × 3 min) and the resulting homogenized material centrifuged at 65,000 *g* for 30 min at 4°C (MSE Superspeed 65). The supernatant was then removed, boiled for approximately 15 min to precipitate soluble protein and then allowed to cool to room temperature. The resulting solution was centrifuged at 65,000 *g* for 30 min at 4°C and the supernatant removed, frozen at −20°C and then freeze-dried. The lyophysylate was then extracted by sonication (5 min) with 2 × 20 volumes (w/v) of Analar grade methanol at room temperature. The methanolic extracts were combined and centrifuged at 4,000 r.p.m. for 5 min (MSE Mistral 3000) to remove any particulate matter and then evaporated to dryness at low pressure. The residual material was dissolved in 10 volumes (w/v) of twice-distilled water and stored at −20°C until required for use.

Membrane preparation

Bovine brains were obtained from the local abattoir immediately after the slaughter of the animal and the cerebral cortices homogenized in 20 volumes of ice cold Tris buffer (50 mmol l^{-1} Tris HCl; pH 7.7 at 25°C) with an OMNI-GEN sealed macro-homogenizer (setting 5; 120 s), to minimize potential health risks associated with aerosol formation. The homogenate was then centrifuged at 20,000 r.p.m. for 10 min at 4°C (MSE Europa 24M). The pellet was resuspended in 20 volumes (w/v) of Tris buffer and re-centrifuged. The final pellet was resuspended in 4.9 volumes (w/v) of 50 mmol l^{-1} Tris buffer for direct use in the binding assay or stored at −20°C. Resuspension of the centrifuged pellet was achieved with an Ultra-turrax homogenizer sited in a laminar airflow hood.

Binding assays

Bovine cerebral cortex membranes (200–300 μg protein) were incubated with increasing concentrations of the three radioligands in the presence and absence of noradrenaline (10 μmol l^{-1}). In another series of experiments the cortical membranes were incubated with either 0.5 nmol l^{-1} [^{3}H]-clonidine, 1 nmol l^{-1} [^{3}H]-idazoxan or 0.2 nmol l^{-1} [^{3}H]-RX-821002

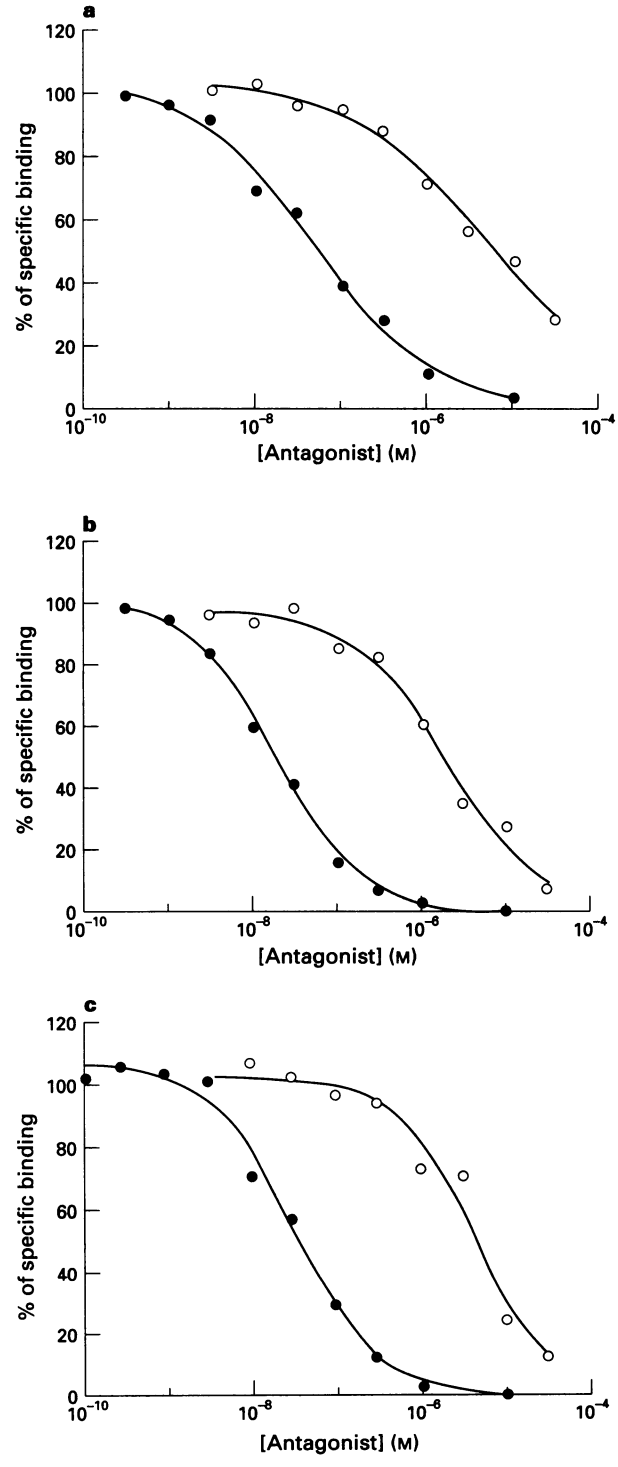

Figure 1 The effect of rauwolscine (●) and corynanthine (○) against the specific binding of (a) 0.5 nmol l^{-1} [^{3}H]-clonidine, (b) 1 nmol l^{-1} [^{3}H]-idazoxan and (c) 0.2 nmol l^{-1} [^{3}H]-RX-821002 to bovine cerebral cortex membranes. Non-specific binding was determined by effect of 10 μmol l^{-1} noradrenaline. The results shown are from duplicate determinations in a single experiment which was repeated on 2 further occasions with the same results.

in the presence and absence of various concentrations of known displacing agents in a final volume of 0.5 ml assay buffer (50 mM Tris HCl; pH 7.4 at 25°C). In addition, the effects of the crude methanolic extracts of bovine brain and bovine lung were also examined against the three selective α_2-adrenoceptor ligands. Non-specific binding was determined in the presence of noradrenaline (10 μmol l^{-1}) and ranged between 5% ([^{3}H]-RX821002) to 15% ([^{3}H]-clonidine) of the total binding. After an incubation period of 60 min at 25°C, bound radioactivity was separated from free by vacuum filtration over Whatman GF/B glass fibre filters using a Brandel cell harvester followed by 2 x 3 ml washes with ice-cold assay buffer. In all experiments the filters were suspended in 4 ml of scintillation cocktail and bound ligand determined by scintillation spectrometry.

Data analysis

Saturation data were analysed with the computer programme InPlot (Graphpad, California, U.S.A.) using a non-linear equation for a rectangular hyperbolic curve. The logarithm of the concentration of either the brain extract, the lung extracts, or the displacing agents, producing 50% inhibition of the radioligand binding (pIC_{50}) was determined by the non-linear least squares method described by DeLean *et al.* (1978) using Kaleidagraph (Synergy Software) on a MacIntosh computer. One unit of CDS is the volume of extract that produces 50% inhibition of 0.5 nmol l^{-1} [^{3}H]-clonidine binding to bovine cerebral cortex membranes in a 1 ml assay (see Atlas & Burstein, 1984a, b), and this was used to calculate the units per wet weight of tissue for each organ. With the exception of the extracts, apparent inhibition constants (K_i) were calculated from the radioligand binding data using the Cheng-Prusoff transformation (Cheng & Prusoff, 1973) and the mean Hill slope (n_H) of the displacement of either [^{3}H]-idazoxan or [^{3}H]-RX-821002 compared with that observed against [^{3}H]-clonidine using Student's unpaired t test. The difference was considered statistically significant if $P<0.05$. All values are shown as the mean ± s.e.mean of n observations.

Drugs

The following drugs and radioligands were used: [^{3}H]-clonidine (2.5 TBq mmol^{-1}, Amersham, UK); [^{3}H] - RX - 821002 (2-(2-methoxy - 1,4 - benzodioxan - 2 - yl) - 2 - imidazoline) 2.11 TBq mmol^{-1}, Amersham, UK); [^{3}H]-idazoxan (1.59 TBq mmol^{-1}, Amersham, UK); (-)-adrenaline bitartrate (Sigma), (-)-noradrenaline bitartrate (Sigma); histamine (Sigma); prazosin hydrochloride (Pfizer), rauwolscine HCl (Roth); UK-14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate, Pfizer), RX-811059 (2-(2-ethoxy-1,4-benzodioxan-2-yl)-2-imidazoline, Reckitt and Coleman), agmatine sulphate (Sigma).

Results

From saturation studies carried out in the presence and absence of noradrenaline (10 μmol l^{-1}) the maximum number of binding sites on bovine cerebral cortex membranes for [^{3}H]-idazoxan (112.3 ± 3.9 fmol mg^{-1} protein, $n=3$) and [^{3}H]-RX-821002 (103.6 ± 9.2 fmol mg^{-1} protein, $n=3$) were significantly greater than that for [^{3}H]-clonidine (62.6 ± 10.7 fmol mg^{-1} protein, $n=3$). Based on the dissociation constant, the rank order of affinity (K_d) for the ligands was [^{3}H]-RX-821002 (0.28 ± 0.07 nM, $n=3$) > [^{3}H]-idazoxan (0.88 ± 0.14 nM, $n=3$) > [^{3}H]-clonidine (1.32 ± 0.49 nM, $n=3$). At these concentrations the non-specific binding varied from 5% ([^{3}H]-RX-821002) to 15% ([^{3}H-clonidine]). As shown in Figure 1 and Table 1 the selective α_2-adrenoceptor antagonist rauwolscine was approximately 100 fold more potent than the selective α_1-adrenoceptor diastereoisomer, corynanthine, against each of the ligands. Also, the pK_i values for the selective α_1-adrenoceptor antagonist, prazosin, against [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002, were less than 6. Taken together, these observations suggest that these [^{3}H]-ligands label prazosin-insensitive, α_2-adrenoceptor binding sites on bovine cerebral cortex membranes.

Table 1 shows the interaction between known agonists

Table 1 pK_i and Hill Slope (n_H) values for various agents against [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 binding to bovine cerebral cortex membranes

	[^{3}H]-Clonidine		*[^{3}H]-Idazoxan*		*[^{3}H]-RX-821002*	
	pK_i RP	n_H	pK_i RP	n_H	pK_i RP	n_H
Adrenaline	8.30 ± 0.13 1	-0.69 ± 0.06	7.53 ± 0.17 0.17	-0.47 ± 0.04*	7.16 ± 0.18 0.07	-0.49 ± 0.02
UK-14304	8.56 ± 0.04 1	-0.89 ± 0.05	7.93 ± 0.08 0.23	-0.63 ± 0.02*	7.51 ± 0.01 0.09	-0.55 ± 0.03**
Rauwolscine	7.37 ± 0.05 1	-0.70 ± 0.03	7.81 ± 0.01 2.75	-0.95 ± 0.04**	7.57 ± 0.04 1.58	-0.94 ± 0.07*
Corynanthine	5.16 ± 0.07 1	-0.52 ± 0.03	5.74 ± 0.03 3.8	-0.81 ± 0.03**	5.32 ± 0.06 1.44	-0.80 ± 0.05*
RX-811059	8.73 ± 0.11 1	-0.80 ± 0.06	9.26 ± 0.03 3.36	-1.02 ± 0.07	9.04 ± 0.02 2.04	-1.00 ± 0.09
Prazosin ($n=2$)	5.25 1	-0.95	5.81 3.6	-0.95	5.62 2.30	-1.09
Agmatine	4.77 ± 0.38 1	-0.82 ± 0.05	4.95 ± 0.17 1.51	-0.84 ± 0.06	4.70 ± 0.19 0.85	-0.90 ± 0.05
Histamine	4.61 ± 0.19 1	-0.84 ± 0.09	4.64 ± 0.09 1.07	-0.88 ± 0.07	4.41 ± 0.10 0.63	-0.75 ± 0.08

Except where indicated the values shown represent the mean ± s.e. mean of 3-5 seperate experiments carried out in duplicate. RP is the potency of the displacing agent against each ligand relative to that observed against [^{3}H]-clonidine. *$P<0.05$ and **$P<0.01$ denote that the Hill slope (n_H) for the displacement of either [^{3}H]-idazoxan or [^{3}H]-RX-821002 from bovine cerebral cortex membranes is significantly different (unpaired Student's t test) from that observed for the ligand against [^{3}H]-clonidine.

and antagonists against [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 binding to bovine cerebral cortex membranes. Adrenaline, UK-14304, rauwolscine, corynanthine, RX-811059 and prazosin produced concentration-dependent inhibition of binding of all three ^{3}H-ligands. Adrenaline and UK-14304 were approximately 5 and 10 fold less potent against [^{3}H]-idazoxan and [^{3}H]-RX-821002, respectively, than against [^{3}H]-clonidine. Also, the Hill slopes of the interaction were significantly lower against [^{3}H]-idazoxan and [^{3}H]-RX-821002 compared to that observed against [^{3}H]-clonidine (Table 1). In contrast, rauwolscine, corynanthine, RX-811059 and prazosin were approximately 2–3 fold more potent against [^{3}H]-RX-821002 and [^{3}H]-idazoxan binding compared to [^{3}H]-clonidine binding. In addition, the Hill slope was steeper against [^{3}H]-RX-821002 and [^{3}H]-idazoxan compared to that observed against [^{3}H]-clonidine; a difference which was statistically significant for both rauwolscine and corynanthine (Table 1). The qualitative difference between the interaction of an agonist (adrenaline) and an antagonist (RX-811059) with the three ^{3}H-ligands is highlighted in Figure 2.

Agmatine and histamine produced a concentration-dependent displacement of [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 binding to bovine cerebral cortex membranes. (Figure 3). As shown in Table 1, the pK_i values, and also the Hill Slope, for agmatine and histamine were independent of the ^{3}H-ligand employed.

The crude methanolic extract from 100 g wt of bovine brain and lung was reconstituted in 10 vol of distilled water. As shown in Figure 4, both extracts produced a concentration-dependent inhibition of [^{3}H]-clonidine binding to bovine cerebral cortex membranes (>90%), with a Hill slope close to unity (see Table 2). Based upon the volume of the extract that produced 50% inhibition of [^{3}H]-clonidine binding (1 unit of CDS, see Table 2), bovine lung contains 3 fold more CDS (17.4 ± 1.7 units g^{-1} wet wt, $n=4$ batches of the extracts) than bovine brain (5.4 ± 0.6 units g^{-1} wet wt, $n=4$ batches of the extract). In contrast to the effect observed against [^{3}H]-clonidine binding, 100 μl ml^{-1} of the lung extract produced only $71.3 \pm 3.0\%$ ($n=4$) and $38.7 \pm 5.2\%$ ($n=4$) inhibition of [^{3}H]-idazoxan and [^{3}H]-RX-821002 binding, respectively. Similarly, the bovine brain extract was not effective against [^{3}H]-idazoxan and [^{3}H]-RX-821002 binding, the inhibition produced by 100 μl ml^{-1} of the extract was $26.5 \pm 4.6\%$ ($n=4$) and $12.3 \pm 1.3\%$ ($n=4$), respectively (Figure 4). As shown in Table 2, both extracts were at least 5 fold more potent against sites labelled by [^{3}H]-clonidine compared to those labelled by [^{3}H]-idazoxan and [^{3}H]-RX-821002.

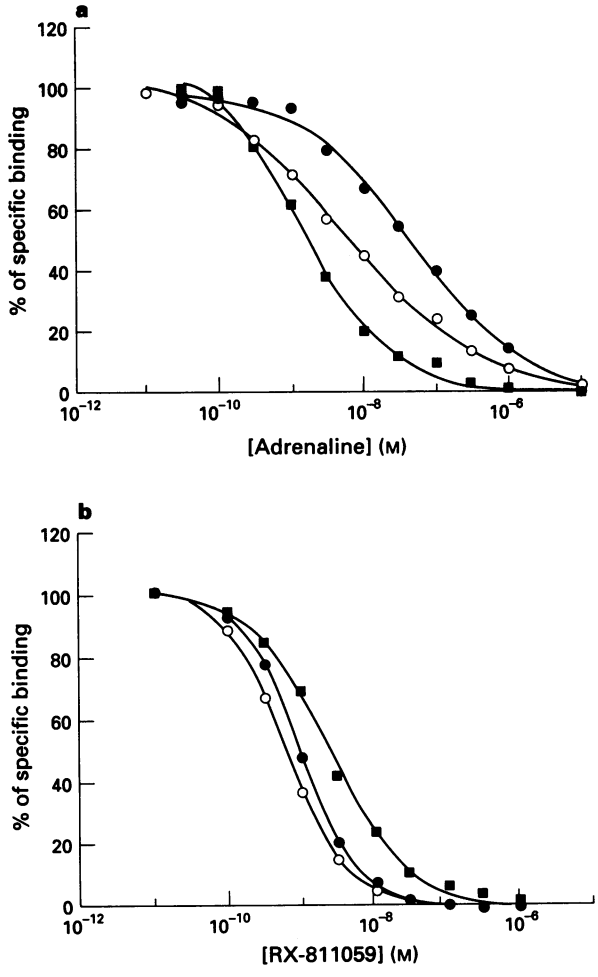

Figure 2 The effect of (a) adrenaline and (b) RX-810059 against the specific binding of 0.5 nmol l^{-1} [^{3}H]-clonidine (■), 1 nmol l^{-1} [^{3}H]-idazoxan (○) and 0.2 nmol l^{-1} [^{3}H]-RX-821002 (●) to bovine cerebral cortex membranes. Non-specific binding was determined by the effect of 10 μmol l^{-1} noradrenaline. The results shown are from duplicate determinations in a single experiment which was repeated on 2 (RX-810059) to 4 (adrenaline) further occasions with similar results.

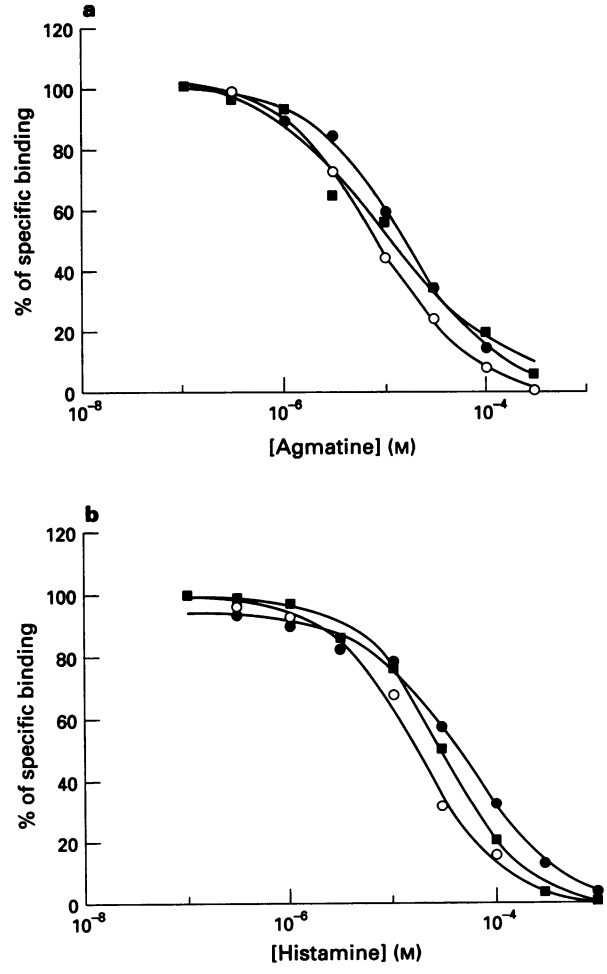

Figure 3 The effect of (a) agmatine and (b) histamine against the specific binding of 0.5 nmol l^{-1} [^{3}H]-clonidine (■), 1 nmol l^{-1} [^{3}H]-idazoxan (○) and 0.2 nmol l^{-1} [^{3}H]-RX-821002 (●) to bovine cerebral cortex membranes. Non-specific binding was determined by the effect of 10 μmol l^{-1} noradrenaline. The results shown are from duplicate determinations in a single experiment which was repeated on 2 (histamine) to 4 (agmatine) further occasions with similar results.

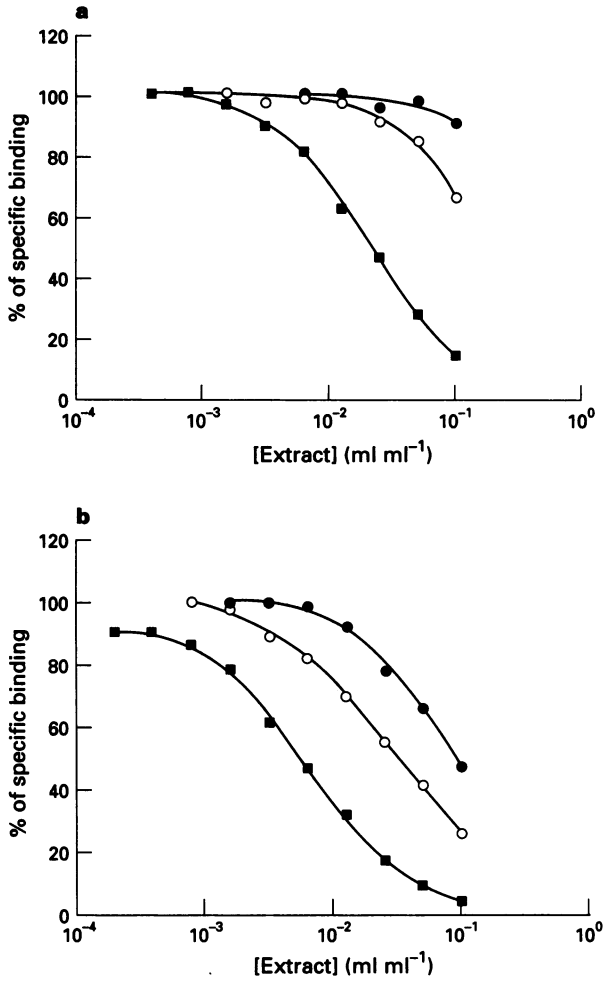

Figure 4 The effect of (a) crude methanolic extracts of bovine brain and (b) crude methanolic extracts of bovine lung against the specific binding of 0.5 nmol l^{-1} [^{3}H]-clonidine (■), 1 nmol l^{-1} [^{3}H]-idazoxan (○) and 0.2 nmol l^{-1} [^{3}H]-RX-821002 (●) to bovine cerebral cortex membranes. Non-specific binding was determined by the effect of 10 μmol l^{-1} noradrenaline. The results shown are from duplicate determinations in a single experiment with the one batch of the lung and brain extract. This was repeated on 3 further occasions, with 3 different batches of the extracts, with similar results. Assays on each batch of the extract were performed in duplicate on three separate occasions. The ordinate scale shows the logarithm of the concentration of the extract (ml) in a 1 ml assay volume.

Discussion

The pharmacological identity of the sites labelled by [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002

The working hypothesis underpinning the present study is that radioligand binding assays, involving the use of a ^{3}H-agonist and a ^{3}H-antagonist, can predict the pharmacological properties of recognized agonists and antagonists (Wilson *et al.*, 1991; MacKinnon *et al.*, 1993). For this to be possible, it is necessary to establish that the radioligands employed identify a pharmacologically homogeneous population of sites. Three observations suggest that [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 label α_2-adrenoceptor binding sites on bovine cerebral cortex membranes that belong to the $\alpha_{2A/D}$ subtype. First, 10 μM noradrenaline displaced 80% to 95% of the total binding of the three ligands, indicating that even for [^{3}H]-idazoxan, non-adrenoceptor, imidazoline binding sites make a minor contribution in this preparation. This contrasts with the rat cerebral cortex, for example, where [^{3}H]-idazoxan, also labels a sizable population of the non-adrenoceptor, imidazoline binding sites (Brown *et al.*, 1990; Hussain *et al.*, 1993; Wallace *et al.*, 1994). Secondly, the selective α_2-adrenoceptor, rauwolscine (Weitzell *et al.*, 1979) caused 100% displacement of the specific binding, and was 100 fold more potent than its diastereoisomer corynanthine, at the sites labelled by each ligand (Starke, 1981). Thirdly, the selective α_1-adrenoceptor antagonist, prazosin exhibited low potency ($pK_i < 6.3$) against each ligand; this makes it unlikely that the sites belong to the B/C (prazosin-sensitive) subgroups of α_2-adrenoceptors (Bylund *et al.*, 1994). Too few agents have been employed in the present study to distinguish conclusively between the A and D subtypes but, as suggested by MacKinnon *et al.* (1994) and Bylund *et al.* (1994), these may simply represent species homologues of the same subtype. If the latter view is correct, then two additional observations lend support to the presence of the D subtype on bovine cerebral cortex membranes. First, the pK_i for rauwolscine (7.57) against [^{3}H]-RX-821002 is consistent with the D subtype (Bylund *et al.*, 1994). Secondly, the α_{2D}-subtype and not the α_{2A}, has been reported on membranes of bovine pineal gland (Simmoneaux *et al.*, 1991).

In an earlier study we reported that the B_{max} for [^{3}H]-clonidine on bovine cerebral cortex membranes was significantly less than that for [^{3}H]-idazoxan, and that the former was more affected by the presence of guanine triphosphate (GTP) (Hussain *et al.*,1993). This, we argued, was consistent with the possibility that [^{3}H]-clonidine preferentially labelled the 'high' affinity state of the α_2-adrenoceptor binding site, while [^{3}H]-idazoxan, an apparently less efficacious partial agonist, was less able to distinguish between the 'low' and 'high' affinity states. Qualitatively similar observations have been made in

Table 2 The effect of crude methanolic extracts of bovine brain and bovine lung against [^{3}H]-clonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 binding to bovine cerebral cortex membranes

	[^{3}H]-clonidine (µl of extract-50% inhibition) RP	n_H	*[^{3}H]-idazoxan* (µl of extract-50% inhibition) RP	*[^{3}H]-RX-821002* (µl of extract-50% inhibition) RP
Brain extract	19.4 ± 2.3 1	-1.01 ± 0.11	NP <0.2	NP <0.2
Lung extract	6.2 ± 0.9 1	-1.08 ± 0.1	44.2 ± 7.6 <0.14	NP <0.06.

NP - not possible to determine a value because 100 µl ml^{-1} of the extract failed to cause more than 50% inhibition. RP - relative potency of the extract against each ligand compared to that observed against [^{3}H]-clonidine. Where the maximum concentration (100 µl ml^{-1}) of extract failed to produce 50% inhibition this value has been estimated. The values shown are the mean ± s.e. mean of a single experiment (performed in duplicate) with 4 different batches of the lung and brain extract.

the present study, where the B_{max} values for both [^{3}H]-idazoxan and [^{3}H]-RX-821002 were significantly greater than that obtained for [^{3}H]-clonidine. However, the B_{max} value for [^{3}H]-RX-82100 was not significantly different from that for [^{3}H]-idazoxan but, in a separate study, was unaffected by the addition of 300 μM GTP (unpublished observation). At present we are unable to reconcile, on the one hand, the ability of the GTP to reduce selectively [^{3}H]-idazoxan binding to bovine cerebral cortex membranes (Hussain *et al.*, 1993), with the similar B_{max} values for [^{3}H]-idazoxan and [^{3}H]-RX-821002 in this preparation (present study). However, for the purposes of the interpretation of the results in this study, we suggest that [^{3}H]-clonidine selectively labels the high affinity state of the population of $\alpha_{2(D)}$-adrenoceptors, while [^{3}H]-idazoxan and [^{3}H]-RX-821002, as antagonists, do not discriminate between the high and low affinity states of the binding site. It is noteworthy that qualitatively similar results with [^{125}I]-*p*-aminoclonidine, [^{3}H]-idazoxan and [^{3}H]-RX-821002 have been reported on the rat cerebral cortex membranes (Wallace *et al.*, 1994).

The value of the radioligand binding assays in predicting agonist or antagonist activity

The three radioligands used to label $\alpha_{2(D)}$-adrenoceptors on bovine cerebral cortex membranes were able to discriminate successfully between known agonists, and antagonists. Adrenaline and UK-14304, exhibited a 5–10 fold greater affinity for the sites labelled by [^{3}H]-clonidine than for those labelled by [^{3}H]-idazoxan and [^{3}H]-RX-821002. On the other hand, the selective α_2-adrenoceptor antagonists rauwolscine and RX-810059 (Mallard *et al.*, 1992) were 2–3 fold more potent against [^{3}H]-idazoxan and [^{3}H]-RX-821002 than against [^{3}H]-clonidine. The predictive value of this model is further underlined by the finding that a similar profile was observed even for antagonists with selectivity for α_1-adrenoceptors (prazosin and corynanthine). These observations are consistent with several earlier findings with platelets (MacKinnon *et al.*, 1993), HT-29 cells (Turner *et al.*, 1985; Bylund *et al.*, 1988; Gleason & Hieble, 1991) and rat cerebral cortex membranes (Wallace *et al.*, 1994), membranes that possess $\alpha_{2A/D}$-adrenoceptor binding sites (see also Wilson *et al.*, 1991). This suggests that the known preference of agonists for the high affinity state of the binding sites can be exploited to identify potential agonists.

The stimulus for the present investigations was our finding that agmatine, a putative clonidine-displacing substance (Li *et al.*, 1994), failed to display detectable biological activity at peripheral and central α_2-adrenoceptors, even though it was able to displace [^{3}H]-clonidine from α_2-adrenoceptor binding sites on both rat and bovine cerebral cortex membranes (Pinthong *et al.*, 1995). Taken together, these observations question the value of employing [^{3}H]-clonidine binding to α_2-adrenoceptors alone as a means of detecting a biologically-active non-catecholamine CDS; as demonstrated by Atlas and coworkers this should be complemented by functional studies to show that this substance has the predicted activity (Diamant & Atlas, 1986; Diamant *et al.*, 1987). Data from this study adds further weight to this view, since agmatine failed to discriminate between [^{3}H]-clonidine and [^{3}H]-RX-821002 binding (Table 1) and, therefore, exhibited a profile unlike that produced by the α_2-adrenoceptor agonists, adrenaline and UK-14304. Furthermore, relative to the affinity exhibited at the sites labelled by [^{3}H]-clonidine, agmatine was less potent against [^{3}H]-idazoxan and [^{3}H]-RX-821002 than might have been expected from the findings with the known antagonists (see Tables 1 and 2). At present we have no satisfactory explanation for the detectable affinity of agmatine for α_2-adrenoceptor binding sites labelled by a ^{3}H-agonist and ^{3}H-antagonists failing to translate into either agonist or antagonist activity in biologically-intact systems. One strategy for examining this paradox may be to examine events intimately linked to receptor activation, e.g. GTPγS binding to membranes (Ito *et al.*, 1994). It is equally possible, however, that agmatine may interact with an allosteric site associated with α_2-adrenoceptors which, under conditions yet to be identified, is of biological significance.

The crude methanolic extracts of bovine lung and brain, previously shown to possess CDS activity (Singh *et al.*, 1995), were significantly more potent against α_2-adrenoceptors labelled by [^{3}H]-clonidine, than against those labelled by [^{3}H]-idazoxan and [^{3}H]-RX-821002. It should be noted, however, that since the IC_{50} values have not been corrected to account for the radioligand concentration, the possibility exists that the estimate of the relative potency (5–15 fold greater affinity for the [^{3}H]-clonidine sites) may have been overestimated. Nonetheless, these observations underline the similar nature of the CDS activity in these extracts, and supports the view that they contain a substance which cannot be accounted for by either agmatine or histamine (Singh *et al.*, 1995), the latter a known contaminant of the crude methanolic extract of bovine lung. Also, the profile exhibited by the crude extracts was remarkably similar to that for the known agonists adrenaline and UK-14304, which raises the possibility that the CDS-activity may possess agonist activity at α_2-adrenoceptors. On a cautionary note, the Hill slope for the extracts against [^{3}H]-clonidine binding to α_2-adrenoceptors was closer to unity than that observed for either of the two known agonists, and this may indicate that the unidentified substance interacts with α_2-adrenoceptors in a novel fashion.

In conclusion, we have demonstrated that α_2-adrenoceptor binding sites on bovine cerebral cortex membranes, when labelled with a ^{3}H-agonist and ^{3}H-antagonists, can successfully discriminate between known agonists and antagonists. Using this model, the putative CDS, agmatine (Li *et al.*, 1994) exhibited a profile qualitatively dissimilar to that observed for either adrenaline or UK-14304, which supports functional studies indicating that agmatine is not an agonist at α_2-adrenoceptors (Pinthong *et al.*, 1995). On the other hand, CDS activity in crude methanolic extracts of bovine brain and lung exhibited a profile consistent with the possibility that it is an agonist at α_2-adrenoceptors and, therefore, not attributable to agmatine. Further purification of the extract, to permit examination of the effect of CDS on functional α_2-adrenoceptors, appears to be warranted.

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