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Regina Alemany · Gabriel Olmos
Jesús A. García-Sevilla

Labelling of I_{2B}-imidazoline receptors by [^{3}H]2-(2-benzofuranyl)-2-imidazoline (2-BFI) in rat brain and liver: characterization, regulation and relation to monoamine oxidase enzymes

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Abstract The novel selective imidazoline radioligand [3H]2-(2-benzofuranyl)-2-imidazoline (2-BFI) was used to characterize and assess further the nature of I_2-imidazoline receptors in rat brain and liver. In the cerebral cortex, 2-BFI displayed high affinity (K_i= 9.8 nM) for a single class of [^{3}H]2-BFI binding sites. Other imidazoline/guanidine compounds (e.g. aganodine, cirazoline and idazoxan) displayed biphasic competition curves, indicating the existence of high (K_{iH}= 2.9-78 nM; R_H= 61-83%) and low (K_{iL}= 4.7-158 μM) affinity sites. The pharmacological profile for [^{3}H]2-BFI binding (aganodine > cirazoline > 2-BFI >> clonidine > amiloride >> efaroxan) was typical of that for I_2-sites. This profile was almost identical to that obtained against [^{3}H]idazoxan (correlation between pK_i values, r = 0.97) which indicated that the sites characterized with [^{3}H]2-BFI in brain corresponded to I_2-imidazoline receptors. The low affinity of amiloride against [^{3}H]2-BFI (K_i= 900 nM) further indicated that these brain I_2-sites belong to the I_{2B}-subtype. [^{3}H]2-BFI binding sites (B_{max}= 72 fmol/mg protein) in brain were differentially modulated by treatment (7 days) with cirazoline (up-regulation: 25%) and the MAO inhibitor phenelzine (down-regulation: 31%), indicating that these I_2-sites are regulated in vivo, as is the case for those labelled by [^{3}H]idazoxan. Chronic treatment with 2-phenylethylamine, a phenelzine metabolite and endogenous amine, did not alter the density of brain of I_2-imidazoline receptors labelled by [^{3}H]idazoxan. Preincubation of liver membranes with the MAO inhibitor clorgyline (10^{-7} M) abolished the binding of [^{3}H]Ro 41-1049 *(N*-(2-aminoethyl)-5-(*m*-fluorophenyl)-4-thiazole carboxamide) to MAO-A, but it did not alter the binding of [^{3}H]Ro 19-6327 *(N*-(2-aminoethyl)-5-chloro-2-pyridine carboxamide) to MAO-B or that of [^{3}H]2-BFI to I_2-sites. At 10^{-4} M it also abolished MAO-B sites, but a substantial proportion of I_2-sites (40%) remained intact. Preincubation of liver membranes at 60 °C also abolished MAO-A/B sites, whereas still 22% of I_2-sites remained. The results indicate that [^{3}H]2-BFI is a good tool for the identification of I_2-imidazoline receptors and suggest further that certain I_2-sites and MAO are different proteins.

Key words [^{3}H]2-(2-benzofuranyl)-2-imidazoline ([^{3}H]2-BFI) · I_{2B}-imidazoline receptor · MAO isoenzymes · Irreversible MAO inhibitors · Rat brain and liver

Introduction

In the last few years, the existence of binding sites specific for imidazol(ine)/guanidine compounds distinct from α_2-adrenoceptors has been shown in a wide variety of species and tissues, including the central nervous system (for a review see Bousquet 1995; French 1995; Reis et al. 1995; Regunathan and Reis 1996). These non-adrenoceptor binding sites, called imidazoline receptors, have been classified into two main types: I_1-imidazoline receptors, labelled with [^{3}H]clonidine and its derivatives, and I_2-imidazoline receptors, traditionally labelled with [^{3}H]idazoxan, which differ not only in their pharmacological profiles but also in their tissue and subcellular distributions (Ernsberger 1992; Michel and Ernsberger 1992). According to the affinity for the guanidide amiloride and the ability to irreversibly bind clorgyline, the I_2-imidazoline receptors have been further subclassified into the I_{2A}- and I_{2B}-subtypes (Diamant et al. 1992; Miralles et al. 1993a; Olmos et al. 1996a).

The interaction of imidazol(ine) ligands with several receptor systems, including α-adrenoceptors, serotonin ($5\text{-}HT_{1A}$) receptors and various cation channels (Tim-

R. Alemany[1] · G. Olmos · J. A. García-Sevilla (✉)
Laboratory of Neuropharmacology,
Department of Biology, University of the Balearic Islands,
Cra. Valldemossa km 7.5, E-07071 Palma de Mallorca, Spain

[1]*Present address*: Institut für Pharmakologie,
Universitätsklinikum Essen, Hufelandstrasse 55,
D-45122, Essen, Germany

mermans and Van Zwieten 1982; Dunne 1991; Lladó et al. 1996; Molderings et al. 1996; Olmos et al. 1996b), and the lack of high affinity and selective I_2 compounds are some of the major obstacles to the further identification and structural characterization of I_2-imidazoline receptors. Recently, the chemical modification of the structure of idazoxan has provided the development of various compounds such as LSL 60101 (2-(2-benzofuranyl)-2-imidazole), BU 224 (2-(4,5-dihydroimidaz-2-yl)-quinoline) and 2-(2-benzofuranyl)-2-imidazoline (2-BFI) with an improved I_2/α_2 selective profile (Hudson et al. 1994, 1995; Menargues et al. 1994; Alemany et al. 1995a). The radiolabelled form of 2-BFI, ([^{3}H]2-BFI), has more recently been synthesized and characterized in the rabbit brain as a new highly selective radioligand for I_2-imidazoline receptors (Lione et al. 1996).

Recent studies demonstrating that the heterologous expression in yeast of cDNA clones for monoamine oxidase (MAO-A and MAO-B isoenzymes) led to the coexpression of [^{3}H]idazoxan binding sites (Tesson et al. 1995), and that some imidazol(ine) drugs, including 2-BFI, can inhibit MAO activity in a non-competitive manner (Carpéné et al. 1995; Tesson et al. 1995), have suggested that I_2-imidazoline receptors are somehow linked to MAO (Parini et al. 1996).

In this context, the present study was designed (1) to characterize I_2-imidazoline receptors with [^{3}H]2-BFI in comparison with [^{3}H]idazoxan in rat brain, (2) to investigate whether chronic treatments with the imidazoline compound cirazoline and the irreversible non-selective MAO inhibitor phenelzine result in regulation of the density of I_2-imidazoline receptors labelled by [^{3}H]2-BFI in rat brain and (3) to seek for possible interactions of [^{3}H]2-BFI with MAO isoenzymes in rat liver.

Materials and methods

Rat cortical and liver membrane preparations. Male Sprague-Dawley rats (250-300 g) were used. The animals received a standard diet with water freely available and were housed at 20 ± 2 °C with a 12 h light/dark cycle. The rats were decapitated and the parietooccipital cortex and liver were rapidly removed into ice-cold Tris-sucrose buffer (5 mM Tris-HCl; 250 mM sucrose; 1 mM $MgCl_2$; pH 7.4) and frozen at -80 °C until required. Cortical and liver membranes (P_2 fractions) were prepared by established methods with modifications (Giralt and García-Sevilla 1989). Briefly, the tissue samples were homogenized in 5 ml of ice-cold Tris-sucrose buffer. The homogenates were centrifuged at 1100 g for 10 min and the supernatants were then recentrifuged at 40000 g for 10 min. The resulting pellet was washed twice with 2 ml of fresh incubation buffer (50 mM Tris-HCl, pH 7.5). The final pellet was resuspended in an appropriate volume of this buffer to a final protein content of 800-1000 µg/ml. Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Drug treatments. Various groups of rats received i.p., every 12 h for 7 days either 0.9 % saline vehicle, cirazoline (1 mg/kg) or the irreversible MAO inhibitor phenelzine (3 mg/kg). In another series of experiments rats were treated i.p., every 12 h for 7 days with 2-phenylethylamine (30 mg/kg), an active metabolite of phenelzine (Dyck et al. 1985; Paetsch et al. 1993) and an endogenous amine as well (Paterson et al. 1990; McManus et al. 1991). Because 2-phenylethylamine is rapidly metabolized by MAO-B (Paterson et al. 1990, 1991), other groups of rats were treated with 2-phenylethylamine (30 mg/kg) together with the reversible MAO-B inhibitor Ro 19-6327 (2 mg/kg) or with Ro 19-6327 (2 mg/kg) alone. Rats were killed 24 h after the last injection and cerebral cortical and liver membranes were prepared as above.

[^{3}H]2-BFI, [^{3}H]idazoxan, [^{3}H]Ro 41-1049 and [^{3}H]Ro 19-6327 binding assays. Total [^{3}H]2-BFI or [^{3}H]idazoxan binding was measured in 0.5 ml-aliquots (50 mM Tris-HCl, pH 7.5) of the cortical and liver membranes, which were incubated with shaking for 30 min at 25 ºC. Binding of [^{3}H]idazoxan to brain I_2-imidazoline receptors was done in the presence of 10^{-6} M (-)-adrenaline and 0.1 % ascorbic acid to prevent binding to α_2-adrenoceptors (Olmos et al. 1992; Miralles et al. 1993a). In agreement with previous studies (Hudson et al. 1995), 2-BFI displayed very low affinity for brain α_2-adrenoceptors (K_i = 11 x 10^{-6} M against [^{3}H]clonidine binding; data not shown) and a K_i ratio of 1528 for α_2-adrenoceptors vs I_2-imidazoline receptors (see Table 1). When 10^{-6} M (-)-adrenaline was included in the assay, it did not affect total [^{3}H]2-BFI binding, suggesting that the radioligand does not label α_2-adrenoceptors in this tissue. Because of this, total [^{3}H]2-BFI binding was defined in the absence of (-)-adrenaline. Nonspecific binding was determined in the presence of 10^{-4} M naphazoline for both radioligands (Olmos et al. 1992). In drug competition studies, membranes were incubated as above with [^{3}H]2-BFI (3 x 10^{-9} M) or with [^{3}H]idazoxan (10^{-8} M) and in the absence or presence of various concentrations of the competing drugs (10^{-10} M to 10^{-3} M; 15 concentrations). Total binding was determined as above and plotted as a function of the drug concentration. In saturation studies, cortical membranes were incubated with eight concentrations of [^{3}H]2-BFI (5 x 10^{-10} M to 2.5 x 10^{-8} M) or [^{3}H]idazoxan (6 x 10^{-10} M to 4 x 10^{-8} M) as above. The specific binding was defined as the difference between total binding ([^{3}H]2-BFI) or total non-adrenoceptor binding ([^{3}H]idazoxan) and nonspecific binding and was plotted as a function of increasing concentrations of the radioligand. Specific binding represented 87 % to 49 % of total [^{3}H]2-BFI binding and 70 % to 40 % of total [^{3}H]idazoxan binding.

In another series of experiments, the effects of in vitro preincubation with clorgyline or those of different preincubation temperatures on [^{3}H]2-BFI (3 x 10^{-9} M) binding to liver I_2-imidazoline receptors and on [^{3}H]Ro 41-1049 (4 x 10^{-9} M) or [^{3}H]Ro 19-6327 (4.5 x 10^{-9} M) binding to liver MAO-A or MAO-B isoenzymes, respectively, were assessed as described previously (Olmos et al. 1993; Miralles et al. 1993b). The pellets resulting from the last centrifugation (see preparation of membranes) were resuspended in 10 ml of fresh incubation buffer (50 mM Tris-HCl, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4) and pooled, after this procedure aliquots of 10 ml were incubated for 30 min at 25 °C in the absence or presence of clorgyline (10^{-9} M to 10^{-4} M) or incubated for 30 min at 25, 37, 50, 55, 60 and 70 °C. Then, the membranes were washed twice with 10 ml of the above incubation buffer and the final pellet was resuspended as above and used in binding experiments. Binding of radioligands to liver I_2-imidazoline receptors, MAO-A or MAO-B was assessed in the same aliquot of membranes for every clorgyline concentration or preincubation temperature. Nonspecific binding was determined in the presence of 10^{-4} M naphazoline ([^{3}H]2-BFI), 10^{-6} M clorgyline ([^{3}H]Ro41-1049) or 10^{-4} M deprenyl ([^{3}H]Ro19-6327).

Incubations were terminated by diluting the samples with 5 ml of ice-cold Tris incubation buffer (4 °C). Bound and free radioligands were separated by vacuum filtration through Whatman GF/C glass fiber filters which had been presoaked with 1 % polyethylenimine (Bruns et al. 1983), using a Brandel 48R cell harvester (Biomedical Research and Development Laboratories, USA). Then the filters were rinsed twice or three times ([^{3}H]2-BFI binding) with 5 ml of incubation buffer, air-dried, transferred to minivials containing 5 ml of OptiPhase 'HiSafe' II cocktail (LKB, England) and counted for radioactivity by liquid scintillation spectrometry at 57 % efficiency (Packard model 1900 TR).

Analysis of binding data and statistics. Analysis of saturation isotherms (K_d, dissociation constant; B_{max}, maximum density of bind-

ing sites) and competition experiments (K_i, inhibition constant) as well as the fitting of data to the appropriate binding models were performed by computer-assisted nonlinear regression using the EBDA-LIGAND programs. All experiments were initially analyzed assuming a one-site model of radioligand binding and then assuming a two-site binding model. The selection between the different binding models was made statistically using the extra sum of squares principle (*F* test) as outlined by Munson and Rodbard (1980). The more complex model was accepted if the *P* value resulting from the *F* test was less than 0.05.

Results are expressed as mean ± SEM or SD. Pearson's correlation coefficients were calculated by the method of least squares. One-way analysis of variance (ANOVA), followed by Scheffé's test, was used for the statistical evaluations. The level of significance was $P = 0.05$.

Drugs. [^{3}H]2-BFI (2-(2-benzofuranyl)-2-imidazoline) (specific activity 64 Ci/mmol; batch 1) and [^{3}H]idazoxan (specific activity, 44 and 49 Ci/mmol; batches 55 and 56) were purchased from Amersham International plc (UK). [^{3}H]Ro 41-1049 (*N*-(2-aminoethyl)-5-(*m*-fluorophenyl)-4-thiazole carboxamide) HCl (specific activity, 30.8 Ci/mmol) and [^{3}H]Ro 19-6327 (*N*-(2-aminoethyl)-5-chloro-2-pyridine carboxamide) HCl (specific activity, 20.2 Ci/mmol) were generous gifts from Dr. J. G. Richards (F. Hoffmann-La Roche Ltd., Switzerland). For binding assays, appropriate amounts of the stock solutions were diluted with distilled and purified water (Milli-Q) containing 2.5 mM HCl and 6 % ethanol. Other drugs (and their sources) included: (-)-adrenaline bitartrate (Sigma Chemical Co., USA); aganodine HCl (Beiersdorf, Germany); agmatine sulfate (Aldrich Chemical Co., USA); amiloride HCl (Sigma); 2-BFI HCl or RX801077 (synthesized by Dr. Plá as LSL 61103 at S.A. Lasa Laboratorios, Spain); bromoxidine (UK 14,304) tartrate (Pfizer, Spain); cirazoline HCl (Synthélabo Recherche, France); clonidine HCl (Boehringer Sohn Ingelheim, Germany); clorgyline HCl (Sigma); efaroxan HCl (Sigma); idazoxan HCl (synthesized by Dr. F. Geijo at S.A. Lasa Laboratorios); LSL 60101 (2-(2-benzofuranyl)-2-imidazole) HCl (synthesized by Dr. F. Plá at S.A. Lasa Laboratorios); (+)-medetomidine HCl (Farmos Group, Finland); moxonidine HCl (Beiersdorf); phenelzine sulphate (Sigma); 2-phenylethylamine HCl (Sigma); Ro 19-6327 HCl (F. Hoffmann-La Roche Ltd.). Other reagents were obtained from Sigma Chemical Co. (USA).

Results

Drug competition studies of [^{3}H]2-BFI and [^{3}H]idazoxan binding in rat brain

Competition experiments were performed using a variety of compounds to assess the pharmacological profile of [^{3}H]2-BFI binding (3×10^{-9} M) to rat brain membranes. 2-BFI showed high affinity (K_i = 9.8 nM) in displacing the specific binding of [^{3}H]2-BFI and produced competing monophasic curves with Hill slopes close to unity, as expected for most homologous displacements (Swillens et al. 1995). However, several imidazol(ine)/guanidine compounds known to have high affinity for I_2-imidazoline receptors such as aganodine, cirazoline, (+)-medetomidine and idazoxan, displayed high affinity and biphasic curves when competing against [^{3}H]2-BFI binding (Hill coefficients significantly less than unity), and computer analysis could resolve this binding into high and low affinity components (Table 1 and Fig. 1). The high affinity component for these drugs (K_{iH} = 2.9-78 nM) represented 61-83 % of bound [^{3}H]2-BFI at 3×10^{-9} M. The imidazol(ine) compounds, bromoxidine, clonidine and LSL 60101 showed moderate affinity (K_{iH} = 113-879 nM, % R_H = 38-60 %) in displacing the binding of [^{3}H]2-BFI in rat brain (Table 1 and Fig. 1). In contrast, other drugs known to have low affinity for I_2-imidazoline receptors such as moxonidine and efaroxan, had also low affinity for a single class of [^{3}H]2-BFI binding sites (K_i = 10 and 225 μM) (Table 1). Similarly, the guanidide amiloride, able to discriminate between I_2-subtypes, and agmatine, a proposed endogenous ligand for imidazoline receptors, displayed also low affinity (K_i = 0.9 and 352 μM) against [^{3}H]2-BFI binding (Table 1).

Table 1 Competition parameters of various drugs on the binding of [^{3}H]2-BFI and [^{3}H]idazoxan in the rat cerebral cortex

	[^{3}H]2-BFI			[^{3}H]idazoxan		
Drugs	K_{iH} (nM)	K_{iL} (μM)	R_H %	K_{iH} (nM)	K_{iL} (μM)	R_H %
Aganodine	2.9	20	61	1.5	0.1	78
Cirazoline	5.3	4.7	77	3.8	2.1	74
2-BFI	9.8	-	100	7.5	80	73
(+)-Medetomidine	50	37	72	45	56	76
Idazoxan	78	158	83	14	–	100
Bromoxidine	113	63	60	96	7.0	74
Clonidine	207	52	54	980	40	56
LSL 60101	879	3.2	38	350	116	79
Amiloride	–	0.9	–	–	5.5	–
Moxonidine	–	10	–	–	5.0	–
Efaroxan	–	225	–	–	58	–
Agmatine	–	352	–	–	342	–

Cortical membranes were incubated at 25 °C for 30 min with [^{3}H]2-BFI (3×10^{-9} M) or with [^{3}H]idazoxan (10^{-8} M in the presence of 10^{-6} M adrenaline) and in the absence or presence of the competing drugs (10^{-10} M to 10^{-3} M, 10-15 concentrations). Binding parameters (K_{iH}, K_{iL} and % R_H, defined as the percentage of high affinity sites for a given drug) were determined directly by simultaneous analysis of 2 to 4 independent experiments for each drug using the EBDA-LIGAND programs. A two-site fit was accepted only if it was significantly better than a one-site binding model ($P < 0.001$; *F* test)

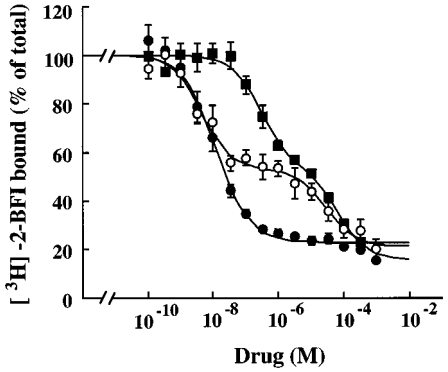

Fig. 1 Inhibition of total [^{3}H]2-BFI binding to rat cerebral cortex membranes by aganodine (○), 2-BFI (●) and clonidine (■). Cortical membranes were incubated at 25 °C for 30 min with [^{3}H]2-BFI (3 x 10^{-9} M) in the absence or presence of various concentrations of the competing drugs (10^{-10} M to 10^{-3} M, 15 concentrations). Total control binding was 4200 dpm. Computer-assisted curve fitting (EBDA-LIGAND programs) demonstrated that for aganodine and clonidine a two site fit was significantly better than a one site fit ($P < 0.001$; F test). Data shown are mean ± SEM of 3 to 4 experiments. See Table 1 for K_i values and other details

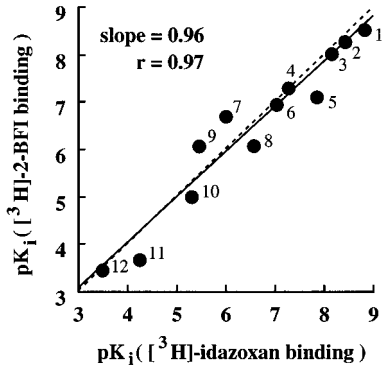

Fig. 2 Correlation between the high or low (amiloride, moxonidine, efaroxan and agmatine) affinity inhibition constants (expressed as pK_i values, i.e., the negative logarithm of the inhibition constant) of various drugs for the binding sites labelled by [^{3}H]2-BFI and for I_{2B}-imidazoline receptors labelled by [^{3}H]idazoxan in the rat cerebral cortex. The identification of drugs is as follows: 1) aganodine, 2) cirazoline, 3) 2-BFI, 4) (+)-medetomidine, 5) idazoxan, 6) bromoxidine (UK 14,304), 7) clonidine, 8) LSL 60101, 9) amiloride, 10) moxonidine, 11) efaroxan and 12) agmatine. The *dotted line* represents the line of identity. The *solid line* represents the regression of the correlation and the data were best described by the equation $y = 0.96x + 0.15$ ($r = 0.97$, $P = 0.0001$). See Table 1 for K_i values and other details

Table 2 Effects of chronic treatment with various drugs on I_{2B}-imidazoline receptors in the rat cerebral cortex

Drug treatment	Dose (mg/kg)	[^{3}H]idazoxan		
		K_d (nM)	B_{max} (fmol/mg protein)	*n*
Saline	–	13.9±1.4	59±2	7
2-Phenylethylamine	30	13.4±1.3	59±5	8
2-Phenylethylamine and Ro 19-6327	30 2	10.8±1.1	48±5	3
Ro 19-6327	2	9.6±2.1	44±7	3

Each drug was administered i.p., every 12 h for 7 days. The rats were killed 24 h after the last injection. Cortical membranes were incubated at 25 °C for 30 min with eight concentrations of [^{3}H]idazoxan (6 x 10^{-10} M to 4 x 10^{-8} M). Total binding of [^{3}H]idazoxan to I_{2B}-imidazoline receptors was defined in the presence of 10^{-6} M adrenaline; non-specific binding was defined in the presence of 10^{-4} M naphazoline. Binding parameters (K_d, B_{max}) were determined directly by computer-assisted nonlinear analysis from untransformed data using the EBDA-LIGAND programs. Each value represents the mean ± SEM of *n* experiments per group with an animal per experiment. One-way ANOVA did not detect any significant change in the binding parameters of [^{3}H]idazoxan to I_{2B}-imidazoline receptors after the various treatments

Parallel competition experiments against [^{3}H]idazoxan binding (10^{-8} M) resulted in the expected pharmacological profile defining I_2-imidazoline receptors in rat brain (Table 1). Moreover, when the potencies of all the compounds tested against [^{3}H]2-BFI binding in the rat cerebral cortex were compared with their potencies against [^{3}H]idazoxan binding in the same tissue (Table 1), a very good correlation was obtained ($r = 0.97$, slope = 0.96) (Fig. 2). This result indicated that the pharmacological profile of [^{3}H]2-BFI binding in rat brain is almost identical to the drug-affinity profile defining I_{2B}-imidazoline receptors labelled by [^{3}H]idazoxan in this tissue (Olmos et al. 1996a).

Regulation of the density of I_2-imidazoline receptors labelled by [^{3}H]2-BFI and [^{3}H]idazoxan in rat brain

Saturation binding studies were performed in the rat cerebral cortex to determine the density and affinity of sites labelled by [^{3}H]2-BFI, in comparison with [^{3}H]idazoxan. These studies revealed that [^{3}H]2-BFI bound with higher affinity (K_d = 4.1 ± 0.3 nM) than [^{3}H]idazoxan (K_d = 13.9 ± 1.4 nM) to a similar number of brain I_2-imidazoline receptors (B_{max} = 72 ± 4 and 59 ± 2 fmol/mg protein for [^{3}H]2-BFI and [^{3}H]idazoxan, respectively) (Fig. 3 and Table 2).

To provide evidence that the sites labelled by [^{3}H]2-BFI (I_2-imidazoline receptors) can be regulated in vivo, the effects of chronic treatment with the specific imidazoline drug cirazoline and the irreversible MAO inhibitor phenelzine were assessed. Chronic treatment (7 days) with cirazoline (1 mg/kg) increased the density of [^{3}H]2-BFI binding sites (B_{max} increased by 25 %, $P < 0.05$) in the rat cerebral cortex without significantly changing the affinity (K_d) of the radioligand for these sites (Fig. 3). In contrast, chronic treatment (7 days) with phenelzine (3 mg/kg) decreased the density of these I_2- sites (B_{max} decreased by 31 %, $P < 0.01$) without changing the affinity for [^{3}H]2-BFI (Fig. 3).

Because phenelzine does not irreversibly bind in vitro to brain I_2-imidazoline receptors labelled by [^{3}H]idazoxan (Alemany et al. 1995b), the possibility that its ac-

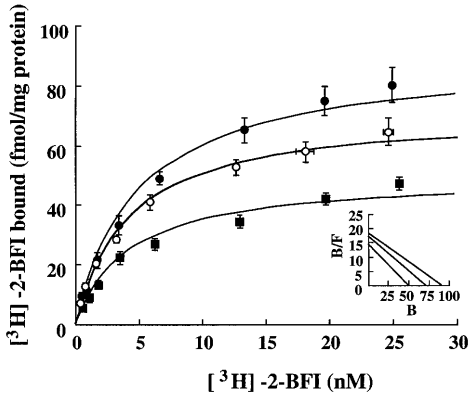

Fig. 3 Saturation curves of the specific binding of [^{3}H]2-BFI to I_{2B}-imidazoline receptors in the rat cerebral cortex after saline (○), or chronic treatment with cirazoline 1 mg/kg (●) or phenelzine 3 mg/kg (■) i.p., every 12 h for 7 days. Cortical membranes were incubated at 25 °C for 30 min with eight concentrations of [^{3}H]2-BFI (5 x 10^{-10} M to 2.5 x 10^{-8} M). Non-specific binding was defined in the presence of 10^{-4} M naphazoline. Nonlinear analysis (EBDA-LIGAND programs) of untransformed data yielded K_d = 4.1 ± 0.3 nM; B_{max} = 72 ± 4 fmol/mg protein for saline-treated rats (n = 9), K_d = 4.9 ± 0.6 nM; B_{max} = 90 ± 3 fmol/mg protein (n = 4) for cirazoline-treated rats and K_d = 4.2 ± 0.8 nM; B_{max} = 50 ± 3 fmol/mg protein (n = 5) for phenelzine-treated rats. *Each point* of the saturation curves is the mean ± SEM (*vertical* and *horizontal bars*) of n experiments per group with an animal per experiment. One way ANOVA followed by a multiple comparison test detected a significant increase (25 %; $P < 0.05$) or decrease (31 %; $P < 0.01$) in B_{max} after chronic treatment with cirazoline or phenelzine, respectively (F [2, 15] = 15.86, P = 0.0002) (ANOVA followed by Scheffé's test). *Inset*: Scatchard plots (same data) showing K_d and B_{max} values similar to those obtained by nonlinear analysis

tive metabolite 2-phenylethylamine could interact with the receptors was investigated. However, chronic treatment (7 days) with 2-phenylethylamine (30 mg/kg) did not induce any significant change in the binding parameters (B_{max}, K_d) of [^{3}H]idazoxan in rat brain (Table 2). Moreover, chronic treatment (7 days) with 2-phenylethylamine (30 mg/kg) together with the MAO-B inhibitor Ro 19-6327 (2 mg/kg), to prevent its metabolization by MAO-B, did not alter significantly the density (B_{max}) or affinity (K_d) of I_2-imidazoline receptors labelled by [^{3}H]idazoxan in rat brain (Table 2).

In vitro effects of preincubation with clorgyline and denaturating temperatures on I_2-imidazoline receptors, MAO-A and MAO-B enzymes in rat liver

[^{3}H]2-BFI, similarly to [^{3}H]idazoxan, also identified I_{2B}-imidazoline receptors in rat liver membranes (Alemany et al. 1995b and data not shown), a tissue enriched with the enzyme MAO. To assess for a possible interaction between the binding of [^{3}H]2-BFI to I_2-imidazoline receptors and MAO-A and/or MAO-B isoenzymes, the effects of the irreversible and selective MAO-A inhibitor clorgyline and of increasing temperature as an index of protein denaturation were investigated in rat liver.

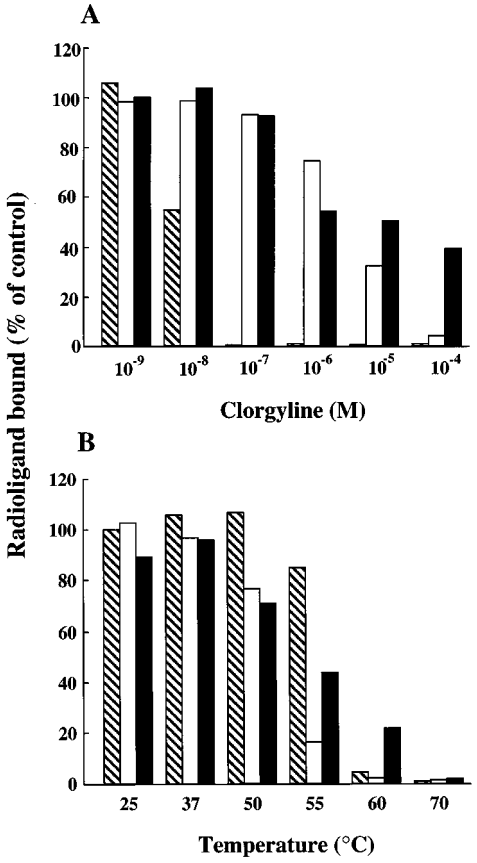

Fig. 4 **A** Effects of preincubation of rat liver membranes in buffer containing the irreversible and selective MAO-A inhibitor clorgyline on the specific binding of [^{3}H]Ro 41-1049 (4 x 10^{-9} M; 19000 dpm) to MAO-A (*hatched columns*), [^{3}H]Ro 19-6327 (4.5 x 10^{-9} M; 17000 dpm) to MAO-B (*empty columns*) and [^{3}H]2-BFI (3 x 10^{-9} M; 20000 dpm) to I_{2B}-imidazoline receptors (*filled columns*). Nonspecific binding was defined in the presence of 10^{-6} M clorgyline ([^{3}H]Ro 41-1049), 10^{-4} M deprenyl ([^{3}H]Ro 19-6327 or 10^{-4} M naphazoline ([^{3}H]2-BFI). Liver membranes were preincubated at 25 °C for 30 min in the absence (control) or presence of various concentrations of clorgyline (10^{-9} M to 10^{-4} M), and then washed twice and prepared for radioligand binding as described in Materials and methods. *Columns* represent means of specific binding of two independent experiments each in duplicate expressed as a percentage of that in control membranes. **B** Effects of increasing preincubation temperature of rat liver membranes on the specific binding of [^{3}H]Ro 41-1049 (4 x 10^{-9} M) to MAO-A (*hatched columns*), [^{3}H]Ro 19-6327 (4.5 x 10^{-9} M) to MAO-B (*empty columns*) and [^{3}H]2-BFI (3 x 10^{-9} M) to I_{2B}-imidazoline receptors (*filled columns*). Liver membranes were preincubated at various temperatures (25-70 °C) for 30 min, and then washed twice and prepared for radioligand binding as above. *Columns* represent means of specific binding of two independent experiments each in duplicate expressed as a percentage of that in control membranes (preincubated at 4 °C and then at 25 °C for the binding assays). For the various experiments **A** and **B**, standard deviations were less than 5 % of the mean reported values

Preincubation of rat liver membranes (30 min at 25 °C) with clorgyline (10^{-7} M) completely abolished the binding of [^{3}H]Ro 41-1049 to MAO-A, but it did not alter the binding of [^{3}H]Ro 19-6327 to MAO-B or that of [^{3}H]2-BFI to I_2-imidazoline receptors (< 10 %) (Fig. 4A). High

concentrations of clorgyline (10^{-6} M to 10^{-4} M) progressively reduced by 25 %, 67 % and 95 %, respectively, the binding of [^{3}H]Ro 19-6327 to liver MAO-B, but they only reduced by 45-60 % the specific binding of [^{3}H]2-BFI to liver I_2-imidazoline receptors (Fig. 4A). Similar results were obtained when [^{3}H]idazoxan was used as a radioligand (specific binding at 10^{-8} M decreased by 48 % after 10^{-4} M clorgyline preincubation) (data not shown).

On the other hand, preincubation of liver membranes for 30 min at high temperatures (55-70 °C) decreased the binding of all radioligands to their respective binding sites (Fig. 4B), but with a different pattern of denaturation. Preincubation of liver membranes at 55 °C only reduced by 15 % the specific binding of [^{3}H]Ro 41-1049 to MAO-A, but it markedly decreased the binding of [^{3}H]Ro 19-6327 to MAO-B (85 %) and that of [^{3}H]2-BFI to I_2-imidazoline receptors (55 %). Preincubation at 60 °C abolished by more than 95 % the specific binding of both [^{3}H]Ro 41-1049 and [^{3}H]Ro 19-6327 to MAO-A and MAO-B isoenzymes, respectively; however, this high denaturating temperature did not completely reduce the specific binding of [^{3}H]2-BFI to liver I_2-imidazoline receptors (22 % of [^{3}H]2-BFI binding remained at 60 °C) (Fig. 4B).

Discussion

Although idazoxan has been the prototypical ligand for the study of I_2-imidazoline receptors, its use is compromised by the fact that it is relatively non-selective and binds with equally high affinity to α_2-adrenoceptors (Mallard et al. 1992; Miralles et al. 1993a). For these reasons, efforts have been invested in developing radioligands with an improved I_2/α_2 selectivity. In this context, the new selective imidazoline radioligand [^{3}H]2-BFI has been recently used to chraracterize I_{2A}-imidazoline receptors in rabbit brain (Lione et al. 1996). The present study demonstrates that [^{3}H]2-BFI is also a good tool for the identification of I_{2B}-imidazoline receptors in rat brain.

In drug competition studies, the excellent correlation found (r = 0.97) when the affinities of a range of compounds for [^{3}H]2-BFI binding to rat brain membranes were compared with their affinities for [^{3}H]idazoxan binding in the same tissue, clearly indicated that the pharmacological profile obtained for [^{3}H]2-BFI binding (aganodine> cirazoline> 2-BFI>> clonidine> amiloride>> efaroxan) was very similar to that found for I_2-imidazoline receptors in various tissues of different species, including the rat brain (Brown et al. 1990; Langin et al. 1990; Wikberg and Uhlén 1990; Zonnenschein et al. 1990; Miralles et al. 1993a; Lione et al. 1996). Therefore, the binding sites characterized with [^{3}H]2-BFI in rat brain correspond to the I_2-imidazoline receptors labelled by [^{3}H]idazoxan in other tissues and species. Moreover, the low affinity displayed by amiloride (K_i = 900 nM) further suggests that the I_2-imidazoline receptors labelled by [^{3}H]2-BFI in rat brain belong to the I_{2B}-subtype, as is the case for those I_2-sites labelled by [^{3}H]idazoxan in the human and rat brains (Miralles et al. 1993a; Olmos et al. 1996a). Agmatine, a proposed endogenous ligand for imidazoline receptors (Li et al. 1994), displaced with very low affinity the binding of [^{3}H]2-BFI (K_i = 352 μM) to I_{2B}-imidazoline receptors. This result is in agreement with previous studies reporting low affinity of agmatine for I_2-imidazoline receptor subtypes in rabbit (I_{2A}-subtype) and rat (I_{2B}-subtype) brains (Lione et al. 1996; Olmos et al. 1996a).

The drug-affinity profile obtained in this study for [^{3}H]2-BFI binding sites in the rat cerebral cortex differed clearly from that typical of I_1-imidazoline receptors labelled by [^{3}H]clonidine or [^{3}H]p-aminoclonidine in human, rat and bovine brains (Ernsberger et al. 1987, 1995; Bricca et al. 1988, 1989). Thus, the imidazoline/guanidine compounds cirazoline and aganodine that displaced with high affinity (K_i in the low nanomolar range) the binding of [^{3}H]2-BFI in rat brain, possess very low affinity for I_1-imidazoline receptors (Ernsberger et al. 1987, 1995), and conversely, the low affinities that moxonidine and efaroxan showed when competing against [^{3}H]2-BFI binding in this tissue (K_i in the micromolar range) contrasted with their high affinities and selectivity for I_1-sites (Bricca et al. 1989; Ernsberger et al. 1995; Separovic et al. 1996). Together, these data indicate that [^{3}H]2-BFI does not recognize I_1-imidazoline receptors in the rat cerebral cortex.

Computer-assissted nonlinear analysis of the competition curves clearly demonstrated that [^{3}H]2-BFI binds to two populations of binding sites with high and low affinity for some of the imidazol(ines)/guanidines tested. One component (R_H) accounts for 38-83 % of [^{3}H]2-BFI binding at 3 x 10^{-9} M and has high affinity for aganodine, cirazoline, 2-BFI and idazoxan, and moderate affinity for bromoxidine (UK 14,304), clonidine and LSL 60101. In contrast, the second binding component (R_L) has at least 1000-fold lower affinity for these compounds (Table 1). These results are in agreement with previous studies which described heterogeneous binding with various selective ([^{125}I]AMIPI, [^{125}I]AZIPI, [^{3}H]RS-45041-190 and [^{3}H]2-BFI) (Ivkovic et al. 1994; MacKinnon et al. 1995b; Lione et al. 1996) and nonselective ([^{3}H]idazoxan) radioligands (Michel et al. 1989; Zonnenschein et al. 1990; Miralles et al. 1993a; Olmos et al. 1996a) to I_{2A}- or I_{2B}-imidazoline receptors in different tissues and species. Therefore, the present results provide further evidence of the heterogeneous nature of these receptors, although it remains unclear whether the multiple sites resolved in competition curves represent distinct proteins or different conformational states of the same receptive site (see Wikberg et al. 1992; Escribá et al. 1994; Olmos et al. 1996a).

The present study also demonstrates that the density of [^{3}H]2-BFI binding sites in the rat cerebral cortex can be differentially regulated in vivo after chronic treatment with cirazoline (25 % induction of up-regulation) and phenelzine (31 % induction of down-regulation). These

results are very similar to those reported for the same drug treatments on the density of I_2-imidazoline receptors using [^{3}H]idazoxan as radioligand (Olmos et al. 1992, 1994; Alemany et al. 1995b), which reinforces the concept that the binding of [^{3}H]2-BFI is associated with I_2-imidazoline receptors. Since repeated treatment with antagonist drugs often results in up-regulation of receptors, the increased density of I_2-imidazoline receptors found after chronic treatment with cirazoline may suggest that this drug could be an antagonist at these sites. However, functional assays would be necessary to demonstrate whether cirazoline and other imidazoline drugs are agonists or antagonists at these receptors. Because phenelzine does not irreversibly bind in vitro to brain or liver I_2-imidazoline receptors, an indirect in vivo mechanism appeared to be responsible for the down-regulation of the density of these receptors observed after chronic treatment with this MAO inhibitor (Alemany et al. 1995b). In this context, the endogenous amine 2-phenylethylamine is also a metabolite of phenelzine and has been proposed as one of the mediators of its antidepressant effects (McManus et al. 1991; Paetsch et al. 1993). It is known that chronic treatment with phenelzine, after irreversible inhibition of MAO-B, is associated with increased synaptic levels of 2-phenylethylamine (Paterson et al. 1990, 1991). Therefore, the possibility that increased levels of 2-phenylethylamine could be responsible for the phenelzine-induced down-regulation of I_2-imidazoline receptors was tested. However, chronic treatments with 2-phenylethylamine alone or 2-phenylethylamine together with the reversible and selective MAO-B inhibitor Ro 19-6327 did not alter the density of I_2-imidazoline receptors labelled by [^{3}H]idazoxan in the rat cerebral cortex. These results discounted any contribution of 2-phenylethylamine in the down-regulation of I_2-imidazoline receptors induced by phenelzine.

Recently, it has been demonstrated that at relatively high concentrations some imidazol(ine) drugs, including the I_2 selective compound 2-BFI, can inhibit in vitro MAO activity (Carpéné et al. 1995; MacKinnon et al. 1995a; Tesson et al. 1995) and also in vivo 2-BFI caused increases in the extracellular levels of noradrenaline in rat brain (Nutt et al. 1995). Moreover, the present study shows that the density of I_2-imidazoline receptors labelled by [^{3}H]2-BFI in rat brain was decreased after chronic treatment with phenelzine. Together these findings may suggest that the observed effect of phenelzine on I_2-imidazoline receptors could be due to a direct interaction of [^{3}H]2-BFI with MAO isoenzymes. However, the reported differential in vitro effects of clorgyline on liver MAO-A or MAO-B isoenzymes and on I_2-imidazoline receptors labelled by [^{3}H]2-BFI discounted the possibility that this radioligand, at nanomolar concentrations ($3x10^{-9}$ M), binds to the catalytic site of MAO isoenzymes. These results are in line with previous pharmacological and biochemical studies indicating that the imidazoline binding sites are not located within the catalytic site or the flavin-adenine dinucleotide prosthetic group of MAO (Carpéné et al. 1995; MacKinnon et al. 1995a; Raddatz et al. 1995; Tesson et al. 1995), although they do not discard the possibility that the I_2-site could be an unknown MAO binding domain different from the active site and that allosterically modulates MAO activity (Tesson et al. 1995; Parini et al. 1996). In this context, however, kinetic analyses of MAO inhibition in rat liver by 2-BFI and other imidazol(ine) compounds, have recently revealed that the drug/enzyme interactions were competitive for MAO-A, and mixed, but with a predominance of the competitive element, for MAO-B (Ozaita et al., 1997). These results suggested that imidazol(ine) drugs, at micromolar concentrations, inhibit MAO-A and MAO-B activity most probably through competitive/catalytic site related mechanisms. Moreover, no direct relationship was found when the affinities of nine imidazol(ine) drugs at I_2-imidazoline receptors were related to their potencies in inhibiting MAO-A or MAO-B activity in rat liver, suggesting furher that inhibition of MAO activity by imidazol(ine) drugs is not related to the postulated I_2-site on MAO (Ozaita et al. 1997). This conclusion is also in line with the present finding of a differential protein denaturating pattern for I_2-imidazoline receptors and MAO isoenzymes. Thus, when liver membranes were preincubated at 60 °C the binding of [^{3}H]Ro 41-1049 or [^{3}H]Ro 19-6327 to MAO-A or MAO-B isoenzymes was abolished, whereas still a 22 % of [^{3}H]2-BFI binding to liver I_2-imidazoline receptors remained intact at this high denaturating temperature. While these results might further suggest that MAO isoenzymes and I_2-imidazoline receptors are different proteins, the possibility that the observed differences could reflect a different temperature sensitivity of the binding domain of the three radioligands at MAO cannot be discarded. Clearly, further work will be needed to clarify this important aspect of I_2-imidazoline sites in relation to MAO.

Although these studies cannot exclude the presence of additional binding sites on MAO (Tesson et al. 1995) that do not affect the activity of the enzyme (see above), the clear relationship observed between drug-induced changes in immunoreactive imidazoline receptor proteins of 29/30- 45- and 66-kDa and density of I_2-sites in rat brain (Escribá et al. 1996) not only indicated the existence of various I_2-imidazoline receptors not related to MAO enzymes, but also that the observed down-regulation of these receptors after chronic treatment with phenelzine (present study; Alemany et al. 1995b) is related to decreased levels of various imidazoline receptor proteins (29/30- 45- and 66-kDa) and not to an irreversible binding of this drug to these receptors.

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