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European Journal of Pharmacology 280 (1995) 205–210

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LSL 60101, a selective ligand for imidazoline I_2 receptors, on glial fibrillary acidic protein concentration

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Received 26 January 1995; revised 29 March 1995; accepted 4 April 1995

Abstract

The concentration of the astrocytic marker, glial fibrillary acidic protein (GFAP) was quantitated by immunoblotting (Western blotting) in the rat brain after treatment with the novel ligand for imidazoline I_2 receptors LSL 60101 [2-(2-benzofuranyl)imidazole] and its 6-methoxy derivative LSL 60125. Chronic (7–21 days), but not acute (1 day) or short-term (3 days), treatment with LSL 60101 (10 mg/kg i.p.) markedly increased (44–49%) GFAP immunoreactivity in the rat cerebral cortex. In contrast, chronic (7 days) treatment with LSL 60125 (10 mg/kg i.p.) did not significantly modify GFAP concentrations. In vitro, both drugs displayed moderate high affinity and high selectivity for imidazoline I_2 receptors versus α_2-adrenoceptors; however, only chronic treatment with LSL 60101 (10 mg/kg i.p.) but not with LSL 60125 (10 mg/kg i.p.) was associated with an up-regulation of imidazoline I_2 receptors. These data indicate that glial imidazoline I_2 receptors may have a direct physiological function related to GFAP expression and that LSL 60101 could be a good tool for the study of the implication of these receptors on astrocyte activation and neuronal regeneration.

Keywords: Imidazoline; Imidazoline receptor; GFAP (glial fibrillary acidic protein); [^{3}H]Idazoxan; Astrocyte

1. Introduction

The imidazoline I_2 receptors are expressed in several tissues (Langin and Lafontan, 1989; Coupry et al., 1989; Zonnenschein et al., 1990; Molderings et al., 1994) including the brain (Mallard et al., 1992; Olmos et al., 1992; Miralles et al., 1993) where they are preponderantly located on glial cells (Regunathan et al., 1993; Olmos et al., 1994). Research on imidazoline I_2 receptors has encountered some difficulties: (1) the diverse effects elicited by the imidazoli(di)ne drugs are difficult to define in the context of known neurotransmitter/hormone receptor systems (i.e. agonistic/antagonistic effects; signal transduction, etc.) and (2) the lack of high affinity and selective I_2 molecules makes it complex to dissociate their actions on these receptors from others related to the interaction with α-adrenoceptors. In this sense, the novel compound LSL 60101 [2-(2-benzofuranyl)imidazole] (Fig. 1) has been shown to induce several biological effects that appear to be related to the interaction with imidazoline receptors, i.e. induction of acute hyperphagia in satiated rats (Menargues et al., 1994), stimulation of insulin release (Cedó et al., 1994) and increase in the levels of certain monoamines in the rat brain (Artiga et al., 1994).

The glial imidazoline I_2 receptors have been shown to be implicated in the regulation of the levels of glial fibrillary acidic protein (GFAP) (Olmos et al., 1994), the main constituent of intermediate filaments of astrocytes (Eng et al., 1971; Eng, 1985). Therefore, the present study was designed (1) to investigate in vitro the interaction of LSL 60101 and its 6-methoxy analogue LSL 60125 (Fig. 1) with brain imidazoline I_2 receptors and α_2-adrenoceptors, (2) to assess whether chronic treatment with these novel ligands could also

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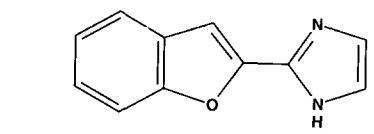
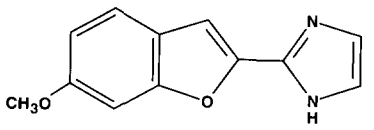
Elsevier Science B.V.
SSDI 0014-2999(95)00214-6



LSL 60101



LSL 60125

Fig. 1. Chemical structures of LSL 60101 [2-(2-benzofuranyl)imidazole] and LSL 60125 [2-(6-methoxybenzofuran 2-yl)imidazole].

modulate in vivo the density of GFAP in the rat brain, and (3) to relate possible changes in GFAP levels with a parallel modulation of the density of brain imidazoline I_2 receptors, as previously described for other highly potent imidazoline I_2 drugs such as cirazoline and idazoxan, but not for imidazoline I_1 drugs such as efaroxan (Olmos et al., 1994).

2. Materials and methods

2.1. Animals and treatments

Male Sprague-Dawley rats (250–300 g) were used. The animals received a standard diet with water freely available and were housed at $20 \pm 2°$ C with a 12 h light/dark cycle. In the chronic (7 days) treatments the animals received i.p. every 12 h either 0.9% saline vehicle, LSL 60101 (1–30 mg/kg), LSL 60125 (10 mg/kg), cirazoline (1 mg/kg) and efaroxan (10 mg/kg). The time-course for the effect of LSL 60101 (10 mg/kg) was studied at days 1, 3, 7, 14 and 21 after treatment. In all treatments the rats were killed 24 h after the last injection.

2.2. Immunoblot analysis of GFAP and quantitation of specific immunoreactivity

Immunoblotting of GFAP was done as previously described (Olmos et al., 1994). Animals were decapitated and brains rapidly removed under ice. 100–150 mg of brain parieto-occipital cortex were homogenised (30 s) with an Ultraturrax homogeniser in 50 volumes of 50 mM Tris buffer pH 6.8 containing 2 mM EDTA, 0.5% Triton X-100 and 2 mM phenylmethylsulfonyl fluoride. The samples were then sonicated (20 s) and centrifuged at $14\,600 \times g$ for 15 min at 4° C. The supernatant was recovered and diluted 1:20 in the previous homogenisation buffer. The protein content in these samples was determined by the method of Bradford (1976), using bovine serum albumin as the standard. An aliquot (200 μl) of the diluted supernatant was mixed with the same volume of electrophoresis loading buffer and boiled. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to nitrocellulose membranes (immunoblotting, Western blotting), incubated with the primary antibody, rabbit anti-cow GFAP (1:5000 dilution) (Dako, Denmark), and then with the secondary antibody, horseradish peroxidase-linked donkey anti-rabbit immunoglobulin, as previously described for other proteins (Escribá et al., 1994). Immunoreactivity was detected with the enhanced chemiluminescence (ECL) Western blot detection system (Amersham International, UK) followed by exposure to Kodak X-AR film (Fig. 3). Omission of the primary antibody was used as a negative control, i.e. immunoreactivity was absent under these conditions. Films were scanned in the image analyzer Bio Image (Millipore, Ann Arbor, MI, USA). After scanning, standard curves were constructed using samples from saline-treated rats (Fig. 3). Quantitation of GFAP in samples from saline-treated and drug-treated rats was done as previously described (Escribá et al., 1994; Olmos et al., 1994). The quantitation procedure was repeated at least 3 times for each sample in different gels. The mean interassay coefficient of variation was 9%.

2.3. [^{3}H]Idazoxan and [^{3}H]RX821002 binding assays

Radioligand binding assays for [^{3}H]idazoxan and [^{3}H]RX821002 (2-methoxy idazoxan) and preparation of P_2 membrane fractions were done as previously described (Miralles et al., 1993). Total [^{3}H]idazoxan and [^{3}H]RX821002 binding was measured in 1.1-ml aliquots (50 mM Tris-HCl, 0.1% ascorbic acid, pH 7.5) of the membranes (0.8–1.2 mg protein) which were incubated with shaking for 30 min at 25° C. Binding of [^{3}H]idazoxan to imidazoline I_2 receptors was always done in the presence of 10^{-6} M (−)-adrenaline to prevent the binding of the radioligand to α_2-adrenoceptors. Nonspecific binding was determined in the presence of 10^{-4} M naphazoline, as previously described (Olmos et al., 1992). In the saturation studies, cortical membranes were incubated with eight concentrations of [^{3}H]idazoxan (6×10^{-10} M to 5×10^{-8} M) as above. In the drug competition studies, cortical membranes were incubated as above with [^{3}H]idazoxan (10^{-8} M) or [^{3}H]RX821002 (10^{-9} M) and in the absence or presence of various concentrations of the competing drugs (10^{-10} M to 10^{-3} M; 15 concentrations). Most imidazoline drugs displace [^{3}H]idazoxan binding from two distinct binding sites of the imidazoline I_2 receptors (Wikberg et al., 1992; Miralles et al., 1993). Incubations were terminated by diluting the

samples with 5 ml of ice-cold Tris incubation buffer (4° C). Membrane-bound ligand was measured by vacuum filtration through Whatman GF/C glass fiber filters which had been presoaked with 0.5% polyethylenimine (Bruns et al., 1983), using a Brandel 48R cell harvester (Biomedical Research and Development Laboratories, USA). Then the filters were rinsed twice with 5 ml of incubation buffer, air-dried, transferred to minivials containing 5 ml of OptiPhase 'HiSafe' II cocktail (LKB, UK) and counted for radioactivity by liquid scintillation spectrometry at 57% efficiency (Packard model 1900 TR). Protein content in these samples was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Analyses of individual saturation isotherms (K_D, dissociation constant; B_{max}, maximum density of binding sites) and competition experiments (K_i, inhibition constant) as well as the fitting of data to the appropriate binding models were performed using the EBDA-LIGAND programs (Munson and Rodbard, 1980; McPherson, 1985).

2.4. Statistics

In GFAP immunoblot quantitation experiments results after treatments are expressed as percent of values of saline-treated rats, as described above, and represent the mean ± S.E.M. In binding experiments, comparisons were done with the binding parameters (K_D, B_{max}) obtained in saturation curves and values of imidazoline I_2 receptors density were then expressed as mean ± S.E.M. of percent of values over saline-treated rats. One-way analysis of variance (ANOVA), followed by Scheffé's test, was used for the statistical evaluations. Correlation coefficients were calculated by the method of least squares. The level of significance was chosen as $P = 0.05$.

2.5. Drugs

Rabbit anti-cow GFAP, a purified immunoglobulin fraction of rabbit antiserum was purchased from Dako (Denmark), and horseradish peroxidase-linked donkey anti-rabbit immunoglobulin was purchased from Amersham International (UK). [^{3}H]Idazoxan (specific activity, 42–46 Ci/mmol; batches 47 and 48) and [^{3}H]RX821002 (2-methoxy idazoxan) (specific activity, 59 Ci/mmol; batch 10) were purchased from Amersham International (UK). [^{3}H]Idazoxan was stored at −30° C and [^{3}H]RX821002 at 4° C. For the 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: cirazoline HCl (Synthélabo Recherche, France); efaroxan HCl (RBI, USA), LSL 60101 [2-(2-benzofuranyl)imidazole HCl] and LSL 60125 [2-(6-methoxybenzofuran 2-yl)imidazole HCl] (synthesized by Dr. F. Pla at S.A. Lasa Laboratories, Spain). Other reagents were obtained from Sigma Chemical Co. (USA).

3. Results

3.1. Effects of LSL 60101 and LSL 60125 on GFAP immunoreactivity in the rat cerebral cortex

After immunoblotting reactive bands of GFAP were found in the rat cerebral cortex with a relative molecular mass of 49 kDa, as previously described for the

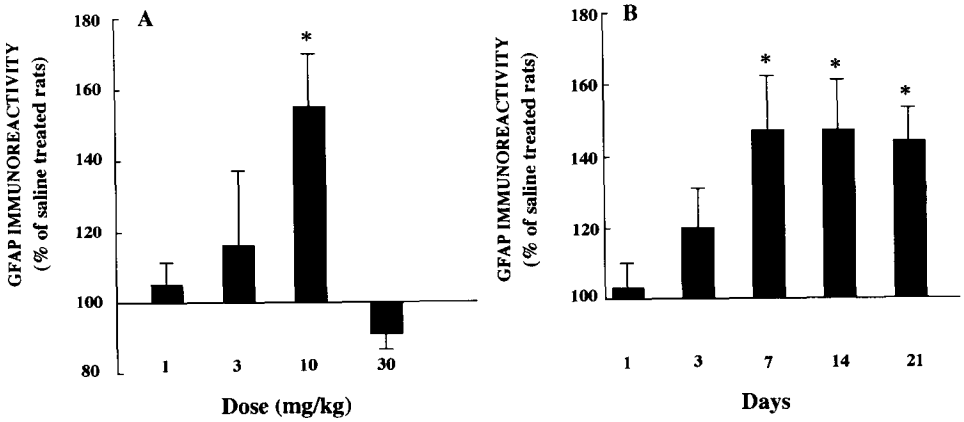


Fig. 2. (A) Effect of chronic treatment (i.p. every 12 h for 7 days) with LSL 60101 (1–30 mg/kg) on glial fibrillary acidic protein (GFAP) immunoreactivity in the rat cerebral cortex. (B) Time-course for the effect of chronic treatment (i.p. every 12 h) with LSL 60101 (10 mg/kg) on GFAP immunoreactivity in the rat cerebral cortex. GFAP immunoreactivity after treatments was expressed as percentage of that from saline-treated rats (controls; data not shown) according to the standard curves described in Materials and methods. Columns represent the mean ± S.E.M. of 3–12 experiments with one animal per experiment. * $P < 0.05$ as compared with saline-treated group (ANOVA followed by Scheffé's test).

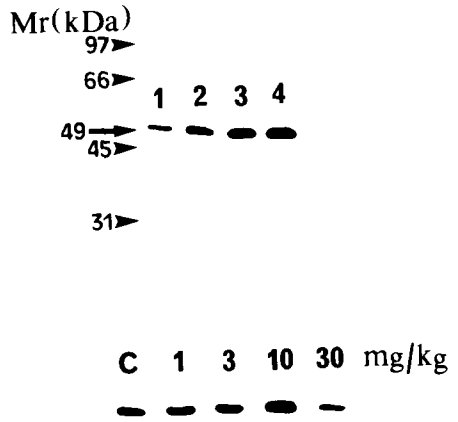


Fig. 3. Upper panel: Western blot of rat brain parieto-occipital cortex showing glial fibrillary acidic protein (GFAP) immunoreactivity signal intensity vs. protein content. The Western blot was performed as described in Materials and methods. The amount of total protein loaded per well was (in ng): 106 (1), 212 (2), 266 (3) and 354 (4). Relative molecular masses (kDa) of marker proteins are given on the left. The chemiluminescent reaction for GFAP immunoreactivity (M_r = 49 kDa) (arrow) appeared to be concentration-dependent in a linear fashion with respect to the protein content. Lower panel: Representative immunoreactive bands using antisera against GFAP in the rat brain after saline (C) or chronic treatment (7 days) with LSL 60101 at the doses indicated. The amount of total protein loaded per well was as follows (in ng): 212 (C); 237 (1 mg/kg); 271 (3 mg/kg); 187 (10 mg/kg) and 186 (30 mg/kg). GFAP immunoreactivities increased significantly after chronic treatment with LSL 60101 (10 mg/kg) (+41%). Samples from the upper and lower panels were taken from the same gel. See Fig. 2A for changes in mean percentage values of GFAP immunoreactivity after the various doses and other details.

monomeric form of this cytoskeletal protein (Eng, 1985). Chronic treatment (7 days) with the imidazole drug LSL 60101 (1–10 mg/kg) dose-dependently increased GFAP immunoreactivity in the rat cerebral cortex (Fig. 2A and Fig. 3), whereas high doses of this compound (30 mg/kg) tended to decrease, although non-significantly, GFAP immunoreactivity (Fig. 2A and Fig. 3). The time-course for the effect of LSL 60101 (10 mg/kg) was then studied. Acute (1 day) or short-term (3 days) treatments failed to modify GFAP levels in the rat cerebral cortex and the increased GFAP immunoreactivity was only observed after chronic (7 days) treatment; this effect was maintained after 14 or 21 days of repeated treatment (GFAP immunoreactivity increased by 44–49%) (Fig. 2B). A similar chronic treatment with LSL 60125 (10 mg/kg for 7 days), the 6-methoxy analogue of LSL 60101, failed to increase significantly GFAP immunoreactivity in the rat cerebral cortex (only 15% of increase) (Fig. 5).

When tested in vitro, both drugs displayed moderate high affinity for imidazoline I_2 receptors and displaced [^{3}H]idazoxan binding to rat cerebral cortex from two distinct binding sites (Table 1 and Fig. 4). In contrast, these two drugs displayed very low affinity for brain α_2-adrenoceptors (K_i in the high micromolar range in competition curves against [^{3}H]RX821002 binding) (Table 1). These data indicate that LSL 60101 and LSL 60125 are rather selective drugs for the interaction with imidazoline I_2 receptors when compared with α_2-adrenoceptors (see Table 1 for K_i ratios).

3.2. Involvement of imidazoline I_2 receptors on the LSL 60101-induced increase of GFAP immunoreactivity

The increases in GFAP immunoreactivity after chronic imidazoline drug treatment has been previously associated with a parallel up-regulation of imidazoline I_2 receptors in the rat cerebral cortex (Olmos et al., 1994). In order to determine if the effects of LSL 60101 on GFAP immunoreactivity could also be mediated through these new putative receptors the effects of the same chronic treatments were studied on the density and affinity of imidazoline I_2 receptors. Chronic treatment (7 days) with LSL 60101 (10 mg/kg) significantly increased by about 40% the density of imidazoline I_2 receptors in the rat cerebral cortex, without significantly changing the affinity (K_D) of [^{3}H]idazoxan for these receptors (Fig. 5). In contrast, a similar chronic treament with LSL 60125 (10 mg/kg for 7 days), the 6-methoxy analogue of LSL 60101, was not associated with significant changes in the density/affinity of imidazoline I_2 receptors (Fig. 5).

Chronic treatment with cirazoline (1 mg/kg i.p. every 12 h for 7 days), a high affinity but moderately selective drug for imidazoline I_2 receptors (Table 1 and Fig. 4), was also shown to be associated with an increase in GFAP immunoreactivity and a parallel up-regulation of brain imidazoline I_2 receptors (Fig. 5).

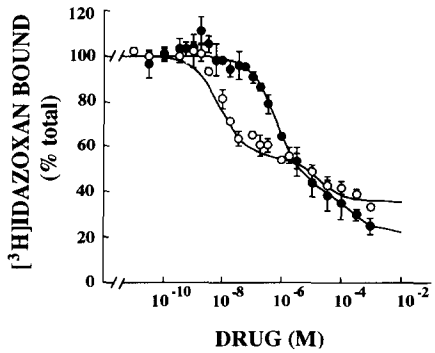


Fig. 4. Inhibition of binding of [^{3}H]idazoxan (in the presence of 10^{-6} M (−)-adrenaline) to imidazoline I_2 receptors in the rat cerebral cortex by cirazoline (○) and LSL 60101 (●). Cortical membranes were incubated at 25° C for 30 min with [^{3}H]idazoxan (10^{-8} M) in the absence or presence of various concentrations of the competing drugs. Total control binding was 2200 dpm. Computer-assisted curve fitting (EBDA-LIGAND programs) demonstrated that in both competition curves a two-site fit was significantly better than a one-sit fit ($P < 0.001$; F-test). Data shown are mean ± S.E.M. of three experiments per drug. See Table 1 for K_i values and other details.

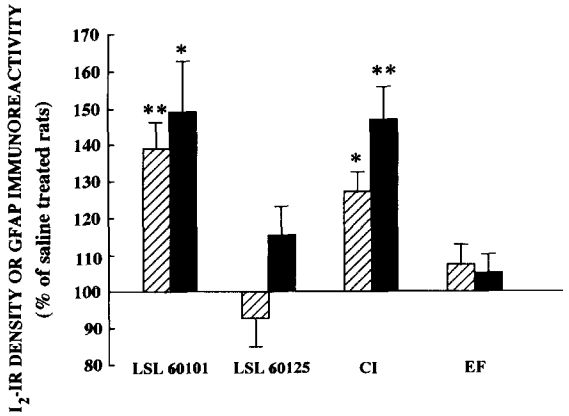


Fig. 5. Effect of chronic treatment (i.p. every 12 h for 7 days) with LSL 60101 (10 mg/kg), LSL 60125 (10 mg/kg), cirazoline (CI) (1 mg/kg) and efaroxan (EF) (10 mg/kg) on the density (B_{max}) of imidazoline I_2 receptors (I_2-IR) (hatched columns) and glial fibrillary acidic protein (GFAP) immunoreactivity (solid columns) in the rat cerebral cortex. Saline-treated rats were used as controls (data not shown). The specific binding of [^{3}H]idazoxan to imidazoline I_2 receptors was determined in P_2 fractions of cortical membranes and was defined as the difference between binding in the presence of 10^{-6} M (−)-adrenaline (total non-adrenoceptor binding) and 10^{-4} M naphazoline (nonspecific binding). Binding parameters (K_D, B_{max}) were determined directly by computer-assisted nonlinear analysis from untransformed data using the EBDA-LIGAND programs (mean values for saline-treated rats: $K_D = 13.3 \pm 0.9$ nM; $B_{max} = 54 \pm 2$ fmol/mg protein; $n = 14$). Imidazoline I_2 receptors density (B_{max}) after treatments is expressed as percentage of that from saline-treated rats. One-way ANOVA followed by a multiple comparison test detected a significant increase in B_{max} for imidazoline I_2 receptors ($F(4,27) = 12.34$; $P = 0.0001$) after chronic treatment with LSL 60101 and cirazoline, but not after chronic LSL 60125 or efaroxan treatments. No significant changes in K_D values were found after treatments (range of K_D values: 13.3 ± 0.9 nM–16.9 ± 2.1 nM). GFAP immunoreactivity data for chronic treatment with LSL 60101 were taken for comparison from Fig. 2 and those for cirazoline and efaroxan from Olmos et al. (1994). Bars represent the mean ± S.E.M. of 3–14 experiments with one animal per experiment. * $P < 0.01$ and ** $P < 0.001$ as compared with saline-treated group (ANOVA followed by Scheffé's test).

A similar chronic treatment (7 days) with efaroxan, a potent α_2-adrenoceptor antagonist and imidazoline I_1 receptor ligand in vitro but displaying very low affinity for imidazoline I_2 receptors (Table 1), did not change GFAP immunoreactivity nor imidazoline I_2 receptor density in the rat cerebral cortex (Fig. 5).

Together these results reinforced the hypothesis that the effects of LSL 60101 on GFAP immunoreactivity could be mediated through the interaction with imidazoline I_2 receptors.

4. Discussion

This study demonstrates that chronic (7 days), but not acute (1 day) or short-term (3 days), treatment with the selective imidazoline I_2 receptor ligand LSL 60101 is associated with increased levels of the astrocyte marker GFAP and that this occurs in parallel with an up-regulation of the density of imidazoline I_2 receptors in the rat brain. These results confirm previous in vitro and in vivo studies relating imidazoline I_2 receptors with the regulation of the expression of GFAP (Regunathan et al., 1993; Olmos et al., 1994). Thus, occupancy of imidazoline I_2 receptors by idazoxan resulted in a marked concentration-dependent increase in mRNA levels for GFAP in rat cultured cortical astrocytes (Regunathan et al., 1993). In these studies the possible implication of α_2-adrenoceptors and imidazoline I_1 receptors had also been discarded (Regunathan et al., 1993; Olmos et al., 1994). Although both LSL 60101 and its 6-methoxy analogue LSL 60125 displayed moderate high affinity and high selectivity for imidazoline I_2 receptors versus α_2-adrenoceptors (Table 1), only LSL 60101 was able to up-regulate GFAP levels and imidazoline I_2 receptors; therefore, it can be suggested that the methoxy group at the 6-position in the benzene ring of LSL 60125 prevents the imidazoline I_2 receptor activation of the signal transduction that leads to a higher expression of GFAP. Alternatively, the methoxy group of LSL 60125 could prevent the drug reaching the brain.

GFAP represents the prototypic marker of astroglial activation (Norton et al., 1992). Reactive astrocytosis is accompanied by an induction and up-regulation of many proteins with potent biological effects (Eddleston

Table 1
Binding parameters of various imidazole/imidazoline drugs to imidazoline I_2 receptors and α_2-adrenoceptors in the rat cerebral cortex

Drug	Imidazoline I_2 receptors (I_2-IR)			α_2-adrenoceptors (α_2R)	Ratio
	K_{iH} (nM)	K_{iL} (μM)	%R_H	K_i (nM)	(α_2R/I_2-IR)
LSL 60101	350	116	79	100148	286
LSL 60125	151	17	60	56150	372
Cirazoline	3.8	2.1	74	307	81
Efaroxan	–	58	–	6.0	0.0001

Cortical membranes were incubated at 25° C for 30 min with [^{3}H]idazoxan (10^{-8} M in the presence of 10^{-6} M (−)-adrenaline; imidazoline I_2 receptors) or [^{3}H]RX821002 (10^{-9} M; α_2-adrenoceptors) and in the absence or presence of the competing drugs (10^{-10} to 10^{-3} M; 15 concentrations). Binding parameters (K_i values) were determined directly by simultaneous analysis of three independent experiments for each drug using the EBDA-LIGAND programs. Computer-assisted curve fitting demonstrated that for LSL 60101, LSL 60125 and cirazoline competition curves against [^{3}H]idazoxan binding a two-site fit was significantly better than a one-site binding model ($P < 0.001$; F test).

and Mucke, 1993). It is possible that activation of imidazoline I_2 receptors by LSL 60101 and other imidazoline drugs (i.e. cirazoline, idazoxan) is related to the induction of reactive astrocytes and up-regulation of the expression of some proteins, including GFAP and the same imidazoline I_2 receptors. It remains controversial if the induced changes are beneficial or detrimental in nature; however, there is a wealth of data indicating that activated astrocytes produce several growth factors that are crucial to a successful regenerative response (Norenberg, 1994). In this context, idazoxan and rilmenidine (an oxazoline structurally related to the imidazolines) have been shown to provide protection from global cerebral ischaemia (Maiese et al., 1992). These findings suggest that highly selective drugs, like LSL 60101, could be good tools for the study of the implication of glial imidazoline I_2 receptors on astrocyte activation and neuronal regeneration.

Acknowledgements

This study was supported by DGICYT Grant PM 91-0069, FIS Grant 93-0641 and by CDTI-S.A. LASA Laboratorios Grant 92-0198, Spain. R.A. was supported by a fellowship from FIS (Fondo de Investigaciones Sanitarias, Spain). J.A.G.S. is a member of the Institut d'Estudis Catalans. Part of this work was presented at the II International Symposium on Imidazoline Receptors (Abstract No. P22), New York, USA, July 1994.

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