ELSEVIER

European Journal of Pharmacology 378 (1999) 213–221

ejp

www.elsevier.nl/locate/ejphar

Extraction of active clonidine-displacing substance from bovine lung and comparison with clonidine-displacing substance extracted from other tissues

Christine A. Parker [a], Alan L. Hudson [a], David J. Nutt [a], Michael P. Dillon [c], Richard M. Eglen [c], Susan L.F. Chan [d], Noel G. Morgan [d], John Crosby [b,*]

[a] *Psychopharmacology Unit, University of Bristol, Bristol BS8 1TS, UK*
[b] *School of Chemistry, University of Bristol School of Chemistry Cantock's Close, Bristol BS8 1TS, UK*
[c] *Neurobiology Unit, Roche Bioscience, Palo Alto, CA 94304-1397, USA*
[d] *Department of Biological Sciences, Keele University, Staffordshire ST5 5BG, UK*

Received 18 February 1999; received in revised form 26 May 1999; accepted 18 June 1999

Abstract

Crude methanolic clonidine-displacing substance (CDS) extracted from bovine lung competed for radioligand binding from α_2-adrenoceptors and I_2-sites present in rat brain membranes, and from I_1-sites present in rat brain and kidney membranes. There was no difference in the competition of [^{3}H]clonidine binding to α_2-adrenoceptors present in either rat or rabbit brain membranes by the crude CDS extract and therefore either tissue could be used to estimate the number of units of CDS present in extracts. Further purification by reverse phase high performance liquid chromatography (RP-HPLC), with UV detection, of extracts obtained from bovine lung, brain and rat brain exhibited similar three-peak profiles, previously reported. Corresponding fractions competed for radioligand binding to α_2-adrenoceptors present in rat brain membranes, eluting between 19 and 23 min, which corresponded with the middle peak of the three-peaks. Therefore, we propose the CDS-like material eluting from all these tissues to be similar. Interestingly, CDS extracted from bovine adrenal glands under the same conditions showed a similar three-peak profile, but did not repeat the displacement of binding just at 19–23 min, but at every time point after 4 min. This suggests this tissue could represent a source of CDS in this species. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: CDS (clonidine-displacing substance); Bovine lung; [^{3}H]Clonidine; RP-HPLC (reverse phase high performance liquid chromatography); α_2-Adrenoceptor

1. Introduction

An endogenous substance, isolated and partially purified from bovine brain, potently inhibits binding of [^{3}H]clonidine to rat cerebral cortical α_2-adrenoceptors (Atlas and Burnstein, 1984). This extract has therefore been termed ''clonidine-displacing substance'' (CDS); where 1 unit of CDS was defined as that amount required to inhibit 50% of specific [^{3}H]clonidine (3 nM) binding to rat α_2-adrenoceptors (Atlas and Burnstein, 1984). It is unlikely that the partially purified extracts reported to date represent a single substance and in fact it has been suggested that there may be several compounds that act as a CDS.

Crude extracts of CDS have been shown to compete for specific binding of [^{3}H]idazoxan from imidazoline (I) sites in the rabbit kidney or rat liver membranes (Zonnenschein et al., 1990). Therefore, crude CDS possesses affinity, not only at α_2-adrenoceptors, but at I-sites as well. CDS has also been discovered peripherally in human cerebrospinal fluid (CSF; Goldberg-Stern et al., 1993), human serum (Kreisberg et al., 1987) and bovine lung (Singh et al., 1995). Consequently, CDS has been suggested to act as a circulating hormone (Meeley et al., 1992). Finally, agmatine (decarboxylated arginine) has been shown to act as a CDS (Li et al., 1994), although it is likely to differ from ''classical'' CDS, both in terms of the HPLC elution profile (Atlas, 1994), but also since it lacks the functional-

* Corresponding author. Tel.: +44-117-928-8445; fax: +44-117-929-8611; E-mail: john.crosby@bristol.ac.uk

0014-2999/99/$ - see front matter © 1999 Elsevier Science B.V. All rights reserved.
PII: S0014-2999(99)00449-5

ity ascribed to classic CDS (Diamant and Atlas, 1986; Felsen et al., 1987).

One of the problems of defining the physiology and pharmacology of classic CDS is that the structure has not been elucidated. A key step in this process is to prepare pure material and to compare CDS isolated from several sources. This study now reports the existence of active crude methanolic CDS obtained from bovine lung, its purification by reverse phase high performance liquid chromatography (RP-HPLC), and its subsequent comparison with active CDS extracts obtained from bovine brain, adrenal gland and rat brain.

2. Materials and methods

2.1. Bovine lung crude CDS preparation

The method used was modified from Singh et al. (1995), since this was a method in the literature which was specific for the extraction of crude CDS from bovine lungs. Bovine lungs were obtained from a local abattoir immediately after slaughter of the animals. Lungs were then chopped into 25 g (wet weight) portions before being stored at −70°C. Prior to extraction, 25 g of lung was allowed to thaw to room temperature before being blended in 10 volumes (w/v) of ultra pure boiling water. The homogenate was then centrifuged at $37{,}000 \times g$ for 30 min, at 4°C. The supernatant was removed and boiled for 15 min to precipitate soluble protein and then allowed to cool to room temperature. This solution was centrifuged at $37{,}000 \times g$ for 30 min, at 4°C, before freezing the supernatant in liquid nitrogen and freeze-drying. The lyophilisate was then extracted by bath sonication (10 min) with 2×20 volumes (w/v) of Analar grade methanol at room temperature. The methanolic extracts were combined and centrifuged at $400 \times g$ for 5 min, at 4°C, to remove any particulate matter, before being evaporated to dryness at low pressure. The residual material was reconstituted into 2 ml ultra pure water immediately prior to use.

2.2. Rat brain crude CDS preparation

The method used was taken from Chan et al. (1997). Briefly, rat whole brains (10) were homogenised, before centrifuging. The supernatant was subsequently denatured by boiling, prior to recentrifugation and lyophilisation of the resulting supernatant. The lyophilisate was reconstituted in distilled water (4 ml), and centrifiltered first through Centricon-10 (10,000 molecular weight cut off), then through Centricon-3 (3000 molecular weight cut off) concentrators. Following lyophilisation, the extract was sonicated in 10 volumes (w/v) methanol. The methanol-insoluble particulate was removed by filtration through Whatman no. 1 filters, prior to freeze-drying and storing at −20°C. Approximately 100 units of CDS were reconstituted into 2 ml ultra pure water immediately prior to use.

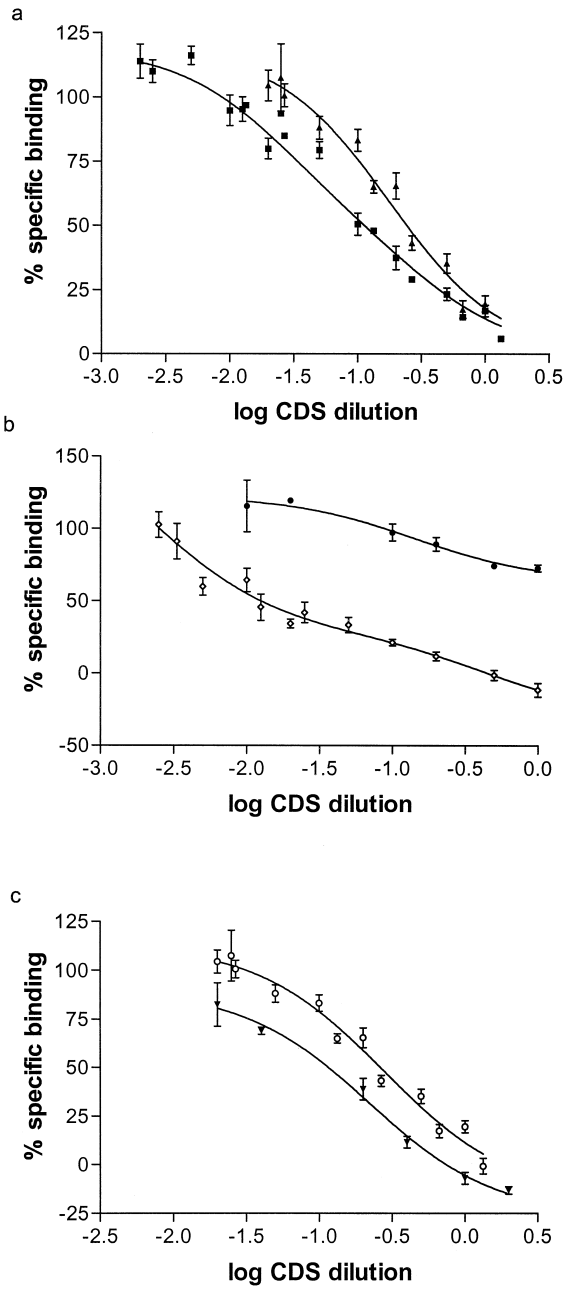

Fig. 1. Bovine lung crude methanolic CDS extract inhibiting binding of (a) [^{3}H]clonidine or [^{3}H]2-BFI from binding to α_2-adrenoceptors or I_2-binding sites present in rat whole brain membranes, respectively. Where the symbols ▲ and ■ indicate [^{3}H]clonidine and [^{3}H]2-BFI binding, respectively. Non-specific binding was defined by rauwolscine for α_2-adrenoceptors and BU224 for I_2-binding sites (both 10 μM), $n = 6$–8 experiments performed in triplicate. (b) [^{3}H]Clonidine from I_1-sites in either rat whole brain or kidney membranes. Where the symbols • and ◇ indicate binding to rat brain and rat kidney membranes, respectively. Non-specific binding was defined by unlabelled clonidine (10 μM), and rauwolscine (10 μM) was included to mask α_2-adrenoceptors, $n = 3$–6 experiments performed in triplicate. (c) [^{3}H]Clonidine from α_2-adrenoceptors present in either rat or rabbit whole brain membranes. Where the symbols ◦ and ▼ indicate binding to rat and rabbit brain membranes, respectively. Non-specific binding was defined by rauwolscine (10 μM), $n = 5$–6 experiments performed in triplicate.

Table 1
Mean and S.E.M. for 50% inhibition of [^{3}H]ligand binding, relative IC_{50}: CDS units* shown. *One unit of CDS in this study is defined as the amount required to inhibit 50% [^{3}H]clonidine (3 nM) from binding to α_2-adrenoceptors present in rat whole brain membranes

	n value	Mean	S.E.M.	Relative IC_{50}: CDS units*
α_2-Adrenoceptors	6	2.934×10^{-1}	6.104×10^{-2}	1
I_1-sites (kidney)	6	2.908×10^{-3}	2.777×10^{-3}	0.01
	6	7.753×10^{-1}	5.307×10^{-1}	2.6
I_1-sites (brain)	2			< 100
I_2-sites	8	9.629×10^{-2}	2.544×10^{-2}	0.3

2.3. Bovine brain crude CDS preparation

The method used was taken from Atlas and Burnstein (1984), and performed at Roche Bioscience, USA. Briefly, calf brains (approximately 300 g, after removal of the cerebellum) were homogenised before centrifugation. The supernatant was denatured by boiling, prior to recentrifugation and lyophilisation of the resulting supernatant. The extract was further extracted by sonication in 20 volumes (w/v) methanol. Following filtration the solute was freeze-dried, and stored at −20°C. The equivalent of one brain's worth of CDS was reconstituted into 1 ml ultra pure water immediately prior to use.

2.4. Bovine adrenal gland crude CDS preparation

Method modified from Singh et al. (1995). A pair of bovine adrenal glands (17–20 g wet weight) were obtained fresh from a local abattoir and stored at −70°C until use. The method of crude CDS extraction was then identical to that in Section 2.1, except the final crude CDS methanolic extract was reconstituted into an appropriate volume of ultra pure water to make a comparable concentration of CDS units to that obtained from 25 g wet weight of bovine lung.

2.5. Rat whole brain and kidney membrane preparations

Method taken from Mallard et al. (1992). Male Wistar rats (200–300 g) were killed by stunning followed by decapitation. Whole brains and kidneys were rapidly dissected over ice and homogenised in 10 volumes (w/v) of buffered sucrose (0.32 M in 50 mM Tris–HCl, pH 7.4 at 4°C) using a motor driven Teflon-glass homogeniser. The homogenate was then centrifuged at $1000 \times g$ for 10 min, at 4°C. The resulting supernatants were pooled and recentrifuged at $31{,}000 \times g$ for 20 min, at 4°C. The supernatants were discarded and each pellet resuspended in 10 volumes of assay buffer (50 mM Tris–HCl, 1 mM $MgCl_2$, pH 7.4 at 4°C). The pellets were then washed twice by repeated centrifugation at $31{,}000 \times g$ for 20 min, at 4°C. The final pellets were stored at −70°C until use. For radioligand binding, pellets were thawed to room temperature and washed twice by centrifugation, the membranes were then resuspended in assay buffer to give 300–450 μg protein per assay tube. The protein content of the membrane preparations was determined by the method of Bradford (1976) utilising Coomassie blue with bovine serum albumin as the standard.

2.6. Rabbit whole brain membrane preparation

Method taken from Lione et al. (1996). New Zealand white rabbits of either sex (1.8–2.5 kg) were killed with an overdose of sodium pentobarbitone (60 mg kg^{-1} i.v.) followed by rapid exsanguination. Whole brains were immediately removed over ice, and the method for preparation identical to the rat whole brain membrane preparation above (Section 2.5).

2.7. Radioligand binding studies

All radioligand binding experiments were performed at 25°C in assay buffer (50 mM Tris–HCl, 1 mM $MgCl_2$, pH 7.4 at 4°C). Competition binding studies were carried out to assess the ability of crude methanolic bovine lung CDS extracts to compete for the binding of various ligands to their corresponding binding sites. Crude methanolic bovine lung CDS extracts were diluted into log concentrations; where log 0 represents 25 g wet weight of bovine lung being extracted and reconstituted into 2 ml ultra pure water, and log 0.125 represents the identical procedure, except being reconstituted into 1.5 ml ultra pure water.

Table 2
Mean and S.E.M. for 50% inhibition of [^{3}H]clonidine binding to α_2-adrenoceptors present in either rat or rabbit whole brain membranes. *One unit of CDS in this study is defined as the amount required to inhibit 50% [^{3}H]clonidine (3 nM) from binding to α_2-adrenoceptors present in rat whole brain membranes

α_2-Adrenoceptors	*n* value	Mean	S.E.M.	Relative IC_{50}: CDS units*
Rat	6	2.934×10^{-1}	6.104×10^{-2}	1
Rabbit	4	2.260×10^{-1}	4.700×10^{-2}	0.77

2.7.1. Competition binding from α_2-adrenoceptors

A competition radioligand binding study was performed to assess the ability of crude methanolic bovine lung CDS extract to compete for [^{3}H]clonidine (3 nM) binding to α_2-adrenoceptors present in rat and rabbit brain membranes, using at least 12 dilutions. Membrane aliquots and [^{3}H]clonidine were incubated in triplicate, to equilibrium (30 min) in a final volume of 0.5 ml. The specific binding

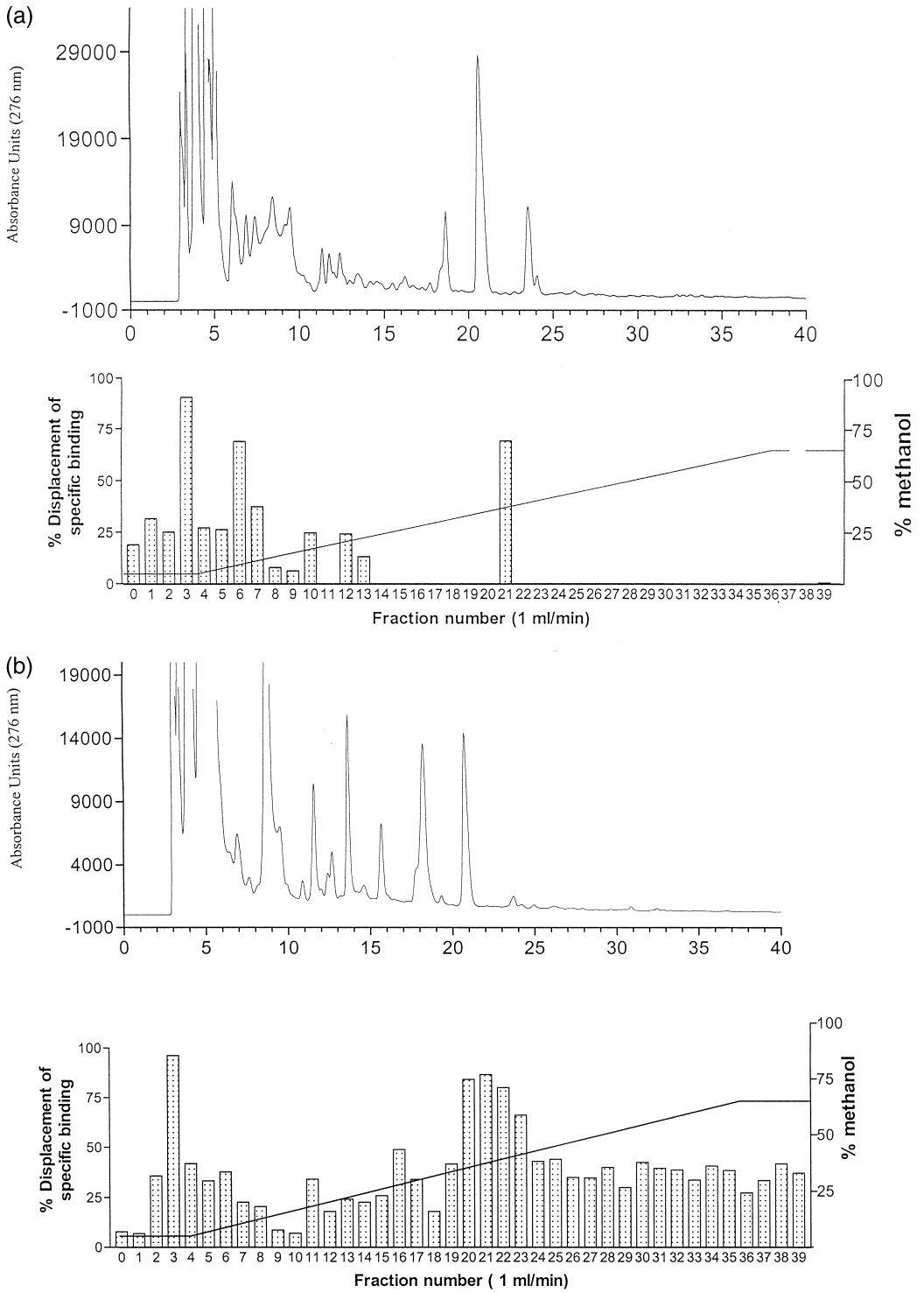

Fig. 2. Typical RP-HPLC traces for CDS extracted from various tissues (upper traces), with corresponding inhibition data for fractions collected at 1 ml min^{-1} intervals (lower traces). Fractions were assayed for their ability to inhibit [^{3}H]clonidine from binding to α_2-adrenoceptors present in rat brain membranes. Where (a), (b) and (c) denote typical RP-HPLC traces and corresponding inhibition data for CDS extracted from bovine lung, bovine brain and rat brain, respectively.

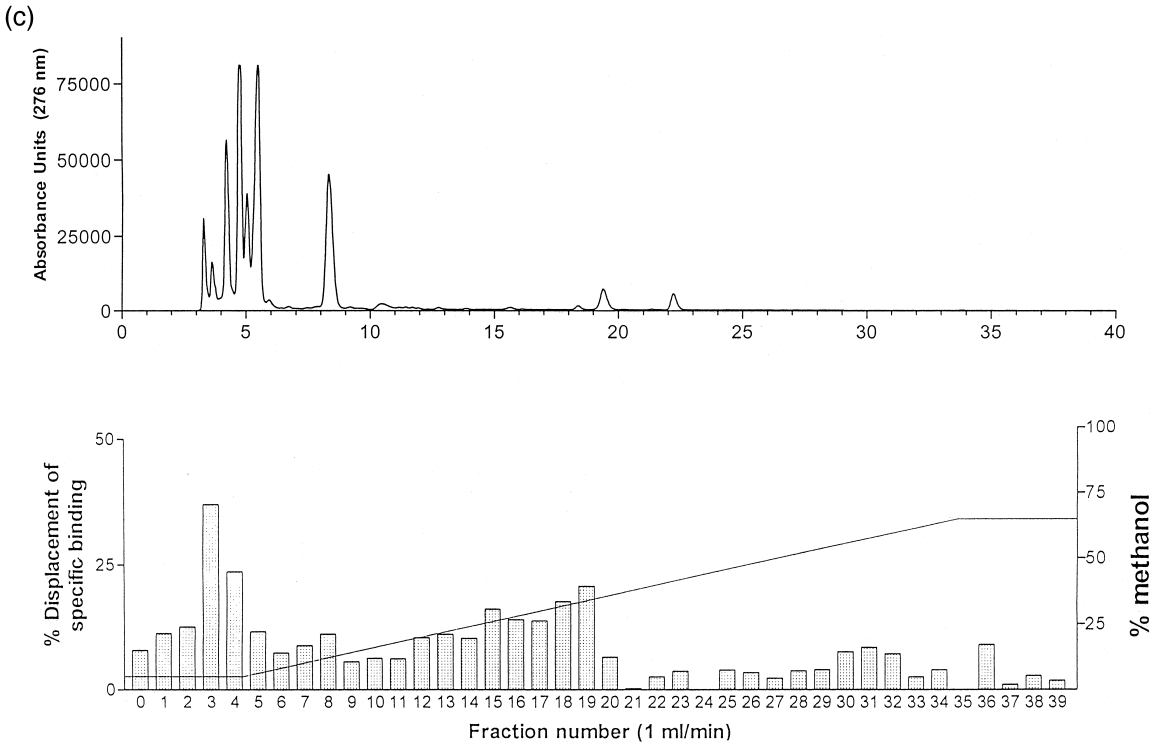

Fig. 2 (continued).

component was determined by addition of rauwolscine (10 μM). Bound radioactivity was separated from free ligand by vacuum assisted rapid filtration through presoaked (0.5% polyethyleneimine) Whatman GF/B filters, using a Brandel M-24 cell harvester. Filters were washed twice with 3 ml of ice-cold assay buffer and the trapped membrane bound radioactivity remaining on the filters was determined by liquid scintillation counting.

2.7.2. Competition binding from imidazoline I_2-binding sites

Competition radioligand binding studies were also performed to assess the ability of crude methanolic bovine lung CDS extract to compete for [^{3}H]2-(2-benzofuranyl)-2-imidazoline (2-BFI; 1 nM) from binding to imidazoline I_2-sites present in either rat or rabbit brain membranes. The procedure was identical to that performed above, except the specific binding component was determined by the addition of BU224 (10 μM).

2.7.3. Competition binding from imidazoline I_1-binding sites

Competition radioligand binding studies were performed to assess the ability of the crude methanolic bovine lung CDS extract to inhibit [^{3}H]clonidine (3 nM, in the presence of rauwolscine (10 μM) to mask the α_2-adrenoceptor component) from binding to imidazoline I_1-sites present in either rat brain or rat kidney membranes. The specific binding component was determined by addition of unlabelled clonidine (10 μM), and the protocol performed as before. The specific component of imidazoline I_1-site binding was the same whether unlabelled rilmenidine or clonidine was used (result not shown). Therefore, unlabelled clonidine was used to define the specific binding component.

2.8. HPLC

The biologically active methanolic extract was applied to a reverse-phase column (RP-C18), and eluted from it using a linear gradient of methanol (5–65%, for 50 min), in 0.05% trifluoroacetic acid (TFA), at room temperature at a flow rate of 1.0 ml min^{-1}. Absorption at 276 nm was recorded. Fractions of 1.0 ml were collected (1–40 min; inclusive) and assayed for their biological activities (see below).

2.9. HPLC fractions used in radioligand binding assays

Fractions collected from the RP-HPLC C18 column were freeze-dried and reconstituted into 50 μl ultra pure water prior to being used to define the ability of each fraction to inhibit radioligand binding to α_2-adrenoceptors. [^{3}H]Clonidine (3 nM), fractions and membrane aliquots were incubated to equilibrium (30 min) in a final volume of 0.5 ml. The specific binding component was defined by rauwolscine (10 μM).

2.10. Analysis of binding data

Data were analysed by the iterative non-linear regression programme (GraphPAD Prism 2.01, 1994) capable of

fitting to a one or two-site model. All experiments were analysed independently.

2.11. Drugs and chemicals

[^{3}H]Clonidine (specific activity = 65 Ci mmol^{-1}) stored at −20°C at a concentration 1 mCi ml^{-1}, from NEN Life Science Products (UK). [^{3}H]2-BFI (specific activity = 69 Ci mmol^{-1}) stored at a concentration of 0.2 mCi ml^{-1}, from Amersham International (UK). Rauwolscine and unlabelled clonidine (Sigma, UK); 2-(4,5-dihydroimidazol-2-yl)quinoline (BU224; Tocris Cookson, UK). All chemicals and reagents were of the highest analytical grade available.

3. Results

3.1. Radioligand binding studies performed using rat and rabbit membranes

3.1.1. Competition from α_2-adrenoceptors and imidazoline I_2-binding sites in rat brain

The competition binding of [^{3}H]clonidine and [^{3}H]2-BFI from α_2-adrenoceptors and I_2-sites, respectively, by bovine lung crude CDS is given in Fig. 1a. Where 1 unit of CDS represents the amount required to displace 50% of [^{3}H]clonidine (3 nM) bound to α_2-adrenoceptors present in rat whole brain membranes. Both radioligands exhibited similar binding curves, however, the crude CDS inhibited more bound radioligand from imidazoline I_2-sites (relative IC_{50}: CDS units 0.3; Table 1) than α_2-adrenoceptors (relative IC_{50}: CDS units 1; Table 1). In this study, we were able to extract 2 units of crude CDS from every 25 g (wet weight) of bovine lung.

3.1.2. Competition binding from imidazoline I_1-binding sites in rat brain and kidney

Bovine lung crude CDS extract inhibited [^{3}H]clonidine from binding to imidazoline I_1-sites present in rat brain and kidney (Fig. 1b). However, there was only a small level of inhibition of [^{3}H]clonidine bound to imidazoline I_1-sites present in rat brain membranes (only 25% at the highest concentration of crude CDS extract). Interestingly, there was complete inhibition of [^{3}H]clonidine bound to imidazoline I_1-sites in the rat kidney, with a two-site linear regression curve (relative IC_{50}: CDS units 0.01 and 2.6; Table 1).

3.1.3. Competition binding from α_2-adrenoceptors in rat and rabbit brain

Fig. 1c shows crude methanolic bovine lung CDS extract competing for [^{3}H]clonidine bound to α_2-adrenoceptors present in either rat or rabbit whole brain membranes. The competition curves from both the rat and rabbit brain paralleled each other, and appeared to possess similar displacement affinities (relative IC_{50}: CDS units 1 and 0.77, respectively; Table 2).

3.2. Typical RP-HPLC trace with corresponding inhibition data

3.2.1. From bovine lung, brain and rat brain

Typical RP-HPLC traces of crude methanolic CDS extracts from bovine lung and brain and rat brain are given in the upper traces of Fig. 2a–c, respectively. Inhibition of [^{3}H]clonidine binding to α_2-adrenoceptors by individual fractions from bovine lung and brain and rat brain are given in the lower traces of Fig. 2a–c, respectively. Clearly, bovine lung CDS extract exhibits an isolated activity at 21 min, which similar to Atlas's findings (Atlas, 1994), appears to elute with the middle peak of the three-peak profile. The bovine brain and rat brain CDS extracts also appeared to exhibit activities at similar points to the lung. However, the activity eluted with the bovine brain was tailed over several minutes, which was not observed with the lung. The rat brain CDS extract also showed a similar RP-HPLC and activity profile to the bovine brain, suggesting that these three tissues may contain similar CDS components. Relative elution times of various catecholamines and other endogenous compounds from the RP-HPLC column are given in Table 3.

In our studies, TFA did not compete for radioligand bound to α_2-adrenoceptors, imidazoline I_1- or imidazoline I_2-sites, and hence can be ruled out as a possible contaminating inhibitor.

3.2.2. From bovine adrenal glands

A typical RP-HPLC trace of crude methanolic CDS extract obtained from bovine adrenal glands (four times more dilute than normal extracts) is shown in Fig. 3 (upper trace). Corresponding inhibition data for [^{3}H]clonidine binding to α_2-adrenoceptors in rabbit brain membranes are also given (lower trace). Again there was a three peak profile exhibited on the RP-HPLC trace similar to that seen with bovine lung CDS. However, in contrast to the bovine lung CDS there appeared to be uniform inhibition of [^{3}H]clonidine binding to α_2-adrenoceptors in rabbit brain membranes with every fraction after 4 min (approximately 60%), and this did not decrease, even when

Table 3
Elution times (in min) of various compounds (1 mg ml^{-1}) from the RP-HPLC column. Methanol gradient 5–65%, for 50 min; in 0.05% TFA at room temperature and a flow rate of 1.0 ml min^{-1}. Absorption at 276 nm was recorded by a UV detector

Compound (1 mg ml^{-1})	Elution time (in min; 5–65% methanol)
Noradrenaline	3.0
Adrenaline	3.0
Histamine	3.0
Agmatine	3.0
5-Hydroxytryptamine	11.5

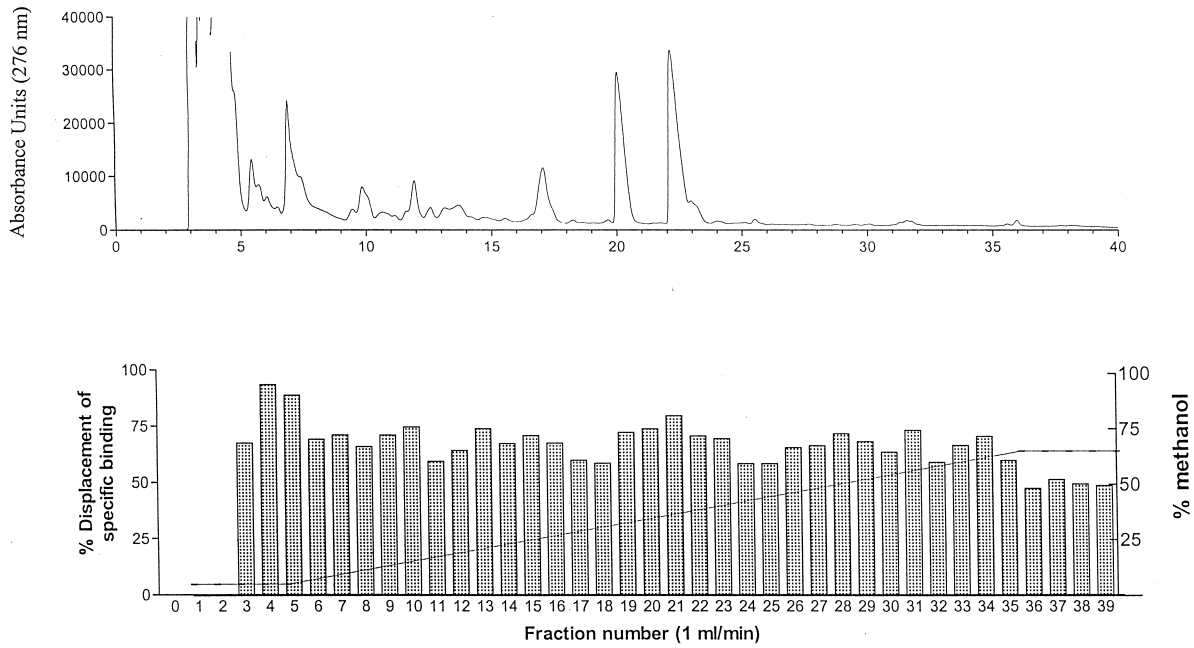

Fig. 3. Typical RP-HPLC trace for CDS extracted from bovine adrenal gland (upper trace), with corresponding inhibition data for fractions collected at 1 ml min^{-1} intervals (lower trace). Fractions assayed for their ability to inhibit [^{3}H]clonidine from binding to α_2-adrenoceptors present in rabbit brain membranes were of 12.5% the concentration of the fractions assayed in Fig. 2.

the extract was diluted eight times (results shown for the extract diluted eight times).

4. Discussion

Crude methanolic CDS has been isolated from various sources including bovine brain (Atlas and Burnstein, 1984), rat brain (Chan et al., 1997), bovine lung (Singh et al., 1995), human CSF (Goldberg-Stern et al., 1993) and human serum (Kreisberg et al., 1987). The crude CDS methanolic extracts have been shown to exhibit contradicting effects, for example, microinjection of extract into the rostroventrolateral medulla (RVLM) of rats causes a lowering of blood pressure in one group (Meeley et al., 1986) and an elevation of blood pressure in another (Bousquet et al., 1986). However, this is hardly surprising since the crude methanolic CDS extract contains within it not only CDS(s), but other active extracts such as bioactive endogenous ligands and dipeptides that have survived the extraction process. Consequently, the only way to progress our understanding of CDS was by further purification, such as by HPLC. HPLC pure CDS (extracted from the bovine brain) has been shown to inhibit the non-adrenergic fast twitch response in the rat vas deferens (Diamant and Atlas, 1986), and contract the smooth muscle located in the rat gastric fundus, but not vascular smooth muscle (Felsen et al., 1987), which collectively suggests that responses to CDS maybe tissue selective, and may represent a variable distribution of its receptor/binding site.

Crude methanolic CDS extracted from bovine lung inhibited the binding of [^{3}H]clonidine and [^{3}H]2-BFI to α_2-adrenoceptors and imidazoline I_2-sites with similar affinities, with the crude CDS exhibiting a marginally higher affinity for I_2-sites than α_2-adrenoceptors. This data supports previous findings by Atlas and Burnstein (1984), where classical CDS was originally isolated was purported to be a non-catecholamine α_2-adrenoceptor agonist. The crude extract also competed for the binding of [^{3}H]clonidine (in the presence of the selective α_2-adrenoceptor antagonist, rauwolscine) to imidazoline I_1-sites present in rat whole brain and kidney. The rat whole kidney has been reported to exhibit a higher number of imidazoline I_1-binding sites (MacKinnon et al., 1993; Parker et al., 1998) than the rat whole brain. Our present competition experiments show that there appeared to be only a minority of imidazoline I_1-binding sites labelled by [^{3}H]clonidine in the rat whole brain which were competed for by the crude CDS extract in a one-site manner. In contrast, crude methanolic CDS inhibited the majority of [^{3}H]clonidine from binding to imidazoline I_1-sites in the rat whole kidney, which was suggested by a higher number of decays per minute (d.p.m.) associated with imidazoline I_1-binding sites present in this tissue when compared with the rat whole brain. [^{3}H]Clonidine binding was shown to be inhibited from rat whole kidney I_1-sites in a two-site manner, by the crude CDS, suggesting the presence of two-distinct isoforms of this binding site, or different affinity states of a single binding protein. It must be borne in mind, however, that the number of imidazoline I_1-sites present in the rat whole brain is very small, and subsequently, the specific binding only exhibited a low level of d.p.m. Therefore, it was very difficult to determine the

relative IC_{50} value for imidazoline I_1-sites in the rat whole brain. Furthermore, the crude methanolic CDS extract does contain not only the CDS-like material, but also other impurities which have survived the extraction process, such as other endogenous ligands, dipeptides and heat stable polypeptides, which may contribute to the displacement observed in these assays.

The crude bovine CDS extract obtained from the bovine lung also inhibited the specific binding of [^{3}H]clonidine from α_2-adrenoceptors present in rabbit whole brain membranes with a similar potency to rat whole brain membranes (see Table 2). The experiments showed that crude CDS exhibited similar apparent affinities for α_2-adrenoceptors in both rat and rabbit whole brains.

Therefore, to investigate the properties of a purer form of the CDS-like material, further purification was performed, utilising the technique of RP-HPLC.

Typical RP-HPLC traces for methanolic CDS obtained from bovine lung, brain or rat brain exhibited the typical three-peak profile characteristic of classical CDS identified by Atlas in 1994 (Atlas, 1994). The corresponding inhibition of [^{3}H]clonidine binding to α_2-adrenoceptors in rat whole brain membranes by each 1 min fraction of the running time is also shown. It is clear that CDS extracted from all three tissues exhibited inhibition of [^{3}H]clonidine binding to α_2-adrenoceptors at 3 and 19–23 min. The inhibition observed at 3 min may correspond to low levels of agmatine (the proposed endogenous ligand (Li et al., 1994)), histamine and/or noradrenaline, all of which are eluted at this time (Table 3). The inhibition observed at 19–23 min, however, directly corresponds with the middle peak of the three-peak profile and may correspond to active CDS(s) under these conditions. It is clear that the three peaks observed between these three tissues are conserved, as is the corresponding inhibition at 19–23 min. Subsequently, this result may reflect similar forms of CDS(s) being extracted from these tissues.

The RP-HPLC analysis of the CDS containing extract isolated from bovine adrenal glands provided an elution pattern similar to that obtained from the lung and brain tissues. However, in contrast to the lung and brain analyses, each HPLC fraction from 4 min onwards exhibited inhibition of [^{3}H]clonidine bound to rabbit brain membrane α_2-adrenoceptors. It is possible that there maybe several interfering substances present in the extract of the adrenal gland, and these may contribute to the inhibition observed at fractions collected after 4 min. In spite of this, it is possible that the bovine adrenal glands contain a greater source of CDS-like material than lung or brain tissues. At present, the nature of the active component(s) extracted from this tissue remains undetermined.

To conclude, we have been able to define with methanolic extracts an active form of crude CDS-like-material from the bovine lung. Subsequently, we have shown that similar extractions with other tissues such as bovine and rat brain also produce CDS(s) with similar properties. Purified CDS obtained from bovine lung, brain and rat brain by RP-HPLC all exhibited the ''three-peak effect'' (UV detection) with corresponding fractions all showing inhibition of radioligand bound to α_2-adrenoceptors at 19–23 min. Therefore, we propose that the CDS-like material eluting from all these tissues may be similar, that is within a species and between species. We also would like to suggest that the adrenal glands may be a source of CDS within the bovine and other species.

References

Atlas, D., 1994. Identifying clonidine-displacing substance. Science 266, 462–463.

Atlas, D., Burnstein, Y., 1984. Isolation and partial purification of a clonidine-displacing endogenous brain substance. Eur. J. Biochem. 144, 287–293.

Bousquet, P., Feldman, J., Atlas, D., 1986. An endogenous, non-catecholamine clonidine antagonist increases mean arterial blood pressure. Eur. J. Pharmacol. 124, 167–170.

Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. 72, 245–254.

Chan, S.L.F., Atlas, D., James, R.F.L., Morgan, N.G., 1997. The effect of the putative endogenous imidazoline receptor ligand clonidine-displacing substance, on insulin secretion from rat and human islets of Langerhans. Br. J. Pharmacol. 120, 926–932.

Diamant, S., Atlas, D., 1986. An endogenous brain substance, CDS (clonidine-displacing substance), inhibits the twitch response of rat vas deferens. Biochem. Biophys. Res. Commun. 134, 184–190.

Felsen, D., Ernsberger, P., Meeley, M., Reis, D.J., 1987. Clonidine displacing substance is biologically active on smooth muscle. Eur. J. Pharmacol. 142, 453–455.

Goldberg-Stern, H., Atlas, D., Schwartz, L., Achiron, A., Ziv, I., Djaldetti, R., Zoldan, Y., Melamed, E., 1993. Detection and measurement of an endogenous clonidine-displacing-substance (CDS) in human cerebrospinal fluid (CSF). Brain Res. 601, 325–328.

Kreisberg, G.A., Diamant, S., Diamant, Y., Atlas, D., 1987. Raised levels of an endogenous non-adrenergic substance in serum of pregnancy-induced hypertension patients. Isr. J. Med. Sci. 23, 1194–1197.

Li, G., Reguanthan, S., Barrow, C.J., Eshraghi, J., Cooper, R., Reis, D.J., 1994. Agmatine: An endogenous clonidine-displacing substance in the brain. Science 263, 966–969.

Lione, L.A., Nutt, D.J., Hudson, A.L., 1996. [^{3}H]2-(2-Benzofuranyl)-2-imidazoline: a new selective high affinity radioligand for the study of rabbit brain imidazoline I_2 receptors. Eur. J. Pharmacol. 304, 221–229.

MacKinnon, A.C., Stewart, M., Olverman, H.J., Spedding, M., Brown, C.M., 1993. [^{3}H] *p*-Aminoclonidine and [^{3}H]idazoxan label different populations of imidazoline sites on rat kidney. Eur. J. Pharmacol. 232, 79–87.

Mallard, N.J., Hudson, A.L., Nutt, D.J., 1992. Characterization and autoradiographical localization of non-adrenoceptor idazoxan binding sites in the rat brain. Br. J. Pharmacol. 106, 1019–1027.

Meeley, M.P., Ernsberger, P.R., Granata, A.R., Reis, D.J., 1986. An endogenous clonidine-displacing substance from bovine brain: receptor binding and hypotensive actions in the ventrolateral medulla. Life Sci. 38, 1119–1126.

Meeley, M.P., Hensley, M.L., Ernsberger, P., Felsen, D., Reis, D.J., 1992. Evidence for a bioactive clonidine-displacing substance in peripheral tissues and serum. Biochem. Pharmacol. 44, 733–740.

Parker, C.A., Nutt, D.J., Hudson, A.L., 1998. [^{3}H]Clonidine binding sites

in rat kidney: a model for putative central imidazoline-1 sites. Br. J. Pharmacol. 125, 76 p.

Singh, G., Hussain, J.F., MacKinnon, A., Brown, C.M., Kendall, D.A., Wilson, V.G., 1995. Evidence for the presence of a non-catecholamine, clonidine-displacing substance in crude, methanolic extracts of bovine brain and lung. Naunyn-Schmiedeberg's Arch. Pharmacol. 351, 17–26.

Zonnenschein, R., Diamant, S., Atlas, D., 1990. Imidazoline receptors in rat liver cells: a novel receptor or a subtype of α_2-adrenoceptors. Eur. J. Pharmacol. 190, 203–215.