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Short communication

Binding of [^{3}H]cirazoline to an imidazoline site in rat brain and kidney membranes

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Abstract

Two classes of high-affinity sites for [^{3}H]cirazoline were characterized in rat brain and kidney membranes. In both tissues, the binding parameters for the high- and low-affinity sites are similar with B_{max} values of ~ 50 fmol/mg protein, K_d ~ 0.6 nM and B_{max} ~ 470 fmol/mg protein, K_d ~ 11 nM respectively. Inhibition studies of [^{3}H]cirazoline binding to the lower affinity site revealed that only guanidinium or imidazoline derivatives compete with the specific binding of this radioligand. Our results suggest that [^{3}H]cirazoline could be used as a novel ligand to label the non-adrenergic imidazoline-preferring sites.

Keywords: Cirazoline; Imidazoline site; α-Adrenoceptor

1. Introduction

Compounds chemically related to the imidazoline structure such as idazoxan, clonidine, or bromoxidine were reported to possess high affinity for α_2-adrenoceptors. Recent studies have demonstrated that such compounds also bind with high affinity to a non-adrenergic site. These studies were carried out in a variety of tissues and using [^{3}H]idazoxan, [^{3}H]clonidine or [^{3}H]*p*-aminoclonidine as radioligands (Ernsberger et al., 1987; Hamilton et al., 1991). It was further demonstrated that this imidazoline-preferring site may be a physical and pharmacological entity distinct from α_2-adrenoceptors (Wang et al., 1992). Wikberg and Uhlén (1990) have demonstrated that cirazoline, an α_1-adrenoceptor agonist with weak partial α_2-adrenoceptor agonist properties, is among the ligands with the highest affinity for [^{3}H]idazoxan-preferring sites; its affinity for the non-adrenergic sites being 500-fold higher than for α_1- and between 50- and 200-fold higher than for α_2-adrenoceptors.

The purpose of this study was to characterize the binding of [^{3}H]cirazoline to rat brain and kidney membrane homogenates. We now report for the first time that [^{3}H]cirazoline labels a non-adrenoceptor site, with pharmacological properties that correspond to those reported for imidazoline recognition sites.

2. Materials and methods

2.1. Materials

Amiloride, benextramine, clonidine, (−)-adrenaline, phentolamine, prazosin, propranolol and yohimbine were purchased from Sigma (Paris, France) and rauwolscine from Laboratoires Sarget (Mérignac, France). Bromoxidine, idazoxan, cirazoline and [^{3}H]cirazoline (25 Ci · mmol^{-1}) were synthesized by the Chemistry Departement of Synthélabo Recherche (Bagneux, France).

2.2. Membrane homogenate preparations

Male Sprague-Dawley rats (200–250 g) were killed by decapitation. Whole brain and kidneys were rapidly removed and homogenized in 20 volumes of ice-cold buffer containing 50 mM Tris/HCl, 5 mM $MgCl_2$ and 5 mM EDTA (pH 7.4 at 4° C) using a Polytron 10.35

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(setting 10 × 10 s bursts). The homogenates were centrifuged at 50 000 × *g* for 15 min at 4° C, and after two other washes and centrifugations, the pellets were resuspended in 5 volumes of Tris/HCl buffer and incubated for 30 min at 25° C in order to remove endogenous ligands. Proteins were determined with the dye-binding BioRad method using a MR 700 Microplate Reader (aliquots of the homogenates were stored at −80° C).

2.3. [^{3}H]Cirazoline binding studies

Brain and kidney membrane homogenates were used under the same experimental conditions. Saturation experiments were performed by incubating the membranes (500 μg protein/ml) in 250 μl Tris/HCl buffer for 30 min at 25° C with increasing concentrations of [^{3}H]cirazoline. Non-specific binding was defined with 10 μM of unlabelled cirazoline. Similar results were obtained in other experiments using bromoxidine (1 μM) to define non-specific binding (data not shown). The membranes were collected by vacuum filtration over Whatman GF/C filters and washed with 3 × 5 ml of ice-cold Tris/HCl buffer. After the addition of 10 ml of scintillation liquid, the radioactivity on the filters was determined using a Packard Tri-carb 4640 counter.

Competition studies were carried out under standard incubation conditions with 5 nM of [^{3}H]cirazoline and a range of concentrations of different drugs (1 pM to 1 mM).

At 5 nM [^{3}H]cirazoline the non-specific binding did not exceed 25% and 30% of total binding for kidney and brain homogenates, respectively. Specific binding represented approximately 0.09% of the total radioligand concentration ($n = 20$).

2.4. Data analysis

The results are expressed as the means ± S.E.M of n experiments performed in triplicate. B_{max}, K_d and K_i parameters were derived from a non-linear regression analysis using the LIGAND program (Munson and Rodbard, 1980). Pseudo-Hill coefficients (n_H) were deduced from the slope of the Hill plot of the inhibition data ($r > 0.92$). The appropriate single- or two-site model fitting was done with the differential F-test.

3. Results

Specific binding studies have shown that [^{3}H]cirazoline, in a range of concentrations from 1 pM to 50 nM, binds to brain and kidney membrane homogenates in a reversible manner (data not shown). Non-specific binding of [^{3}H]cirazoline was a linear function of the radioligand concentration (Fig. 1). Saturation analysis revealed two classes of high-affinity binding sites for each homogenate preparation ($P < 0.001$; $n = 4$ using the differential F-test). The binding parameters of rat brain and kidney homogenate membranes were not significantly different. For the higher affinity binding sites, the dissociation constant, K_d, of [^{3}H]cirazoline on brain membranes was 0.45 ± 0.19 nM and the maximal binding density, B_{max}, was 54.60 ± 3.76 fmol/mg protein. Likewise, using kidney homogenates, the K_d was 0.70 ± 0.14 nM and the B_{max} was 41.6 ± 4.9 fmol/mg protein. The maximal binding capacity of the low-affinity binding sites was 458 ± 14 fmol/mg protein and 483 ± 16 fmol/mg protein using rat brain and kidney membranes respectively. The dissociation constant of

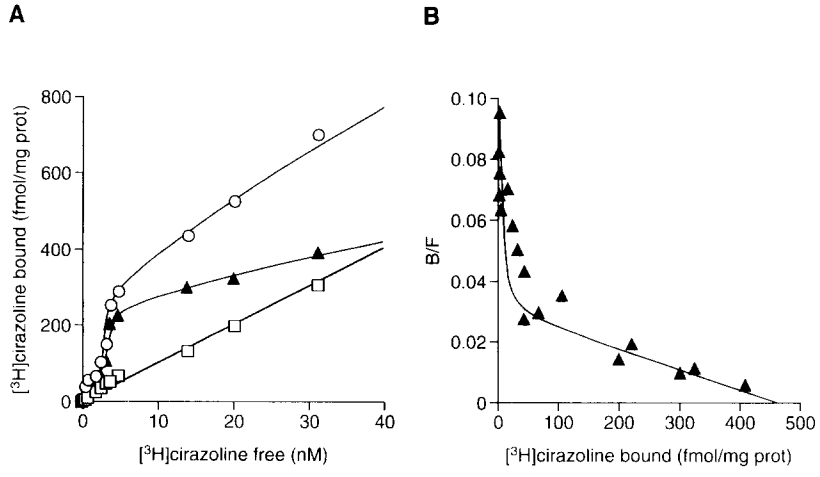

Fig. 1. (A) Saturation analysis of [^{3}H]cirazoline binding (1 pM to 50 nM) to kidney membrane homogenates: (○) total [^{3}H]cirazoline binding, (▲) specific binding and (□) non-specific binding (in the presence of 10 μM of unlabelled cirazoline). (B) The Scatchard plot of [^{3}H]cirazoline specific binding indicating two classes of sites. The data from this experiment are B_{max} = 43.39 fmol/mg protein, K_d = 0.46 nM and B_{max} = 452 fmol/mg protein, K_d = 13.16 nM for the high- and low-affinity binding sites respectively (see Materials and methods for details).

Table 1
Inhibition of [^{3}H]cirazoline binding by drugs

Drugs	Brain		Kidney	
	K_i (μM)	n_H	K_i (μM)	n_H
Cirazoline	0.015 ± 0.003	(1.05 ± 0.07)	0.038 ± 0.004	(1.19 ± 0.05)
Clonidine	0.050 ± 0.006	(0.92 ± 0.11)	0.012 ± 0.004	(1.11 ± 0.22)
Bromoxidine	0.027 ± 0.003	(1.27 ± 0.09)	0.026 ± 0.004	(1.21 ± 0.09)
Idazoxan	0.112 ± 0.010	(0.93 ± 0.13)	0.240 ± 0.012	(1.06 ± 0.17)
Amiloride	0.214 ± 0.024	(1.14 ± 0.06)	0.105 ± 0.031	(1.12 ± 0.10)
Rauwolscine	32.5 ± 10.5	n.d.	26.7 ± 12.3	n.d.
Phentolamine	14.0 ± 5.4	n.d.	14.8 ± 7.0	n.d.
Benextramine	17.1 ± 3.5	n.d.	46.3 ± 12.2	n.d.
(−)-Adrenaline	635 ± 213	n.d.	230 ± 92	n.d.
Propranolol	395 ± 160	n.d.	418 ± 176	n.d.
Yohimbine	232 ± 46	n.d.	293 ± 99	n.d.
Prazosin	1772 ± 242	n.d.	1514 ± 384	n.d.

Inhibition studies of [^{3}H]cirazoline (5 nM) binding to brain and kidney membranes were performed with imidazoline, guanidinium, phenylethylamine and alkaloid compounds. Non-specific binding was determined using 10 μM of unlabelled cirazoline. The dissociation constant, K_i, of each drug is expressed in μM with the Hill coefficient, n_H, in parentheses. The data represent the means ± S.E.M. of 4 experiments (see Materials and methods for details, n.d.: not determined).

[^{3}H]cirazoline was 8.98 ± 1.50 nM with brain homogenates and 13.2 ± 2.1 nM with kidney membranes.

Competition studies of [^{3}H]cirazoline binding to kidney and brain homogenates were performed with alkaloid, phenylethylamine, imidazoline and guanidinium derivatives (Table 1). These studies were carried out with 5 nM of [^{3}H]cirazoline, conditions where the higher affinity site is occupied and therefore the results mainly represent the pharmacological profile of the low-affinity binding sites. Catecholamines (adrenaline) and non-imidazoline adrenoceptor ligands such as benextramine, prazosin, propranolol, rauwolscine or yohimbine, did not compete with [^{3}H]cirazoline binding ($K_i > 10$ μM).

Under our experimental conditions, only guanidinium and imidazoline derivatives except phentolamine ($K_i > 14$ μM) had a high affinity for [^{3}H]cirazoline binding sites. Unlabelled cirazoline, clonidine and bromoxidine were found to have the highest affinity for [^{3}H]cirazoline binding ($K_i < 0.05$ μM). None of the compounds tested showed significant differences in their affinity for [^{3}H]cirazoline binding sites in either brain or kidney homogenates.

4. Discussion

Previous studies have demonstrated that cirazoline has the highest affinity for the non-adrenoreceptor imidazoline recognition site with an inhibition constant of 0.5–1 nM, compared to other imidazoline and guanidinium derivatives (Wikberg and Uhlén, 1990; Hamilton et al., 1991). Studies to characterize the non-adrenergic imidazoline binding site are usually performed with radioligands such as [^{3}H]clonidine, [^{3}H]*p*-aminoclonidine, [^{3}H]bromoxidine or [^{3}H]idazoxan which are less specific for imidazoline binding sites than cirazoline (Coupry et al., 1987; Ernsberger et al., 1987; Wikberg and Uhlén, 1990; Hamilton et al., 1991). Therefore, such studies were carried out in the presence of adrenaline, noradrenaline or phentolamine in order to occupy the α_2-adrenoceptor sites. In most of these binding studies, the relative affinity to α_2-adrenoceptors and the density of the sites depend on the radioligand used (Coupry et al., 1987; Ernsberger et al., 1987; Zonnenschein et al., 1990; Hamilton et al., 1991). Using the highly selective ligand, [^{3}H]cirazoline, we have now characterized non-adrenergic imidazoline binding sites in rat brain and kidney membrane homogenates in the absence of any additional drugs in the incubation medium to occupy the α_2-adrenoceptors.

Saturation experiments, performed with a range of [^{3}H]cirazoline concentrations from (1 pM to 50 nM) did not reveal significant differences between brain and kidney preparations. In both tissues, the dissociation constants of the high and low affinity binding sites are about ~0.6 nM and ~11 nM respectively. The receptor density of the high-affinity sites is ~50 fmol/mg protein while the B_{max} of the low affinity sites is ~470 fmol/mg protein.

The two categories of [^{3}H]cirazoline binding sites characterized above have similar affinities to the two classes of imidazoline-preferring binding sites in the human nucleus reticularis lateralis found by Greney and coworkers using [^{3}H]idazoxan (Greney et al., 1992). In addition, the dissociation constants of the low-affinity [^{3}H]cirazoline binding sites are in the same range as the K_d values observed using [^{3}H]clonidine, [^{3}H]*p*-aminoclonidine or [^{3}H]idazoxan (Ernsberger et al., 1987; Zonnenschein et al., 1990; Hamilton et al., 1991). However, in both tissues the receptor density of

[^{3}H]cirazoline binding sites is different from what was reported by other authors (Coupry et al., 1987; Ernsberger et al., 1987; Zonnenschein et al., 1990; Hamilton et al., 1991; Greney et al., 1992). These differences may be due to the radioligand used to characterize imidazoline-preferring sites and to the different experimental conditions.

Competition experiments were performed using 5 nM of [^{3}H]cirazoline, a concentration at which saturation of the high-affinity binding site is obtained. Under these conditions two groups of compounds could be distinguished. The alkaloid and phenylethylamine derivatives showed very low affinity (> 10 μM).

This includes adrenoceptor ligands of different chemical classes and activities such as (−)-adrenaline (α- and β-adrenoceptor agonist), prazosin (α_1-adrenoceptor antagonist), benextramine, rauwolscine and yohimbine (α_2-adrenoceptor antagonists), as well as propranolol, a β-adrenoceptor antagonist (Table 1). The second group included the guanidinium and imidazoline derivatives. Phentolamine had low affinity for [^{3}H]cirazoline at these binding sites despite its imidazoline moiety ($K_i > 14$ μM). A similar low affinity of phentolamine for imidazoline sites has been observed by other authors using [^{3}H]bromoxidine or [^{3}H]idazoxan as radioligands (Langin and Lafontan, 1989; Hamilton et al., 1991). Idazoxan and amiloride possess similar intermediate affinity ($K_i \sim 0.15$ μM) and the highest affinities were observed with bromoxidine, clonidine and cirazoline ($K_i \sim 10$–50 nM). This observation, that non-imidazoline α- or β-adrenoceptor agonists or antagonists fail to displace [^{3}H]cirazoline binding, clearly suggests that [^{3}H]cirazoline labels imidazoline binding sites which are non-adrenergic. The decreasing order of inhibitor potencies in brain membranes was cirazoline > bromoxidine > clonidine > idazoxan > amiloride while in membranes from kidney preparations it was clonidine > bromoxidine > cirazoline > amiloride > idazoxan. It is now evident that imidazoline-preferring sites can be classified into at least two groups (Reis et al., 1992). The I_1 types, so-called clonidine-preferring sites, bind [^{3}H]*p*-aminoclonidine and [^{3}H]clonidine preferentially showing low affinity for amiloride. The I_2 types or idazoxan-preferring sites are labelled by [^{3}H]idazoxan and can be subdivided into two subtypes such as I_{2a} and I_{2b}, possessing, respectively, high and low affinity for amiloride. The high potencies of clonidine and bromoxidine to inhibit [^{3}H]cirazoline binding would support the classification as I_1 sites. However, the guanidinium compound, amiloride, displaces the [^{3}H]cirazoline binding sites with affinities similar to that for idazoxan and therefore these sites appear to be different from the sites labelled by [^{3}H]*p*-aminoclonidine (Michel and Insel, 1989). On the other hand, the high affinity of unlabelled cirazoline and the potencies of idazoxan and amiloride would indicate instead that we are dealing with I_2 sites. Taken together these results suggest that [^{3}H]cirazoline may not distinguish between the I_1 and I_2 sites, or that this site represents another, as yet non-characterized subtype of imidazoline-preferring site.

In summary, [^{3}H]cirazoline is a novel high-affinity radioligand which specifically labels imidazoline-guanidinium recognition sites without significant α- or β-adrenoceptor binding on rat brain and kidney membranes. These special properties make [^{3}H]cirazoline a very useful ligand for further studies of imidazoline recognition sites. In addition cirazoline appears to be a useful tool for in vitro and in vivo characterization of the function associated to imidazoline recognition sites.

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