Br. J. Pharmacol. (1993), **110**, 1537–1543

Relevance of the use of [^{3}H]-clonidine to identify imidazoline receptors in the rabbit brainstem

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1 [^{3}H]-clonidine binding was investigated in membranes isolated from the ventral medulla oblongata of the rabbit where clonidine produced a hypotensive effect which was not mediated by adrenoceptors. [^{3}H]-clonidine specific binding, as defined by the difference between the binding of [^{3}H]-clonidine in the presence and in the absence of 10 μM cirazoline, occurred at two sites: a high affinity site with a $K_D = 2.9 \pm 0.7$ nM and a B_{max} of 40 ± 8 fmol mg^{-1} protein and a low affinity site with a $K_D = 18.2 \pm 0.4$ nM and a B_{max} of 66 ± 14 fmol mg^{-1} protein.

2 The high affinity sites being catecholamine-sensitive were identified as α_2-adrenoceptors. The low affinity binding of [^{3}H]-clonidine was insensitive to catecholamines, as well as to other α_2-adrenoceptor specific probes, and could be inhibited with high affinity only by compounds which lowered blood pressure when directly injected in the nucleus reticularis lateralis of the ventral brainstem, or by antagonists.

3 It was concluded that in the ventral medulla of the rabbit, [^{3}H]-clonidine labelled α_2-adrenoceptors and imidazoline receptors (IRs). Only the latter were related to the hypotensive effects of clonidine and rilmenidine directly injected into the rostroventrolateral medulla oblongata (RVLM) of the rabbit. The methodological problems regarding the study of IRs with [^{3}H]-clonidine are discussed.

Keywords: Imidazoline receptor; clonidine; binding; nucleus reticularis lateralis

Introduction

The main site for the vasodepressor response to clonidine and related compounds has been located within the medullary nucleus reticularis lateralis (NRL) of the rostroventrolateral medulla (RVLM) region of various species (Bousquet *et al.*, 1981; Wolf & Mohrland, 1984; Ernsberger *et al.*, 1990). When stereotaxically injected in this region, the imidazolines proved hypotensive whereas catecholamines and phenylethylamines did not influence the arterial blood pressure whatever their selectivity for the different subtypes of α-adrenoceptors. Thus it has been suggested that the hypotensive effect of clonidine-like compounds was mediated via imidazoline receptors (IRs) (Bousquet *et al.*, 1984).

Since the existence of IRs was first proposed, numerous biochemical data have confirmed the existence of specific binding sites which could account for the properties of imidazolines. The presence of IRs was detected in the bovine and human ventrolateral medulla with [^{3}H]-*p*-aminoclonidine and [^{3}H]-clonidine (Meeley *et al.*, 1986; Bricca *et al.*, 1988) and in the rabbit kidney and forebrain (Hamilton *et al.*, 1991). Idazoxan, also an antagonist of the effect of clonidine-like drugs on IRs, has been reported to label non-catecholamine binding sites both in the brain and in various peripheral tissues of several species. Such sites were named IGRS, IRs and NAIBS (Bousquet *et al.*, 1984; Coupry *et al.*, 1987; Hamilton *et al.*, 1988; Yablonsky *et al.*, 1988; Langin & Lafontan, 1989; Michel *et al.*, 1989; Wikberg *et al.*, 1991).

Recently, we observed that the rabbit was very sensitive to the hypotensive effect of clonidine and rilmenidine, a compound structurally related to imidazolines (Feldman *et al.*, 1990). When stereotaxically injected into the rabbit NRL area, clonidine and rilmenidine exhibited potent hypotensive actions whereas noradrenaline had no influence on blood pressure, confirming that, as in the cat, the central hypotensive effect of clonidine and clonidine-like drugs was related to their interaction with IRs in the rabbit NRL area (Feldman *et al.*, 1990). Imidazoline binding sites have already been detected in the rabbit forebrain (Hamilton *et al.*, 1988; 1991; Yakubu *et al.*, 1988); but IRs have not yet been investigated in the rabbit RVLM where clonidine and related compounds induced their hypotensive effect. Moreover, clonidine and rilmenidine displayed low if no affinity for [^{3}H]-idazoxan labelled imidazoline specific binding sites (Hamilton *et al.*, 1991). This led various authors to suggest that [^{3}H]-idazoxan and [^{3}H]-clonidine labelled different classes of IRs (Tesson & Parini, 1991; Wikberg *et al.*, 1991). Therefore it was of importance to characterize IRs in the rabbit NRL region of the medulla oblongata, with [^{3}H]-clonidine, the reference hypotensive drug.

Clonidine was the prototype of the imidazoline substances inducing hypotension originating within the RVLM of the brainstem. The study of the mechanism of action of that drug allowed the development of the IR concept. Yet, there were only few studies in which this radiolabelled substance was used as a probe for the specific analysis of these receptors. Indeed, numerous binding studies have been performed with [^{3}H]-clonidine but to label α_2-adrenoceptors (U'Prichard, 1981). Previously, we used this tritiated substance in various binding studies performed on bovine and human brain membrane preparations (Bricca *et al.*, 1988; 1989a). However, because preliminary studies showed that α_2-adrenoceptors and IRs co-existed within the RVLM, we aimed to characterize precisely the properties of that binding in a species particularly sensitive to the hypotensive effects of clonidine and analogous substances. We present here the characteristics of [^{3}H]-clonidine binding in rabbit RVLM membrane preparations.

Methods

Tissues

Brains from ZYKA strain rabbits were obtained from a local slaughterhouse. The animals were killed by electric shock. The brains were rapidly removed and the brainstem isolated

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by trans-section at the rostral and caudal limits of the ponto-medullary junction and the pyramidal decussatio. Samples were flash frozen in liquid nitrogen, transported on dry ice to the laboratory and stored up to 3 weeks without damage at − 20°C until membranes were prepared.

The pia mater was removed from the ventral medullary surface and the ventral part of the medulla, including the NRL region, isolated by discarding the dorsal part of the medulla.

Membrane preparation

Membranes were prepared essentially as described previously (Bricca *et al.*, 1989a) from at least 20 pooled brainstems. All steps were performed at 4°C unless otherwise specified. The tissues were homogenized for 30 s in 20 volumes (w/v) of ice-cold buffer (50 mM Tris-HCl, 5 mM EDTA, pH = 7.5) with a polytron PT12. The homogenate was centrifuged for 10 min at 500 *g*. The first sediment was discarded and the supernatant was centrifuged for 12 min at 45,000 *g*. The resulting pellets (P2) were washed twice with 50 mM ice-cold Tris-HCl buffer (pH = 7.5) and recentrifuged as above. To allow the dissociation of endogenous ligands, membranes were incubated at 25°C for 30 min between the two washes. The P2 pellets were stored for a maximum of 10 days at − 20°C before use.

Binding assay

Binding tests, run in duplicate at 25°C, were performed in a final volume of 1 ml containing 0.2 to 0.6 mg protein, [^{3}H]-clonidine and competitors in variable concentrations. The binding buffer was: 50 mM Tris-HCl, 1 mM $MgCl_2$, pH = 7.5. Kinetic experiments were carried out in the presence of 6 nM [^{3}H]-clonidine. Once equilibrium was reached, dissociation was measured at various time intervals after the addition of unlabelled clonidine to a final concentration of 10 μM. In equilibrium experiments samples were incubated for 45 min. Saturation curves were constructed with data obtained with 10 to 12 different concentrations of [^{3}H]-clonidine ranging from 0.1 to 38 nM. Competition experiments were performed with 5 to 6 nM [^{3}H]-clonidine and 9–11 different concentrations of each competitor. In all experiments involving catecholamines, 0.005% ascorbic acid was present. Non specific binding was determined with 10 μM cirazoline. In saturation experiments, two measurements of the non specific binding were performed for each concentration of [^{3}H]-clonidine, either with 10 μM (−)-noradrenaline or with 10 μM cirazoline. Whether the binding studies were performed immediately after membrane preparation or after storage of the pellets, the results were superimposable.

Protein assay

Protein concentrations were determined according to the method of Bradford (1976) with bovine serum albumin as standard.

Calculation and statistical analysis

Data were analysed by linear regression and iterative computerized non-linear curve fitting (Munson & Rodbard, 1980; McPherson, 1985). IC_{50} and slopes were obtained by non linear curve fitting of the experimental data to a sigmoidal curve by means of the EBDA programme. Saturation curves were analysed according to Scatchard (1949) and by non-linear curve fitting with the aid of the LIGAND programme (Munson & Rodbard, 1980). Student's paired *t* test was used to compare mean K_D and B_{max} values. Kinetic constants were obtained through the KINETIC programme (McPherson, 1985).

Chemicals

[^{3}H]-clonidine (specific activity 48 to 61 Ci mmol^{-1}) was obtained from New England Nuclear. (−)-Adrenaline, (−)-noradrenaline, clonidine, dopamine, histamine, 5-hydroxytryptamine, tolazoline and yohimbine were purchased from Sigma. Rilmenidine, (±)-idazoxan and (−)-idazoxan were from Servier. Cirazoline was from LERS-Synthélabo, BDF-6143 (4-chloro-2-(2-imidazoline-2-yl-amino-isoindole)HCl) and moxonidine from Beirsdorf, UK-14304 (bromoxidine) was from Pfizer, phentolamine from Ciba-Geigy, oxymetazoline and α-methylnoradrenaline from Farmex, guanfacine from Sandoz, guanoxabenz from Roussel-Uclaf, B-HT 920 (5-allyl-2-amino-5,6,7,8-tetrahydrothiazolo-[4,5-d]azépine diHCL) from Karl Thomae GMBH and phenoxybenzamine from Röhm Pharma.

Results

[^{3}H]-clonidine binding kinetics

[^{3}H]-clonidine specific binding, determined with cirazoline, reached equilibrium within 30 min in association experiments and was stable for at least 2 h (Figure 1). The specific binding was completely reversed by addition of unlabelled clonidine. Using the KINETIC programme of McPherson (1985), the data fitted a bi-exponential model ($P < 0.01$) in both association and dissociation experiments (Table 1). The K_D values calculated for [^{3}H]-clonidine binding from kinetic constants were in good agreement with the data obtained from the equilibrium experiments (Tables 1 and 2).

Saturation of [^{3}H]-clonidine binding

[^{3}H]-clonidine specific binding was saturable whether the non specific binding was measured in the presence of 10 μM noradrenaline or cirazoline (Figure 2a). The non specific binding of 7 nM [^{3}H]-clonidine determined in the presence of either 10 μM noradrenaline or 10 μM cirazoline represented 56 ± 3% and 27 ± 4% respectively of the total binding. Cirazoline alone or a mixture of cirazoline and noradrenaline inhibited the [^{3}H]-clonidine binding to the same extent indicating that noradrenaline-sensitive sites were part of the cirazoline-sensitive population. These data confirmed that, under our experimental conditions, 10 μM cirazoline inhibited [^{3}H]-clonidine binding to both noradrenaline-sensitive and insensitive sites. [^{3}H]-clonidine binding sites sensitive to (−)-noradrenaline will be referred to as α-adrenoceptor binding and those insensitive to (−)-noradrenaline and sensitive to

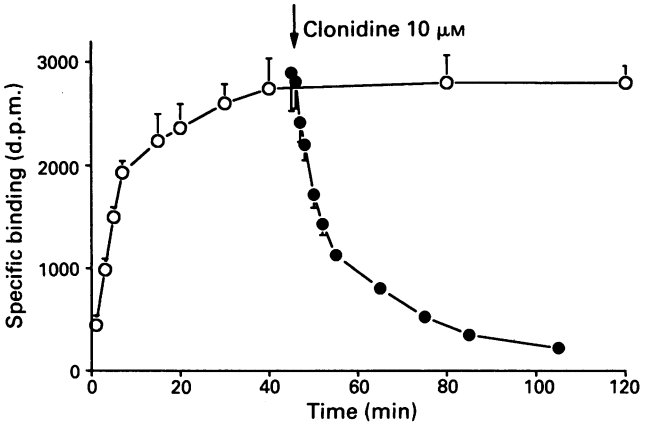

Figure 1 Kinetics of 6 nM [^{3}H]-clonidine binding to rabbit ventral medulla membranes at 25°C. Non specific binding was measured in the presence of 10 μM cirazoline subtracted from the total binding. Dissociation was obtained after 45 min equilibrium by addition of unlabelled clonidine to reach a final concentration of 10 μM. Values are means ± s.e.mean of 3 association or 4 dissociation independent experiments performed in duplicate.

Table 1 Kinetic constants of 6 nM [^{3}H]-clonidine binding to ventral medulla membranes from the rabbit brain

	Site 1	*Site 2*
k_{obs} (min^{-1})	0.098 ± 0.003	0.322 ± 0.015
k_{-1} (min^{-1})	0.036 ± 0.005	0.253 ± 0.004
K_D (nM)	3.5 ± 0.3	22 ± 0.1

Constants were calculated with the aid of a bi-exponential model computerized curve fitting procedure (McPherson, 1985). K_D were calculated according to the equation $K_D = k_{-1}/k_1$ with $k_1 = (k_{obs} - k_{-1})/[L]$. [L] being the concentration of the radioligand. Values are means ± s.e.mean of 3 association and 4 dissociation experiments.

Table 2 LIGAND analysis of data pooled from 5 experiments according to either a one- or two-site model

	One site analysis	*Two sites analysis*	
		Site 1	*Site 2*
IR + α-adrenoceptors			
K_D (nM)	6.9 ± 0.6	2.9 ± 2.9	19.8 ± 3.4
B_{max} (fmol mg^{-1} prot.)	89 ± 7	26 ± 64	94 ± 32
α-Adrenoceptors			
K_D (nM)	2.9 ± 0.7	2.9 ± 0.7	Set to
B_{max} (fmol mg^{-1} prot.)	39 ± 7	40 ± 8	zero
IR			
K_D (nM)	18.2 ± 0.4	Set to	18.2 ± 0.4
B_{max} (fmol mg^{-1} prot.)	66 ± 12	zero	66 ± 14

The specific binding was defined either as the difference between the binding of [^{3}H]-clonidine alone and the binding in the presence of 10 μM cirazoline (α-adrenoceptors ± IR), or binding of [^{3}H]-clonidine alone and binding in the presence of 10 μM noradrenaline (α-adrenoceptors), or binding of [^{3}H]-clonidine in the presence of 10 μM noradrenaline and binding in the presence of 10 μM cirazoline (IR). 'Set to zero' indicates that the data did not fit with a second site according to the computer programme.

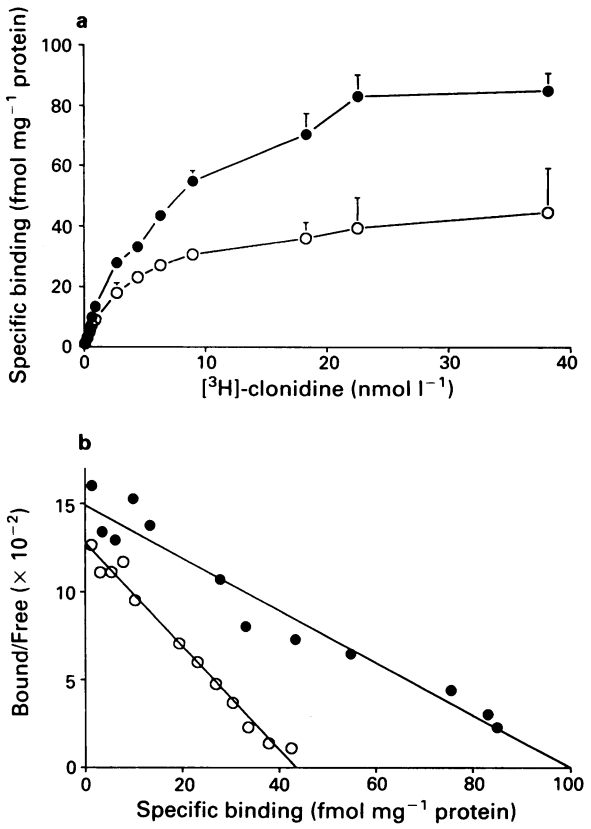

Figure 2 Saturation isotherms of [^{3}H]-clonidine specific binding to rabbit ventral medulla membranes. Specific binding was defined as the difference between binding of [^{3}H]-clonidine alone and binding in the presence of either 10 μM noradrenaline (○) or 10 μM cirazoline (●). (a) Values are means ± s.e.mean of 5 independent experiments performed in duplicate. (b) Scatchard transformation of the same data.

cirazoline defined as IRs. Thus the IRs represented the difference between [^{3}H]-clonidine binding measured in the presence of 10 μM (−)-noradrenaline and that measured in the presence of 10 μM cirazoline. When non specific binding was evaluated with (−)-noradrenaline or cirazoline, Scatchard analysis gave different K_D and B_{max} values (Figure 2b). For α-adrenoceptors, [^{3}H]-clonidine had a $K_D = 4.3 \pm 1.1$ nM for a $B_{max} = 47 \pm 6$ fmol mg^{-1} protein ($n = 5$). For cirazoline-sensitive sites (α-adrenoceptors + IRs) the K_D of [^{3}H]-clonidine was 8.3 ± 1.5 nM and the $B_{max} = 96 \pm 6$ fmol mg^{-1} protein. The differences were statistically significant ($P < 0.05$). In all cases the Hill coefficient was close to 1.

The difference in the K_D values for α-adrenoceptors and cirazoline-sensitive sites indicated that [^{3}H]-clonidine affinity was lower for IRs than for the α-adrenoceptors. To investigate further this point, a LIGAND analysis of the data pooled from five experiments was performed (Table 2).

(i) When specific binding was defined as the difference between the binding of [^{3}H]-clonidine in the presence of 10 μM cirazoline (α-adrenceptors + IRs) and in the absence of cirazoline, data matched both one- and two site models without significant statistical differences ($P = 0.09$). A one site model revealed a K_D and B_{max} in close agreement with the results of the Scatchard analysis ($K_D = 6.9 \pm 0.6$ nM, B_{max} of 89 ± 7.5 fmol mg^{-1} protein). The results obtained for a two site model matched those obtained in kinetic experiments (Table 2).

(ii) When the specific binding was defined as the difference between [^{3}H]-clonidine binding in the absence of (−)-noradrenaline and in the presence of 10 μM (−)-noradrenaline (α-adrenoceptors), the computerized iterative non-linear analysis of the binding data matched only a one site model with K_D and B_{max} close to the high affinity site data obtained with the LIGAND analysis for the total cirazoline specific binding or the Scatchard analysis of the α-adrenoceptors.

(iii) (−)-Noradrenaline-insensitive cirazoline-sensitive binding (IRs) was also analysed. The LIGAND programme matched only a one-site model with K_D and B_{max} values close to the low affinity site obtained with the LIGAND analysis of the total cirazoline specific binding.

The two sites thus defined closely matched those observed in kinetic experiments (Table 1 and 2).

Competition experiments

To check the selectivity of [^{3}H]-clonidine binding, competition experiments were performed with various compounds. IRs represented 20 to 30% of the total 5 to 6 nM [^{3}H]-clonidine cirazoline specific binding (Figure 3). Because the presence of an excess of noradrenaline usually led to a high non specific binding (about 65%), competition experiments were routinely analysed on the total specific binding (α-adrenoceptors + IRs). Substances were classified according to the maximum displacement observed at a concentration of 20 μM and three groups of compounds were revealed (Tables 3 and 4).

Group I: substances, including histamine and 5-hydroxytryptamine, totally unable to inhibit [^{3}H]-clonidine binding.

Group II: all the catecholamines and the compounds inhibiting 64 to 77% of the total [^{3}H]-clonidine specific binding with a distinct plateau in the competition curves. The

IC_{50} ranged from 6.5 ± 1 nM for (−)-adrenaline to 570 ± 190 nM for yohimbine. Substances, such as yohimbine or phenoxybenzamine, considered as α-adrenoceptor probes behaved like catecholamines (Table 3). Within this group the competition slopes were close to 1, with the exception of moxonidine, yohimbine and to a lesser extent α-methylnoradrenaline (Table 3).

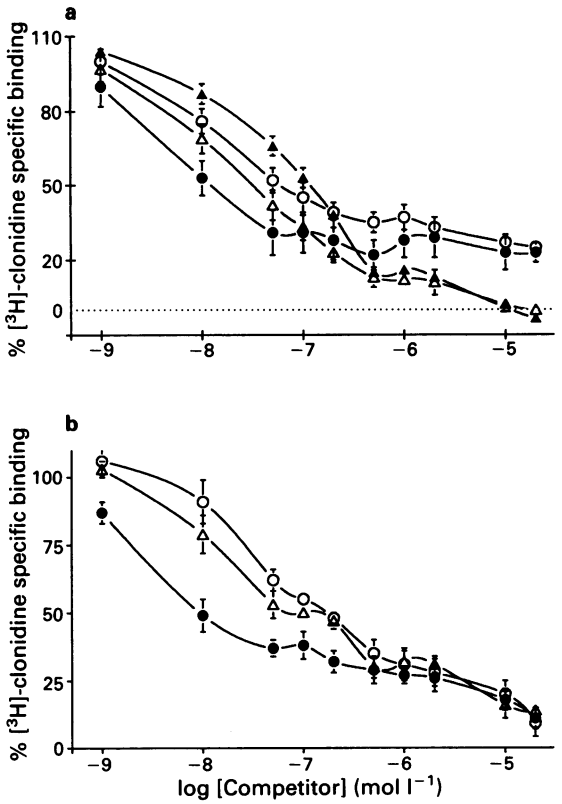

Figure 3 Competition curves between 5 to 7 nM [^{3}H]-clonidine and various compounds. Values are means ± s.e.mean of 4 to 6 independent experiments performed in duplicate. Non specific binding was defined as the binding measured in the presence of 10 μM cirazoline. In experiments with catecholamines, 0.005% ascorbic acid was present, without any effect on the [^{3}H]-clonidine binding. (a) (Δ) Clonidine; (▲) cirazoline; (●) (−)-adrenaline; (○) noradrenaline. (b) (○) Guanfacine; (Δ) phentolamine; (●) oxymetazoline.

Table 3 Maximum inhibition of [^{3}H]-clonidine specific binding observed with a 20 μM concentration of the compounds tested

	IC_{50} (nM)	*Slope*	I_{max} (%)	n
Group I				
Histamine			5 ± 5	4
5-Hydroxytryptamine			7 ± 7	4
Group II				
(−)-Adrenaline	6.5 ± 1	1.00 ± 0.10	77 ± 4	4
(−)-Noradrenaline	22 ± 6	0.80 ± 0.08	75 ± 3	5
α-Methylnoradrenaline	49 ± 15	0.72 ± 0.06	72 ± 3	5
Dopamine	643 ± 122	0.90 ± 0.08	72 ± 7	5
Phenoxybenzamine	21 ± 3	1.00 ± 0.10	64 ± 5	3
Moxonidine	81 ± 9	0.56 ± 0.03	77 ± 1	5
Yohimbine	568 ± 188	0.60 ± 0.07	68 ± 6	5

Specific binding was defined as the difference between binding of [^{3}H]-clonidine alone and binding in the presence of 10 μM cirazoline. In all cases there was a clear cut plateau of inhibition (Figure 3). Values were means ± s.e.mean of *n* independent experiments. IC_{50} and slopes were obtained by non linear curve fitting of experimental data to sigmoïdal curve with the aid of the EBDA programme. Non specific binding was considered as the maximal displacement observed with each compound.

Group III: compounds inhibiting almost completely the [^{3}H]-clonidine binding (α-adrenoceptors + IRs). This group consisted of the imidazolines and related compounds including oxazolines. Other non imidazoline substances, such as B-HT 920 or guanoxabenz, also inhibited this binding almost completely (Table 4). The order of potency of the drugs according to their IC_{50} was only possible to establish for the most abundant sites (i.e. α-adrenoceptors). Indeed, information as to the affinity of the substances for the IRs might only be obtained through comparison of the slopes of the competition curves. In contrast to group I compounds, only a few substances, (±)-idazoxan, cirazoline, ST 587, tolazoline and guanoxabenz, exhibited competition slopes close to 1 (Table 4), indicating that the affinities of these compounds for α-adrenoceptors and IRs should be of the same order of magnitude: (−)-idazoxan ≈ cirazoline > ST587 > tolazoline ≈ guanoxabenz. In all other cases, slopes differed markedly from 1 and the only information available was that the compounds were active at both sites within the concentration-range. Some substances like phentolamine, oxymetazoline and, to a lesser extent, guanfacine exhibited a maximum inhibition at the highest concentration used (20 μM), which was intermediate between those observed for group II and group III in spite of a low IC_{50} (Table 4). Competition curves obtained with these drugs were biphasic (Figure 3b). These data together with those of the two sites analysis of these curves (Table 5) confirmed that these compounds have low affinities for IRs (μM range) and high affinities for α_2-adrenoceptors (nM range).

To characterize further the low affinity noradrenaline insensitive binding, paired competition experiments were performed with cirazoline and a high [^{3}H]-clonidine concentration (20 nM) in the absence and in the presence of an excess of (−)-noradrenaline (30 μM). When the high concentration of [^{3}H]-clonidine was used, the IRs represented about 50% of the total specific binding whereas it was only 20–30% when the low concentration (6 nM) was used. The IC_{50} of cirazoline and slopes of the competition curves were 4,100 ± 1,200 nM with a slope of 0.76 ± 0.06 and 150 ± 74 nM with a slope of 0.72 ± 0.10 respectively in the absence and in the presence of

Table 4 Inhibition of 5 to 7 nM [^{3}H]-clonidine binding to membranes prepared from the ventral medulla of the rabbit brain: competition curves were established with 9 to 11 concentrations of each competitor

	IC_{50} (nM)	*Slope*	I_{max} (%)	n
Group III				
(±)-Idazoxan	85 ± 8	0.80 ± 0.26	106 ± 3	4
Cirazoline	100 ± 19	0.90 ± 0.13	103 ± 2	4
ST 587	625 ± 173	0.90 ± 0.13	101 ± 1	5
Tolazoline	1200 ± 600	0.90 ± 0.26	94 ± 3	5
Rilmenidine	113 ± 8	0.77 ± 0.11	98 ± 3	4
Guanoxabenz	1450 ± 320	1.00 ± 0.19	95 ± 3	4
BDF 6143	12 ± 3	0.50 ± 0.08	106 ± 3	5
UK-14304	22 ± 7	0.43 ± 0.11	104 ± 2	5
Clonidine	34 ± 10	0.61 ± 0.05	100 ± 2	5
B-HT 920	98 ± 15	0.66 ± 0.06	97 ± 3	4
(−)-Idazoxan	256 ± 28	0.60 ± 0.10	98 ± 4	4
Oxymetazoline	9.1 ± 3	0.50 ± 0.12	86 ± 4	6
Phentolamine	59 ± 28	0.70 ± 0.21	86 ± 2	4
Guanfacine	122 ± 29	0.54 ± 0.30	91 ± 5	6

IC_{50} and slopes, calculated by non linear curve fitting of experimental data to a sigmoid curve (EBDA programme, McPherson, 1985), were means ± s.e.mean of *n* independent experiments performed in duplicate. I_{max} represented the maximum specific inhibition observed with a 20 μM concentration of the inhibitor. Specific binding was defined as the difference between [^{3}H]-clonidine binding alone and binding measured in the presence of 10 μM cirazoline.

Table 5 Two sites analysis of competition curves for various compounds with competition slope lower than one (see Table 4)

	Large compartment			*Small compartment*	
	Size (%)	IC_{50} (nM)	K_i (nM)	IC_{50} (nM)	K_i (nM)
BDF 6143	65	2.5	0.8	316	242.5
UK- 14304	73	4	1.3	1995	1531.0
B-HT 920	64	40	13.0	631	484.3
(−)-Idazoxan	57	63	20.5	1584	1215.6
Phentolamine	66	19.9	6.5	3981	3055.2
Guanfacine	77	50	16.3	3162	2426.7
Oxymetazoline	78	4	1.3	3162	2426.7

Two sites analysis of mean competition curves were performed with the aid of Graph Pad software. K_i were derived from IC_{50} values according to Cheng & Prusoff (1972) considering the large compartment as the α-adrenoceptor one with a K_D of clonidine of 2.9 nM and the small one being the imidazoline specific one with a K_D of 19.8 nM.

(−)-noradrenaline ($n = 6$), suggesting that cirazoline had a higher affinity for the noradrenaline-insensitive binding sites of [^{3}H]-clonidine than for α_2-adrenoceptors.

Discussion

Since the existence of IRs was proposed on the basis of the discrepancies between the mechanisms of action of substances acting on α-adrenoceptors with an imidazoline structure and those with a phenylethylamine structure (Bousquet *et al.*, 1984), a great deal of data has subsequently confirmed this dichotomy. Binding experiments have demonstrated the existence of imidazoline specific sites independent of α-adrenoceptors and pharmacological experiments have described functional properties specific for imidazolines (Meeley *et al.*, 1986; Coupry *et al.*, 1987; Ernsberger *et al.*, 1987; 1990; Convents *et al.*, 1988; Wikberg, 1988; Bricca *et al.*, 1989a; Schultz & Hasselblatt, 1989; Zonnenschein *et al.*, 1990; Göthert & Moldering, 1991; Tibiriça *et al.*, 1991). However, the biochemical data and the pharmacological properties were never available from the same tissue and/or species. We had previously shown that the rabbit was very sensitive to clonidine-like substances the hypotensive effect of which was related to the interaction with IRs in the NRL region of the ventral brainstem (Feldman *et al.*, 1990), suggesting the presence of a high concentration of IRs. Therefore, it was of interest to characterize the [^{3}H]-clonidine binding in this species in brain areas where clonidine-like substances induced their vasodepressor effect. Here, we report evidence for the existence of two distinct [^{3}H]-clonidine binding sites, α-adrenoceptors and IRs, in the rabbit RVLM the latter being supposed to be specifically associated with the hypotensive properties of clonidine and related drugs.

In equilibrium experiments, [^{3}H]-clonidine specific binding defined by noradrenaline or cirazoline was saturable, but the maximum specific binding observed under each condition was markedly different. α-Adrenoceptors were less numerous than cirazoline-sensitive sites; however, in each case, saturation was reached, indicating that [^{3}H]-clonidine binding to IRs was also saturable. [^{3}H]-clonidine binding to α_2-adrenoceptors was of high affinity whereas IRs exhibited a lower affinity for [^{3}H]-clonidine as previously suggested (Bricca *et al.*, 1989b; Hamilton *et al.*, 1991).

Both sites could only be clearly revealed in kinetic experiments and by the use of different drugs to define the non specific binding. Each time it was possible (i.e. when the competitors disclosed two compartments) the Cheng and Prusoff equation was applied; K_i values were then calculated for each of them (Table 5). These data confirmed that all of the ligands fitting with these criteria exhibited their highest affinities for the α-adrenoceptor compartment as compared to their affinities for the IR compartment.

Several results indicated that α-adrenoceptors could be identified as α_2-adrenoceptors. First, group II compounds, which inhibited binding as much as (−)-noradrenaline, included specific α-adrenoceptor probes such as phenoxybenzamine and yohimbine. The rather low potency of yohimbine for inhibition of [^{3}H]-clonidine binding to α_2-adrenoceptors found in this study had already been observed in previous studies with [^{3}H]-clonidine; this observation might be related to the fact that yohimbine is an antagonist at a receptor where clonidine is an agonist (Hornung *et al.*, 1979; Salama *et al.*, 1982). Second, IC_{50} values measured for the different drugs mainly reflected the inhibitory potency at α-adrenoceptors. The order of potencies of the compounds considered altogether was close to that expected for α_2-adrenoceptors. Although moxonidine has an imidazoline moiety, it behaved in our binding assays as a selective α-adrenoceptor competitor. It did not inhibit [^{3}H]-clonidine binding more than noradrenaline itself, thus corroborating the pharmacological data indicating that this compound was a potent α_2-adrenoceptor agent (Armah, 1988; Armah *et al.*, 1988). Moreover, in the rabbit brainstem, according to Schlicker *et al.* (1990), moxonidine decreased blood pressure by acting on presynaptic noradrenergic structures i.e. by a completely different mechanism from that of clonidine (Connor & Finch, 1981; Bousquet *et al.*, 1983). This confirmed that the imidazoline structure alone was not a sufficient requirement for interaction with the IRs. That was already suggested by several groups who described highly selective α_2-adrenoceptor properties for RX 821002, the close analogue of idazoxan (Saulnier-Blache *et al.*, 1989; Langin *et al.*, 1990; Vauquelin *et al.*, 1990).

The specific IR binding was usually inhibited by imidazoline compounds or very close analogues such as rilmenidine, an oxazoline. But in this binding also, at least one exotic drug, B-HT 920 a thiazoloazepine, devoid of the imidazoline moiety, exhibited competitive properties. Just like clonidine and rilmenidine, B-HT 920 was able to induce a hypotensive effect when microinjected in the NRL (Bousquet *et al.*, 1983; 1984; Feldman *et al.*, 1990). Thus, the ability to inhibit [^{3}H]-clonidine binding to IRs paralleled the hypotensive activity. Moreover, the sensitivity to various chemical classes (Laduron, 1988), one of the criteria required to define a receptor which the IR did not yet fulfil, has been satisfied with the ability of B-HT 920 to inhibit [^{3}H]-clonidine binding to IRs.

Yohimbine and phenoxybenzamine were unable to compete with [^{3}H]-clonidine binding to IRs. Oxymetazoline and phentolamine proved rather weak and incomplete competitors for imidazoline binding sites. This had already been observed in other binding studies on IRs in peripheral tissues such as kidney, adipocytes as in the central nervous system (Bricca *et al.*, 1989a; Langin & Lafontan, 1989; Michel *et al.*, 1989). Therefore, these compounds were probably not the most adequate tools to define non specific binding in such studies on IRs using [^{3}H]-clonidine. In the present study, we have shown that cirazoline had a higher affinity for the IRs than for α-adrenoceptors. Cirazoline was consistently the most potent agent for preventing drug binding to imidazoline specific binding sites (MacKinnon *et al.*, 1989; Parini *et al.*, 1989; Bricca *et al.*, 1989a; Brown *et al.*, 1990; Wikberg & Uhlen, 1990). Furthermore, in rat and rabbit brain or human platelets, cirazoline has been shown to have a K_i for α_2-adrenoceptors of about 50 to 300 nM consistent with the inhibition constant observed in the present study (Van Meel *et al.*, 1981; Brown *et al.*, 1990; Diamant *et al.*, 1992). In any case, under our experimental conditions, a concentration of cirazoline of 10 μM was sufficient to prevent all [^{3}H]-clonidine bindings to IRs, thus making it the most reliable compound for this purpose.

The existence of two sites labelled by [^{3}H]-clonidine was confirmed in kinetic experiments where the experimental data fitted a bi-exponential model significantly better. In addition, the K_D calculated from kinetic parameters closely matched those obtained from saturation experiments (Table 1). The kinetic experiments also demonstrated that the binding of [^{3}H]-clonidine to both compartments defined by cirazoline was reversible.

We have observed that the percentage contribution of the IRs to total binding was increased by restricting the sample more specifically to the NRL area. This point also outlines the discrete distribution of IRs within the brainstem. Although [^{3}H]-clonidine was at a higher concentration in competition experiments (5 to 7 versus 1 to 3 nM), the proportion of IRs labelled in the rabbit brainstem with [^{3}H]-clonidine or [^{3}H]-*p*-aminoclonidine was lower than that found in the human brainstem (Meeley *et al.*, 1986; Bricca *et al.*, 1989a). This may be due to species differences.

In order to correlate the binding affinity of the imidazolines to the IRs and their hypotensive potency when microinjected within the NRL, precise measurements of the affinities were required. However, under the present conditions IC_{50} values mainly reflected the competitive potency for α_2-adrenoceptors which were considerably more numerous. The only compounds for which potency at IRs could be evaluated were those with competition slopes near 1. Only 5 compounds out of the 14 competitors tested satisfied this condition. Only cirazoline and ST 587 induced hypotension when injected into the NRL area, wheras idazoxan and tolazoline were considered to be antagonists. The very weak hypotensive effect of guanoxabenz when microinjected in the NRL might be due to its low affinity for the IR.

Further hypotensive competitors with adequate Hill numbers have to be analysed to confirm the possibility of the expected correlation. A previous attempt by Ernsberger *et al.* (1990) to correlate affinity for IRs and hypotensive activity in this area included compounds such as cimetidine and imidazole acetic acid which were completely inactive in our study. Moreover, these authors gave K_i values and this was impossible in our study for the reasons mentioned above.

In conclusion, we have demonstrated that:

(1) In the rabbit, a species very sensitive to the cardiovascular effects of imidazolines, [^{3}H]-clonidine binding experiments revealed the existence of binding sites specifically sensitive to imidazolines within the RVLM region supposed to be involved in their hypotensive effect as well as the presence of classical α_2-adrenoceptors.

(2) Despite a rather low affinity for the imidazoline specific receptors, [^{3}H]-clonidine remains one of the most efficient tools to study these receptors.

(3) Since both receptors were present in the membrane preparations, the only compounds for which affinity for IRs could be approximated in the absence of selective ligands were those with competition slopes close to 1. Only a few competitors exhibited this property, namely: cirazoline, ST 587, tolazoline, idazoxan and guanoxabenz.

(4) The ability of B-HT 920 to inhibit [^{3}H]-clonidine binding to IRs, allowed this binding site to fulfill an important criterion of a receptor; that is that substances of different chemical classes with the same pharmacological properties should be able to interact with the receptor.

We are grateful to Beiersdorf, Lers-Synthélabo and Servier Laboratories for the gifts of the drugs. We also wish to thank Dr Langley, INSERM Unit 44 - Strasbourg, for careful revision of this text.

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(Received April 23, 1993
Revised August 5, 1993
Accepted August 12, 1993)