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Identification and characterization of imidazoline-binding sites from calf striatum

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Abstract

'Non-adrenoceptor'-binding sites for [^{3}H]clonidine (I_1-sites) and [^{3}H]idazoxan (I_2-sites) are identified in calf striatum membranes. The pharmacological profile of both subtypes was investigated by competition binding with the imidazolines idazoxan, cirazoline, Bu 224 (2-(4,5-dihydroimidaz-2-yl)-quinoline) and Bu 239 (2-(4,5-dihydroimidaz-2-yl)-quinoxaline); the guanidino derivatives clonidine, moxonidine, guanabenz, amiloride and agmatine; the oxazoline rilmenidine and the imidazole histamine. The competition experiments indicate that both populations of imidazoline-binding sites in calf striatum consist of a high- (H) and a low-affinity (L) compartment. The monoamine oxidase (MAO) inhibitors pargyline (non-selective) and deprenyl (MAO-B-selective) have micromolar affinity for the I_1-sites and much lower affinity for the I_2-sites. The venom of the marine snail *Conus geographus* is the most potent of the 13 tested *Conus* venom preparations. None of the tested venoms is able to discriminate between both sites.

Keywords: Imidazoline-binding site; Striatum; (Calf); Monoamine oxidase; *Conus* venom

1. Introduction

Imidazoline-binding sites were discovered in the mid-1980s in the nucleus reticularis lateralis, a locus in the rostral ventrolateral part of the medulla oblongata (Bousquet et al., 1984), and have since been identified in different regions from the central nervous system (De Vos et al., 1994) as well as in peripheral tissues from various species (for a review, see Bousquet, 1996). Imidazoline-binding sites are now recognised to constitute a heterogenous family of sites with at least two members: the I_1-sites which are usually labeled by [^{3}H]clonidine and [^{3}H]*p*-amino-clonidine and I_2-sites which are preferentially labeled by [^{3}H]idazoxan. These subtypes not only differ from each other with respect to ligand selectivity (Ernsberger, 1992), but also on basis of their regional distribution (De Vos et al., 1994), subcellular distribution (Ernsberger et al., 1995; Limon et al., 1992; Piletz and Sletten, 1993; Tesson et al., 1991), structural properties (Greney et al., 1994a; Limon et al., 1992; Wang et al., 1992) and function (Buccafusco et al., 1995; Ernsberger et al., 1990; Olmos et al., 1994; Penner and Smyth, 1994)

I_1-sites (imidazoline receptors (IR) (Bricca et al., 1993), I_1-receptor (Piletz and Sletten, 1993)) display high affinity towards clonidine (Ernsberger, 1992), idazoxan (Bricca et al., 1993; De Vos et al., 1994) and cirazoline (Piletz and Sletten, 1993), and low affinity towards guanabenz and amiloride (Michel and Insel, 1989). These sites are involved in the centrally controlled vascular effects produced by locally administered imidazoline/guanidine compounds (Buccafusco et al., 1995; Ernsberger et al., 1993) and in insulin secretion from pancreatic β-cells (Berdeu et al., 1994). I_1-sites seem to be located at plasma membrane (Ernsberger et al., 1995) and the apparent molecular mass of the putative human brain I_1-site is of 43 kDa. Their recently reported purification opens new prospects for their biochemical characterization (Greney et al., 1994a).

I_2-sites (imidazoline-guanidinium-receptive sites (IGRS) (Tesson et al., 1991), non-adrenergic idazoxan-binding

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sites (NAIBS) (De Vos et al., 1994)) display high affinity for idazoxan (Ernsberger, 1992), guanabenz (Miralles et al., 1993; Wikberg et al., 1992) and cirazoline (Greney et al., 1994b), and medium-to-low affinity for clonidine (Ernsberger, 1992). Subtyping of I_2-sites has been suggested based on differential sensitivity towards the guanidine compound amiloride (I_{2A}-sites, high affinity towards amiloride; I_{2B}-sites, low affinity) (Michel and Insel, 1989). The functions of the I_2BS remain unclear, but it has been proposed that they play an active role in various physiological processes, like the regulation of glial fibrillary acidic protein synthesis in astrocytes (Olmos et al., 1994), natriuresis from renal proximal tubule cells (Bidet et al., 1990) and feeding behaviour (Jackson et al., 1995; McKinnon et al., 1995). The concentration of the I_2-sites is increased in frontal cortex of depressed suicide victims (Meana et al., 1993) and brains from patients with Alzheimer's disease (Ruiz et al., 1993) and they can be down-regulated by chronic treatment with antidepressant monoamine oxidase inhibitors (Alemany et al., 1995; Olmos et al., 1993). This reinforces the hypothesized correlation between I_2BS and the mitochondrial enzyme monoamine oxidase (MAO; EC 1.4.3.4) (Sastre and Garcia-Sevilla, 1993; Tesson et al., 1995). Several similarities between I_2-sites and monoamine oxidase have indeed been identified; i.e., subcellular (Tesson et al., 1991) and regional distribution (Sastre and Garcia-Sevilla, 1993) and amino-acid sequence homology between peptides from I_2-sites and several monoamine oxidase enzymes (Tesson et al., 1995).

The regional distribution of I_1- and I_2-sites in the human central nervous system has been investigated using quantitative autoradiography (De Vos et al., 1994) and the striatum was identified as one of the regions with the highest densities of both sites. Membrane preparations from this region were also found to contain appreciable levels of I_1- and I_2-sites and these preparations can conveniently be used to investigate the pharmacological profile of both sites in the human (Flamez et al., 1996). Imidazoline-binding sites have also been investigated in the bovine rostral ventrolateral medulla (Ernsberger et al., 1993, 1994; Haxhiu et al., 1994) and, in the present study, we identify and characterize them in membrane preparations of calf striatum using various imidazoline and guanidino compounds (for their pharmacological characterization), monoamine oxidase inhibitors (correlation between imidazoline-binding sites and monoamine oxidase) and different *Conus* venom preparations. The venoms of these tropical marine snails, belonging to the family of the *Conidae*, are mixtures of peptides that interact with diverse physiological targets, including ion channels and hormone and neurotransmittor receptors (Czerwiec et al., 1989, 1993; De Vos et al., 1991; Olivera et al., 1985, 1990, 1991). The present findings emphasize the many similarities between imidazoline-binding sites in the human and the bovine brain.

2. Materials and methods

2.1. Chemicals

[^{3}H]Idazoxan (1,4-[6,7(*n*)-^{3}H]benzodioxan-2yl)-2-imidazoline hydrocloride; 73 Ci/mmol) was obtained from Amersham (Little Chalfont, UK) and [^{3}H]clonidine [benzene ring-^{3}H]clonidine hydrocloride; 63.5 Ci/mmol) from New England Nuclear (Boston, MA, USA). *R*-Deprenyl · HCl (*R*-*N*-α-dimethyl-*N*-2-propynyl-benzeneethanamine hydrochloride), Ro 16-6491 · HCl (*N*-(2-aminoethyl)-4-chlorobenzamide hydrochloride), Ro 41-1049 · HCl (*N*-(2-aminoethyl)-5-(3-fluorophenyl)-4-thiazolecarboxamide hydrochloride) and RX 821002 (2-[2-(2-methoxy-1,4-benzodioxanyl)]imidazoline hydrochloride) were obtained from Research Biochemicals International (Natick, MA, USA). (*R*)-Adrenaline bitartrate, bovine serum albumin (BSA) (fraction V), clorgyline · HCl (*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxy)-propylamine hydrochloride) and pargyline · HCl (*N*-methyl-*N*-propynylbenzylamine hydrochloride) were obtained from Sigma (St. Louis, MO, USA). The following were generous gifts: Bu 224 · HCl (2-(4,5-dihydroimidaz-2-yl)-quinoline hydrochloride) and Bu 239 · HCl (2-(4,5-dihydroimidaz-2-yl)-quinoxaline hydrochloride) (Dr. D. Nutt, Bristol, UK), cirazoline (Synthélabo, France), *R*/*S*-idazoxan ((*R*/*S*)-2-(1,4-benzodioxan-2-yl)-2-imidazoline hydrochloride (Pierre Fabre Medicaments, France) and rilmenidine ((*N*-dicyclopropylmethyl)-amino-2-oxazoline) (Institut de Recherche Servier, France). All other chemicals were of the highest grade commercially available.

2.2. Membrane preparation

Calf brains were obtained from a local slaughterhouse within 2 h post-mortem and kept on ice until dissection. Brain samples were homogenized with an Ultraturrax and Potter Elvejhem homogenizer in 50 mM Tris-HCl (pH 7.4)/2 mM $MgCl_2$ at 4°C. All subsequent steps were carried out at 4°C. The homogenate was centrifuged at 30 000 × *g* for 20 min and pellets were resuspended in the same buffer. This procedure was repeated twice and the final pellet was resuspended in the above-mentioned buffer containing 10% glycerol (v/v). Membrane suspension was divided in 1-ml batches and stored in liquid nitrogen until use. Protein concentrations were determined according to the SopaChem total protein assay (Sopar Biochem) using bovine serum albumin as a standard.

2.3. Conus venom preparations

Specimens were taken alive: *C. aulicus*, *C. canonicus*, *C. eburneus* (var. *polyglotta*), *C. geographus*, *C. litteratus*, *C. marmoreus*, *C. mercator*, *C. miles*, *C. namocanus*, *C. rattus* and *C. vexillum* from the Philippines (near Cebu); *C. pulcher* from Senegal (near Dakar); and *C. anemone*

from south-western Australia. The gastropods were frozen, shipped to Brussels in dry ice via air and stored at −20°C until use. The following steps were carried out at 0–4°C. The venom ducts of *C. anemone*, *C. pulcher* and *C. tessulatus* were dissected out of the animals, the venom was squeezed out of the duct and homogenised in 10 volumes (w/v) 30 mM ammonium acetate with a Polytron mixer and sonicated 3 times for 10 s in a Soniprep 150 sonicator. Whole ducts were homogenised and sonicated for the other species. Suspensions were centrifugated at $9000 \times g$ for 10 min and the resulting supernatants stored at −20°C. Protein concentrations were determined according to the SopaChem total protein assay (Sopar Biochem) using bovine serum albumin as a standard.

2.4. Radioligand-binding assays

Incubations were performed in plastic 96-well plates in a final volume of 200 μl. Membrane suspensions (100 μl, typically about 100 μg protein) were incubated with the radioligand at 37°C for 15 min. RX 821002 (final concentration 5 μM) was added to the assay to mask α_2-adrenoceptors. In saturation experiments, final concentrations of [^{3}H]idazoxan or [^{3}H]clonidine ranging from 1 to 100 nM were used. In competition binding studies, [^{3}H]idazoxan was used in a final concentration of 7 nM and [^{3}H]clonidine in a final concentration of 8 nM. Concentrations of competitors typically ranged from 0.1 nM to 100 μM for drugs and from 0.1 to 100 μg protein/ml for *Conus* venoms. Specific binding was defined as control binding (i.e., in the presence of 5 μM RX821002 and no other competitor) minus non-specific binding (in the presence of 5 μM RX821002 and 100 μM cirazoline). Assay conditions for kinetic experiments were identical to those from competition studies. In kinetic association experiments, binding was monitored for periods up to 60 min. In dissociation experiments, membranes were incubated with radioligand for 15 min at 37°C and dissociation was started by adding 100 μM cirazoline (final concentration) to the assay mixture. Binding was measured at different time intervals up to 30 min.

Incubations were terminated by rapid filtration (under vacuum) of the samples through glass fibre filters (Whatman GF/C Printed Filtermat B from Wallac, pre-washed with ice-cold buffer) using a Skatron Cell Harvester. Filters were washed 4 times with ice-cold buffer (twice for 2 s followed by twice for 1 s), dried for 10 s in the harvester and in an oven for 1 h at 40°C. Filters were then sealed together with MeltiLex in a sample bag and radioactivity was counted in a BetaplateTM.

2.5. Binding data analysis

Binding experiments were performed in triplicate unless stated otherwise. Binding curves were calculated using non-linear curve-fitting (with binding parameters obtained by iteration with the Solver function from Microsoft Excel 3.0). Binding parameters were determined from the calculated curves using the same program. Association and dissociation constants were calculated using non-linear-regression analysis from GraphPad Prism. Values are expressed as means ± S.E.M.

3. Results

3.1. Identification of non-adrenergic [^{3}H]clonidine and [^{3}H]idazoxan imidazoline-binding sites

Competition binding experiments on [^{3}H]idazoxan and [^{3}H]clonidine binding in calf striatum membranes yielded clear biphasic curves with the α_2-agonist adrenaline and the selective α_2-antagonist RX 821002 (Fig. 1A,B). Part of the [^{3}H]clonidine binding is displaced with high affinity by adrenaline ($IC_{50H} = 81 \pm 19$ nM, 41 ± 7%) and RX 821002 ($IC_{50H} = 21 \pm 5$ nM, 47 ± 2%) (Table 1). For [^{3}H]idazoxan, 38 ± 2% of the sites have high affinity for adrenaline ($IC_{50H} = 270 \pm 95$ nM) and 46 ± 2% for RX 821002 ($IC_{50H} = 63 \pm 17$ nM). These high-affinity sites correspond to α_2-adrenoceptors. When these sites are shielded with 5 μM RX821002, cirazoline is still capable of displacing part of the remaining binding (see below). These 'non-adrenergic' sites which are displaced by 100 μM cirazoline were defined as I_1-sites (for [^{3}H]clonidine) and I_2-sites (for [^{3}H]idazoxan), respectively. These sites have low to very low affinity for RX 821002 and adrenaline (Fig. 1).

Saturation binding experiments yielded the following parameters: estimated K_d of [^{3}H]clonidine for the I_1-sites

Table 1
Parameters of adrenaline and RX 821002 for competition with [^{3}H]clonidine and [^{3}H]idazoxan binding in calf striatum membranes

	[^{3}H]Clonidine (8 nM)			[^{3}H]Idazoxan (7 nM)		
	$\alpha_2 + I_1$-sites			$\alpha_2 + I_2$-sites		
Competitor	IC_{50H} (nM)	IC_{50L} (nM)	% R_H	IC_{50H} (nM)	IC_{50L} (nM)	% R_H
(*R*)-Adrenaline	81 ± 19	$> 10^4$	41 ± 7	270 ± 95	$> 10^4$	38 ± 2
RX 821002	21 ± 5	$> 10^4$	47 ± 2	63 ± 17	$> 10^4$	46 ± 2

[^{3}H]Clonidine and [^{3}H]idazoxan binding represents binding to both α_2-adrenoceptors and imidazoline binding sites. The IC_{50} values and relative amounts of high-affinity sites (% R_H) refer to the competition curves in Fig. 1A,B. Values are means ± S.E.M. of 3 experiments.

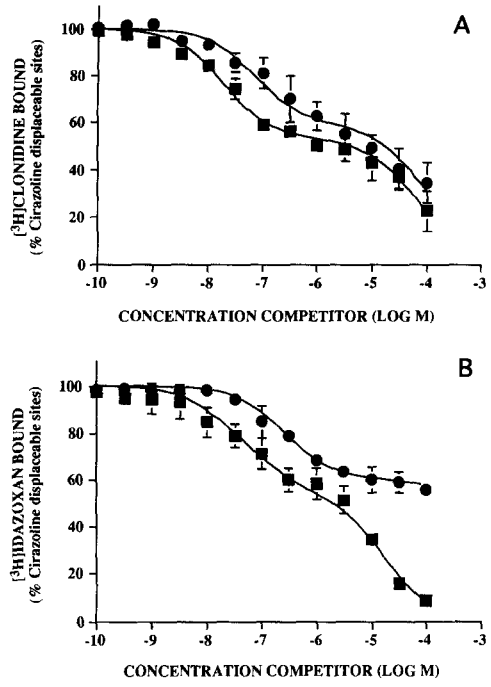

Fig. 1. Adrenaline and RX 821002 competition binding on [^{3}H]clonidine and [^{3}H]idazoxan binding to α_2-adrenoceptors and imidazoline-binding sites in calf striatum membranes. Membranes were incubated with 8 nM [^{3}H]clonidine (A) and 7 nM [^{3}H]idazoxan (B) and increasing concentrations of (*R*)-adrenaline (●) and RX 821002 (■). Binding is expressed in percentage control binding, i.e. binding in the presence of buffer only. Non-specific binding was measured in the presence of 0.1 mM cirazoline. Table 1 lists the IC_{50} values from the different curves. Values are means and bars are S.E.M. of 3 experiments.

$= 67 \pm 22$ nM and $B_{max} = 156 \pm 7$ fmol/mg protein ($n = 3$); estimated K_d of [^{3}H]idazoxan for the I_2-sites = 87 ± 26 nM and and $B_{max} = 203 \pm 22$ fmol/mg protein ($n = 3$) (Table 2). The accuracy of these values is limited by the fact that the highest used concentrations of radioligand (100 nM) were still in the range of the estimated K_d. The K_d values, calculated on basis of association and dissociation rate constants measured in kinetic experiments (Fig. 2A,B) are in reasonable agreement with those estimated from the saturation experiments (Table 2).

Table 2
Binding parameters for [^{3}H]clonidine (I_1-sites) and [^{3}H]idazoxan (I_2-sites) in calf striatum membranes

	I_1-sites	I_2-sites
Kinetic experiments		
k_{-1} (min^{-1})	0.22±0.08	1.4±0.1
k_{+1} (10^6 $M^{-1} \cdot min^{-1}$)	3.1±0.3	30±4
K_d (nM)	70	47
Saturation experiments		
K_d (nM)	67±22	87±26
B_{max} (fmol/mg)	156±7	203±22
n_H	1.0±0.1	1.0±0.1

α_2-Adrenoceptors were masked by the addition of 5 μM RX 821002. Estimated K_d values obtained in saturation experiments are compared to values calculated from association and dissociation contants obtained in kinetic experiments. The maximal amount of binding sites (B_{max}) and the Hill coefficients (n_H) are calculated from saturation experiments. Values are means ± S.E.M. of 3 experiments.

3.2. Pharmacological characteristics of the imidazoline-binding sites

Competition binding experiments were performed with: (a) the imidazoline compounds cirazoline, idazoxan, Bu 224 (2-(4,5-dihydroimidaz-2-yl)-quinoline) and Bu 239 (2-(4,5-dihydroimidaz-2-yl)-quinoxaline); (b) the guanidino derivatives clonidine, guanabenz, moxonidine, amiloride and agmatine (i.e., decarboxylated arginine); (c) the oxazoline compound rilmenidine; and (d) the imidazole compound histamine (Figs. 3–5). Table 3 lists their K_i values for the I_1- and I_2-sites.

Cirazoline/[^{3}H]clonidine and cirazoline/[^{3}H]idazoxan competition binding curves are very similar. They are both shallow and the displacement is only maximal at 100 μM cirazoline. When this concentration of cirazoline is used to determine the non-specific binding, the competition curves are readily analysed in terms of two sites. The majority of the I_1- and I_2-sites possess high affinity for cirazoline

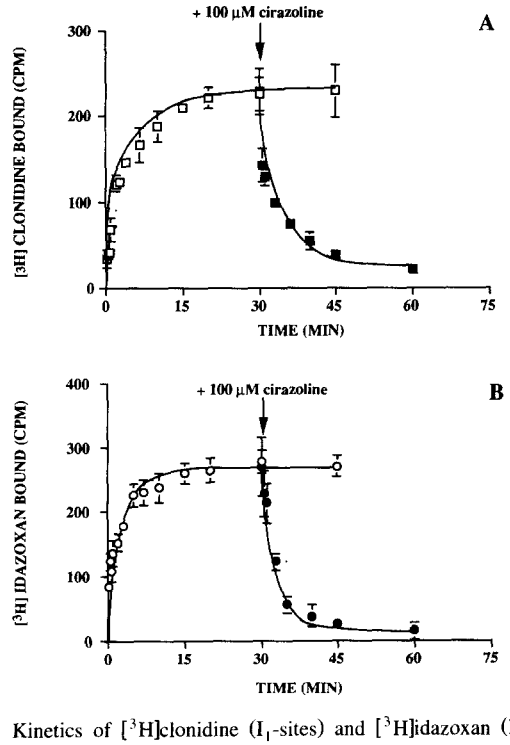

Fig. 2. Kinetics of [^{3}H]clonidine (I_1-sites) and [^{3}H]idazoxan (I_2-sites) binding to imidazoline-binding sites in calf striatum. (A) Time course of association (□) and dissociation (■) of [^{3}H]clonidine (8 nM). (B) Time course of association (○) and dissociation (●) of [^{3}H]idazoxan (7 nM). Binding to α_2-adrenoceptors was masked by the addition of 5 μM RX821002. Binding is expressed in cpm-specific binding (i.e. binding in the presence of 5 μM RX821002 minus non-specific binding measured in the presence of 0.1 mM cirazoline). Table 2 lists the association and dissociation rate constants from the curves. Values are means and bars are S.E.M. of 3 experiments.

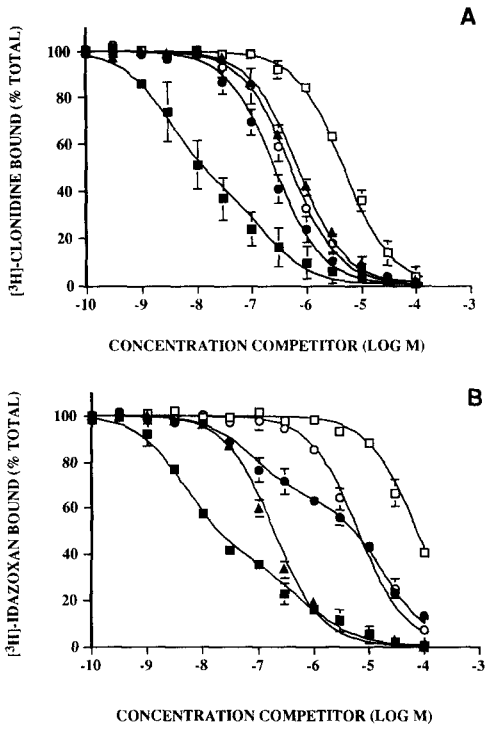

Fig. 3. Competition binding on [^{3}H]clonidine (I_1-sites) and [^{3}H]idazoxan (I_2-sites) binding in calf striatum membranes. Calf striatum membranes were incubated with 8 nM [^{3}H]clonidine (A) and 7 nM [^{3}H]idazoxan (B) and increasing concentrations of cirazoline (■), clonidine (●), (*R*/*S*)-idazoxan (▲), rilmenidine (○) and moxonidine (□). Binding to α_2-adrenoceptors was masked by the addition of 5 μM RX821002. Binding is expressed in percentage of control binding (i.e. binding in the presence of 5 μM RX821002 and buffer minus non-specific binding measured in the presence of 0.1 mM cirazoline). Table 3 lists the K_i values from the different curves. Values are means and bars are S.E.M. of 3 experiments.

(65 ± 1 and 61 ± 5% of the displaceable sites, respectively) and the K_{iH} values are in the nanomolar range (K_{iH} = 3.9 ± 0.35 and 4.6 ± 0.1 nM, respectively) (Table 3, Fig. 3A,B). The remaining displaceable sites possess 50- and 250-fold, respectively, lower affinity for cirazoline (Table 3).

Idazoxan/[^{3}H]clonidine and idazoxan/[^{3}H]idazoxan competition curves are steep (n_H = 0.86 ± 0.04 and 0.88 ± 0.03, respectively) and can be analysed in terms of a single population of sites; the K_i values are 570 ± 180 and 200 ± 25 nM, respectively (Fig. 3A,B, Table 3). The competition binding characteristics of the newly synthesized imidazoline compounds Bu 224 and Bu 239 are quite similar to those of cirazoline. For both the I_1- and I_2-sites, these compounds display biphasic curves with about 65% of the sites displaying high affinity (Fig. 4A,B). Bu 224 does not discriminate between the high-affinity sites of I_1- and I_2-sites: K_{iH} = 31 ± 8 and 31 ± 9 nM, respectively. On the other hand, there is a limited selectivity of Bu 239 for the high-affinity sites of I_2-sites: K_{iH} = 230 ± 90 nM for competing with [^{3}H]clonidine vs. K_{iH} = 71 ± 8 nM for competing with [^{3}H]idazoxan (Table 3).

Clonidine, the prototype of the guanidino compounds, displays steep competition binding for the [^{3}H]clonidine-labeled I_1-sites (n_H = 0.93 ± 0.01, K_i = 220 ± 50 nM) whereas the clonidine/[^{3}H]idazoxan competition binding curve is clearly shallow and best analysed in terms of two sites (K_{iH} = 190 ± 5 nM, % R_H = 62 ± 4%) (Fig. 3A,B, Table 3). Guanabenz has a similar behaviour towards both subtypes of the calf striatum imidazoline-binding sites as clonidine. The I_1-sites behave as a single population of sites for guanabenz and its potency (K_i = 240 ± 30 nM) is similar to the potency of clonidine. The I_2-sites behave as a heterogenous population of sites for guanabenz (K_{iH} = 87 ± 4 nM, R_H = 63 ± 2%) (Fig. 4A,B, Table 3). Amiloride, on the other hand, is able to divide the I_1- and I_2-sites into two populations with different affinity for this compound; 65 ± 4% of I_1-sites display high, but micromolar affinity (K_{iH} = 1550 ± 450 nM) and 63 ± 2% of the I_2-sites display high affinity (K_{iH} = 500 ± 70 nM). The guanidino derivative, moxonidine, is regarded to be a highly selective I_1-agonist that mediates its effects through I_1-sites in the central nervous sytem (Haxhiu et al., 1994). Yet, moxonidine displays only micromolar affinity for the I_1-sites in calf striatum membranes (K_i = 5.0 ± 0.9 μM) and its K_i value for the I_2-sites exceeds the limit of detection. Agmatine (decarboxylated arginine) has been suggested to be the endogenous ligand for IBS (Li et al., 1994). However, this compound has a very low affinity for

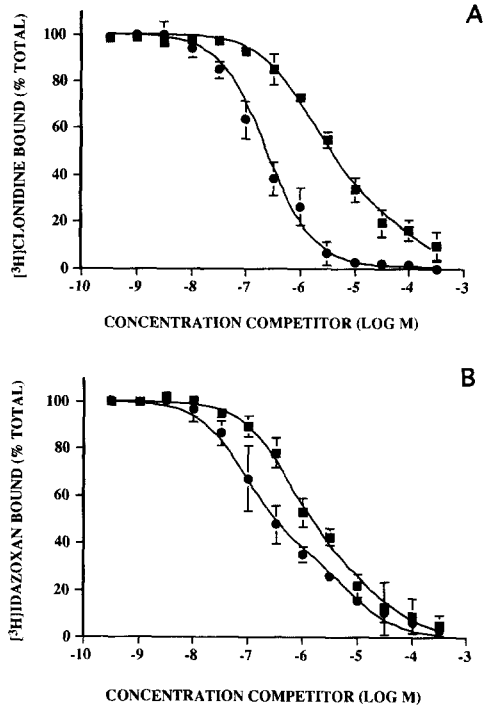

Fig. 4. Competition binding on [^{3}H]clonidine (I_1-sites) and [^{3}H]idazoxan (I_2-sites) binding in calf striatum membranes. Calf striatum membranes were incubated with 8 nM [^{3}H]clonidine (A) and 7 nM [^{3}H]idazoxan (B) and increasing concentrations of amiloride (■) and guanabenz (●). Binding to α_2-adrenoceptors was masked by addition of 5 μM RX821002. Binding was measured and expressed as in Fig. 3. Table 3 lists the K_i values from the different curves. Values are means and bars are S.E.M. of 3 experiments.

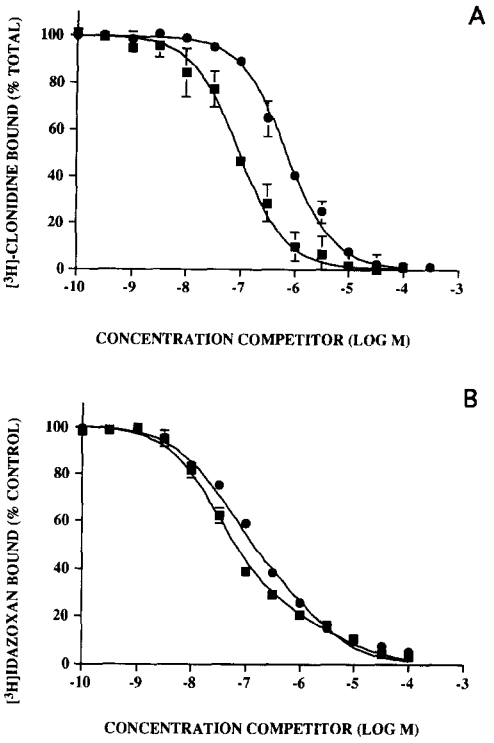

Fig. 5. Competition binding on [^{3}H]clonidine (I_1-sites) and [^{3}H]idazoxan (I_2-sites) binding in calf striatum membranes. Calf striatum membranes were incubated with 8 nM [^{3}H]clonidine (A) and 7 nM [^{3}H]idazoxan (B) and increasing concentrations of Bu 224 (■) and Bu 239 (●). Binding was measured and expressed as in Fig. 3. Table 3 lists the K_i values from the different curves. Values are means and bars are S.E.M. of 3 experiments.

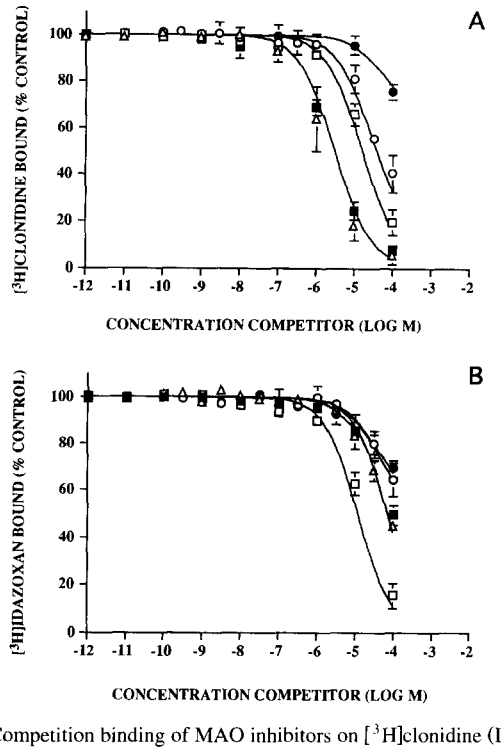

Fig. 6. Competition binding of MAO inhibitors on [^{3}H]clonidine (I_1-sites) and [^{3}H]idazoxan (I_2-sites) binding in calf striatum membranes. Calf striatum membranes were incubated with 8 nM [^{3}H]clonidine (A) and 7 nM [^{3}H]idazoxan (B) and increasing concentrations of pargyline (■), clorgyline (□), (*R*)-deprenyl (△), Ro 16-6491 (○) and Ro 41-1049 (●). Binding was measured and expressed as in Fig. 3. Table 4 lists the K_i values from the different curves. Values are means and bars are S.E.M. of 3 experiments.

I_1-sites as well as I_2-sites ($K_i > 100$ μM) under our assay conditions.

The oxazoline compound rilmenidine displays steep competition binding curves for the I_1- and I_2-sites ($n_H = 0.83 \pm 0.06$ and 0.84 ± 0.01, respectively) which can be analysed in terms of one single site (Fig. 3A,B). Of the compounds tested, rilmenidine is the most discriminatory one: it displaces [^{3}H]clonidine binding with a 10-fold higher affinity ($K_i = 590 \pm 130$ nM) than [^{3}H]idazoxan binding ($K_i = 6900 \pm 850$ nM). The imidazole histamine has a very low affinity for the I_1- and I_2-sites ($K_i > 100$ μM).

Table 3
Competition binding with [^{3}H]clonidine (I_1-sites) and [^{3}H]idazoxan (I_2-sites) binding in calf striatum membranes

	[^{3}H]Clonidine (8 nM) I_1-sites				[^{3}H]Idazoxan (7 nM) I_2-sites			
Competitor	$K_{i1\ site}$ (nM)	K_{iH} (nM)	K_{iL} (nM)	% R_H	$K_{i1\ site}$ (nM)	K_{iH} (nM)	K_{iL} (nM)	% R_H
Cirazoline		3.9 ± 0.4	190 ± 40	65 ± 1		4.6 ± 0.9	1200 ± 670	61 ± 5
(*R,S*)-Idazoxan	570 ± 180			100	200 ± 25			100
Clonidine	220 ± 50			100		190 ± 52	> 10^4	62 ± 4
Rilmenidine	590 ± 130			100	6900 ± 850			100
Moxonidine	5000 ± 880			100	> 10^4			
Guanabenz	240 ± 30			100		87 ± 4	7600 ± 2000	63 ± 2
Amiloride		1550 ± 450	> 10^4	65 ± 4		500 ± 70	> 10^4	63 ± 2
Bu 224		31 ± 8	1700 ± 300	61 ± 3		31 ± 9	8300 ± 3200	68 ± 8
Bu 239		230 ± 90	6400 ± 700	66 ± 4		71 ± 8	3200 ± 850	69 ± 4
Agmatine	> 10^4				> 10^4			
Histamine	> 10^4				> 10^4			

The K_i values and relative amounts of high-affinity sites (% R_H) refer to the competition curves in Fig. 3A,B for cirazoline, (*R/S*)-idazoxan, clonidine, rilmenidine and moxonidine, Fig. 4A,B for guanabenz and amiloride and Fig. 5A,B for Bu 224 and Bu 239. Binding to α_2-adrenoceptors was masked by the addition of 5 μM RX821002. Values are means ± S.E.M. of 3 experiments.

Table 4
Parameters of irreversible (pargyline, clorgyline and (*R*)-deprenyl) and reversible (Ro 16-6491 and Ro 41-1049) MAO-inhibitors for competing with [^{3}H]clonidine (I_1-sites) and [^{3}H]idazoxan (I_2-sites) labeled sites in calf striatum membranes

		[^{3}H]Clonidine (8 nM) I_1-sites		[^{3}H]Idazoxan (7 nM) I_2-sites	
	Competitor	K_i (μM)	n_{H*}	K_i (μM)	n_{H*}
Non-selective	Pargyline	2.7 ± 0.9	0.8 ± 0.1	57 ± 25	
MAO-A	Clorgyline	28 ± 9	0.95 ± 0.1	13 ± 3	0.8 ± 0.1
	Ro 41-1049	> 100		> 100	
MAO-B	*R*-Deprenyl	2.5 ± 0.6	0.9 ± 0.1	51 ± 24	
	Ro 16-6491	44 ± 12		> 100	

The K_i and n_H values refer to the competition curves in Fig. 5A,B. Binding to α_2-adrenoceptors was shielded by the addition of 5 μM RX 821002. Values are means ± S.E.M. of 3 experiments.
* Hill coefficients are given for compounds that displaced more > 80% of control binding at a concentration of 0.1 mM.

3.3. Imidazoline-binding sites and monoamine oxidase inhibitors

[^{3}H]idazoxan-binding sites have been related to the mitochondrial enzyme monoamine oxidase, and evidence for this correlation has increased with ongoing research. To investigate the relation between imidazoline-binding sites from calf striatum and monoamine oxidase, we tested different selective (monoamine oxidase-A (MAO-A): clorgyline, Ro 41-1049 (*N*-(2-aminoethyl)-5-(3-fluorophenyl)-4-thiazolecarboxamide); monoamine oxidase-B (MAO-B): *R*-deprenyl, Ro 16-6491 (*N*-(2-aminoethyl)-4-chlorobenzamide)) monoamine oxidase inhibitors on [^{3}H]idazoxan and [^{3}H]clonidine binding (Fig. 6A,B, Table 4). Competition curves of the sufficiently potent monoamine oxidase inhibitors all fit with a one-site model.

The non-selective inhibitor pargyline and the MAO-B-selective inhibitors deprenyl and Ro 16-16491 show a selectivity for the I_1-sites. The affinity of the two most potent compounds, the irreversible propargylamines pargyline and deprenyl, for the I_1-sites is only in the micromolar range (K_i = 2.7 ± 0.9 and 2.5 ± 0.6 μM, respectively) but their affinity ratio between I_1- and I_2-sites exceeds 40-fold for pargyline and is about 20-fold for deprenyl. No such ratio could be calculated for Ro 16-16491 because of its low affinity for I_1-sites and very low affinity for I_2-sites.

The tested MAO-A-selective inhibitors are unable to discriminate between both sites. Clorgyline possesses comparable affinity for the I_1- and I_2-sites (K_i = 28 ± 9 and 13 ± 3 μM, respectively) while Ro 41-1049 has a very low affinity for both sites (K_i > 100 μM).

3.4. Imidazoline-binding sites: effect of crude Conus venom preparations

13 different crude *Conus* venom preparations were tested for their capability to interact with I_1- and I_2-sites in

Table 5
Parameters of crude *Conus* venoms for competing with [^{3}H]clonidine (I_1-sites) and [^{3}H]idazoxan (I_2-sites) binding in calf striatum membranes and [^{3}H]idazoxan binding in calf retina membranes

Species	Calf striatum		Calf retina			
	I_1-sites	I_2-sites	α_2 [b]	I_2/I_1 [c]	α_2/I_1 [c]	α_2/I_2 [c]
	IC_{50} [a]	IC_{50} [a]	IC_{50} [a]			
C. geografus	23 ± 6	55 ± 8	18 ± 1	2.5	1	0.3
C. vexillum	31 ± 9	135 ± 46	52 ± 9	4.5	2	0.4
C. litteratus	70 ± 25	96 ± 5	–	1.5	> 3.6	> 2.6
C. eburneus	73 ± 15	78 ± 15	18 ± 2	1	0.2	0.2
C. rattus	82 ± 28	170 ± 20	–	2	> 3	> 1.5
C. aulicus	93 ± 37	100 ± 10	18 ± 2	1	0.2	0.2
C. mercator	95 ± 15	213 ± 66	102 ± 14	2	1	0.5
C. namocanus	108 ± 43	220 ± 60	147 ± 12	2	1.5	0.7
C. pulcher	113 ± 58	170 ± 30	–	1.5	> 2	> 1.5
C. marmoreus	123 ± 19	112 ± 38	–	1	> 2	> 2.2
C. anemone	155 ± 15	245 ± 55	–	1.5	> 1.5	> 1
C. miles	–	–	–	–	–	–
C. canonicus	–	87 ± 4	57 ± 2	< 0.3	< 0.2	0.7

[a] IC_{50} values are given in μg protein/ml.
[b] Calf retina membranes contain < 10% of imidazoline binding sites (Czerwiec, unpublished results) and [^{3}H]idazoxan binding can be considered as occurring to α_2-adrenoceptors alone (Convents et al., 1987).
[c] I_2/I_1, α_2/I_1, α_2/I_1 refers to the ratio of IC_{50} values for the respective binding sites and receptors. –, IC_{50} value > 250 μg protein/ml.

calf striatum and with α_2-adrenoceptors ([^{3}H]idazoxan binding) in calf retina membranes (Table 5). The amount of I_1- and I_2-sites is negligeable (< 10% of the α_2-adrenoceptors) in this latter tissue. Only two venoms display relatively high potency ($IC_{50} \leq 30$ μg protein/ml) for displacing [^{3}H]clonidine binding to the I_1BS: *C. geografus* (23 ± 6 μg protein/ml) and *C. vexillum* (31 ± 9 μg protein/ml). These venoms are 2- and 4-fold, respectively, less potent in displacing [^{3}H]idazoxan binding to the I_2-sites but about equally potent in displacing [^{3}H]idazoxan binding to the α_2-adrenoceptors ($IC_{50} = 18 \pm 1$ and 52 ± 9 μg protein/ml, respectively). The other venoms display IC_{50} values of > 70 μg protein/ml for both I_1- and I_2-sites and are not capable of relevant discrimination between these two sites. Some of these latter venoms show preference for the α_2-adrenoceptors: *C. eburneus* (18 ± 2 μg protein/ml) and *C. aulicus* (18 ± 2 μg protein/ml).

4. Discussion

Imidazoline-binding sites are commonly defined as the non-adrenergic, non-serotonergic, cirazoline-displaceable binding sites of radioligands, such as [^{3}H]clonidine and [^{3}H]*p*-aminoclonidine (for I_1-sites) and [^{3}H]idazoxan (for I_2-sites) (Ernsberger, 1992). These sites have already been identified in different regions from the central nervous sytem in human and rat (De Vos et al., 1994; King et al., 1995) and quantitative autoradiographic studies revealed that both I_1- and I_2-sites are present at fairly high concentrations in the human striatum (De Vos et al., 1994). Moreover, their pharmacological profile appears to be similar to that of the imidazoline-binding sites from the human nucleus reticularis lateralis (Bricca et al., 1994). In the present study, we show that I_1- and I_2-sites are also present in membrane preparations from calf striatum and that they show a more distinct heterogeneity with respect to several compounds as in human striatum.

Saturation binding experiments with [^{3}H]clonidine and competition binding experiments with unlabeled clonidine (Tables 1 and 3) indicate that the I_1-sites in calf striatum behave as a single class of sites with regard to this ligand. However, competition binding curves of cirazoline, Bu 224, Bu 239 and amiloride are clearly biphasic, with about 65% of the sites displaying high affinity for these compounds (Table 3) and control experiments wherein the control binding was measured in the presence of 0.1 μM cirazoline (data not shown) indicate that the sites with low affinity for cirazoline also display low affinity for the other compounds. The I_1-sites in calf striatum are, therefore, likely to comprise two distinct populations of sites, one with high affinity and one with low affinity for these latter compounds. They will be further denoted as 'I_1(H)' and 'I_1(L)'. The I_1-sites have already been shown to be heterogeneous with respect to cirazoline in the human nucleus reticularis lateralis (Bricca et al., 1994) but studies in human striatum revealed the presence of a single population of I_1-sites with high affinity for cirazoline (Bricca et al., 1994; De Vos et al., 1994). The present findings on calf striatum indicate the existence of species-related differences.

The binding profile of the I_1(H)-sites in calf striatum is in reasonable agreement with those reported for the I_1-sites in human striatum (De Vos et al., 1994; Flamez et al., 1996). The potencies of compounds, such as Bu 224 and cirazoline, and of the centrally acting hypotensive drugs clonidine, rilmenidine and moxonidine are indeed very similar for both sites. The largest differences occur for idazoxan and Bu 239, which were about 10 times less potent for the I_1(H)-sites. Based on these similarities, it is reasonable to assume that I_1(H)-sites in calf striatum correrspond to I_1-sites in human striatum and that differences in the potency of compounds, such as idazoxan and Bu 239, are species-related. The difference between the binding profile of the I_1(L)-sites in calf striatum and I_1-sites in human striatum is more pronounced, especially because of the very low affinity of the I_1(L)-sites for compounds, such as cirazoline, Bu 224 and Bu 239. Although this suggests that the I_1(H)-sites are unrelated to the I_1(L)-sites in calf striatum, it cannot yet be excluded that both represent different conformational states of a same molecule or molecular complex. Further investigations, including the isolation and detailed characterization of both binding sites, are clearly needed to solve this ambiguity.

The antihypertensive compound moxonidine has received special attention in previous binding studies on I_1-sites because it has been claimed to exert its actions through centrally located I_1-sites and to display considerable selectivity towards the I_1-sites over the α_2-adrenoceptors (Ernsberger et al., 1993). The experiments of Ernsberger and coworkers on the [^{3}H]clonidine-labeled I_1-sites in the bovine rostral ventrolateral medulla detect indeed high affinity for this compound. However, results obtained in human brain with moxonidine are not in line since neither in the human nucleus reticularis lateralis nor in the human striatum that high-affinity site is found for this compound (Bricca et al., 1994). Similar to the findings in human striatum, this present study indicates that moxonidine displays only low affinity in for the I_1-sites in calf striatum.

The I_2-sites in calf striatum behave as a single class of sites for idazoxan, but the existence of two or more populations is readily revealed by the shallow competition binding curves of cirazoline, Bu 224, Bu 239, amiloride as well as of clonidine and guanabenz (Tables 1 and 3), with about 65% of the sites displaying high affinity for these compounds (Table 3). In fact, except for rilmenidine, all tested imidazoline and guanidino derivatives detect heterogeneity of the I_2-sites. It is, however, possible that due to the very low affinity of rilmenidine, a second population with an even lower affinity remains undetected.

Heterogeneity of the I_2-sites has formerly also been

reported to occur in various tissues, including human and rat cortex (Flamez et al., 1996; Miralles et al., 1993), and the heterogeneity of these sites with respect to clonidine has gained special attention. This heterogeneity was in marked contrast with the uniformly high affinity of the I_1-sites for idazoxan and several models have been proposed to explain both findings. One of the proposed models regards the I_2-sites as a collection of non-adrenergic binding sites which include I_1-sites (De Vos et al., 1994). In an alternative model, it was considered that allosteric interactions occur between multiple binding sites on one macromolecular complex in which idazoxan controls the affinity for clonidine but not the reverse (De Vos et al., 1994; Rangel et al., 1993; Wikberg et al., 1992).

A striking difference between the I_2-sites in calf and human striatum is that the latter has uniformly high affinity for compounds, such as cirazoline, Bu 224 and Bu 239. The I_2-sites in calf striatum appear, therefore, to be even more complex than those in the human. Taken together, the present data suggest that [^{3}H]clonidine and [^{3}H]idazoxan bind to non-adrenergic sites in calf striatum which correspond to the I_1- and I_2-sites in human striatum as well as to an additional site which is characterized by its high affinity for both radioligands but by its low affinity for compounds, such as cirazoline. As these sites have not been characterized thoroughly, it is presently unclear whether they correspond to the same molecular entity and even whether they have any pharmacological significance.

Both isoforms of the mitochondrial enzyme monoamine oxidase (i.e., MAO-A and MAO-B) are present in most regions from the CNS (Saura et al., 1992) and a link between them and I_2-sites has been recognised, based on several observations on the subcellular and regional distribution of [^{3}H]idazoxan-binding sites (Sastre and Garcia-Sevilla, 1993; Tesson et al., 1991). The onset of studies investigating localization of the I_2-sites was the observed association of these sites with the outer mitochondrial membrane (Tesson et al., 1991). Further studies revealed that densities of the I_2-sites in mitochondria correlate well with monoamine oxidase-activity in human and rat liver, and that age- and pathology-dependent density changes of I_2-sites follow those of the enzyme (Halbreich et al., 1993; Sastre and Garcia-Sevilla, 1993). Enzymatic and molecular biology studies have recently reinforced the hypothesis that I_2-sites are located on monoamine oxidase (Carpéné et al., 1994; Tesson et al., 1995). In contrast, very little is known about the effect of monoamine oxidase inhibitors on I_1-sites since the above studies focused exclusively on I_2-sites. Evaluating the behaviour of monoamine oxidase inhibitors could, therefore, provide clues for the relationship between the [^{3}H]clonidine and [^{3}H]idazoxan-labeled imidazoline-binding sites in calf striatum as well as between these sites and monoamine oxidase enzymes.

Competition studies with MAO-A- and MAO-B-selective and non-selective monoamine oxidase inhibitors yielded two striking results. First, in those instances where the inhibitors were potent enough to yield a near-complete competition curve at the investigated concentration range (up to 0.1 mM), the curves were steep and best analysed according to a one-site model (Table 4). This indicates that the populations of I_1- and I_2-sites with different binding affinities for compounds, such as cirazoline, display the same binding profile for at least certain monoamine oxidase inhibitors. Second, two of the investigated monoamine oxidase blockers (the irreversible non-selective and MAO-B-selective propargylamines pargyline and deprenyl) were about 20 times more potent in displacing [^{3}H]clonidine binding as compared to [^{3}H]idazoxan binding and the MAO-A-selective propargylamine clorgyline had equal potency. These findings clearly indicate that I_1-sites may be even more sensitive to certain monoamine oxidase inhibitors than the I_2-sites in calf striatum and, hence, that there could be a link between monoamine oxidase and I_1-sites. This could even be expected if one assumes that I_1- and I_2-sites make part of the same molecular complex. However, the correlation between imidazoline-binding sites and monoamine oxidase is not unequivocal because of weak potency of the MAO-A inhibitor Ro 16-6491 and of the MAO-B inhibitor Ro 41-1094. Discrepancies in the behaviour of monoamine oxidase inhibitors on the [^{3}H]idazoxan binding and, reciprocally, of imidazolines on monoamine oxidase activity have been observed before (Alemany et al., 1995; Tesson et al., 1995). Several explanations have been proposed for these divergent results which, however, all share the common prerogative that the I_2-sites do not coincide with the catalytic enzymatic site (Olmos et al., 1993; Sastre and Garcia-Sevilla, 1993; Tesson et al., 1995).

Venoms and natural toxins from various origin have often played a key role in the identification and classification process of membrane-bound receptors and ion channels. They have proven to be particularly succesful discriminatory tools for the division of hormone and neurotransmitter receptors into different subtypes. In certain instances, their discriminatory power may well exceed that of other natural or synthetic ligands. This is well illustrated in the case of muscarinic receptors for which synthetic ligands, such as pirenzepine, provide a much poorer distinction between the M1- and M2-subtypes than the venom of the marine snail *Conus tessulatus* (Czerwiec et al., 1993). The above considerations have prompted us to explore the ability of venom preparations from various *Conus* species to interact with I_1- and I_2-sites in calf striatum.

From the 13 different venom preparations that were tested, only those from *C. geografus* and *C. vexillum* were reasonably potent competitors for [^{3}H]clonidine and [^{3}H]idazoxan binding. These venoms were unable to discriminate between the different populations of I_1- and I_2-sites and, as they were only slightly less potent for displacing [^{3}H]idazoxan binding, they also provided only little discrimination between I_1- and I_2-sites in calf stria-

tum. Yet, some of these venoms (i.e., from *C. eburneus* and *C. aulicus*) provided a clear-cut preference for α_2-adrenoceptors in calf retina. This confirms previous reports which suggested that imidazoline-binding sites and α_2-adrenoceptors are unrelated.

In conclusion, competition binding studies with classical imidazoline-binding site ligands, monoamine oxidase inhibitors and *Conus* venoms reveal that the [^{3}H]clonidine-labeled I_1-sites and the [^{3}H]idazoxan-labeled I_2-sites in calf striatum are each composed of at least two populations. The I_1(H)- and I_2(H)-sites are very similar to their counterparts in human striatum. The additional sites (i.e., I_1(L) and I_2(L)) display low affinity for compounds, such as cirazoline, Bu 224 and Bu 239, but they cannot be discriminated from the imidazoline binding (H)-sites with the monamine oxidase inhibitors and with venom preparations from various *Conus* species. The presence of such additional sites in calf striatum stresses the importance of considering the investigated species when comparing data from different studies on imidazoline-binding sites. With respect to these different imidazoline-binding sites, it is clear that further investigations, including their isolation and detailed characterization, are needed to find out whether they are related to each other as well as to monoamine oxidase.

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