British Journal of Pharmacology (1996) 118, 2029–2036

Pharmacological modulation of immunoreactive imidazoline receptor proteins in rat brain: relationship with non-adrenoceptor [^{3}H]-idazoxan binding sites

Pablo V. Escribá, Regina Alemany, Magdalena Sastre, Gabriel Olmos, Andrés Ozaita & [1]Jesús 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 The densities of various imidazoline receptor proteins (with apparent molecular masses of ≈29/30 – 45- and 66-kDa) were quantitated by immunoblotting in the rat cerebral cortex after various drug treatments. The modulation of these imidazoline receptor proteins was then compared with the changes in the density of non-adrenoceptor [^{3}H]-idazoxan binding sites (I_2-sites) induced by the same drug treatments.

2 Chronic treatment (7 days) with the I_2-selective imidazol(in)e drugs idazoxan (10 mg kg^{-1}), cirazoline (1 mg kg^{-1}) and LSL 60101 (10 mg kg^{-1}) differentially increased the immunoreactivity of imidazoline receptor proteins. The levels of the 29/30-kDa protein were increased by idazoxan and LSL 60101 (23%), the levels of the 45-kDa protein only by cirazoline (44%) and those of the 66-kDa protein only by idazoxan (50%). These drug treatments also increased the density of I_2-sites (32–42%).

3 Chronic treatment (7 days) with efaroxan (10 mg kg^{-1}), RX821002 (10 mg kg^{-1}) and yohimbine (10 mg kg^{-1}), which possess very low affinity for I_2-imidazoline receptors, did not alter either the immunoreactivity of imidazoline receptor proteins or the density of I_2-sites.

4 Chronic treatment (7 days) with the monoamine oxidase (MAO) inhibitors clorgyline (10 mg kg^{-1}) and phenelzine (10 mg kg^{-1}) decreased the immunoreactivity of the 29/30-kDa (17–24%), 45-kDa (19%) and 66-kDa (23–31%) imidazoline receptor proteins. The alkylating agent N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (1.6 mg kg^{-1}, 6 h) also decreased the levels of the three imidazoline receptor proteins (20–47%). These drug treatments consistently decreased the density of I_2-sites (31–57%).

5 Significant correlations were found when the mean percentage changes in immunoreactivity of imidazoline receptor proteins were related to the mean percentage changes in the density of I_2-sites after the various drug treatments ($r=0.92$ for the 29/30-kDa protein, $r=0.69$ for the 45-kDa protein and $r=0.75$ for the 66-kDa protein).

6 In the rat cerebral cortex the I_2-imidazoline receptor labelled by [^{3}H]-idazoxan is heterogeneous in nature and the related imidazoline receptor proteins (29/30-, 45- and 66-kDa) detected by immunoblotting contribute differentially to the modulation of I_2-sites after drug treatment.

Keywords: Imidazoline receptor proteins; [^{3}H]-idazoxan; I_2-sites; rat brain

Introduction

Various imidazol(in)e/guanidinium compounds, such as clonidine and idazoxan interact with membrane proteins distinct from α_2-adrenoceptors. These proteins, called imidazoline receptors, mediate various effects which do not originate from the activation of α_2-adrenoceptors, such as regulation of blood pressure (Bousquet *et al.*, 1984), stimulation of insulin secretion (Schulz & Hasselblat, 1989), inhibition of noradrenaline release (Göthert & Molderings, 1991), stimulation of locus coeruleus noradrenergic neurones (Pineda *et al.*, 1993), activation of the catecholamine biosynthetic enzymes (Evinger *et al.*, 1995) and the induction of the expression of glial fibrillary acidic protein (Regunathan *et al.*, 1993; Olmos *et al.*, 1994a), among others. Based on the rank order of affinity for different ligands, imidazoline receptors have been classified into two main types: the clonidine-preferring receptor (I_1-type) and the idazoxan-preferring receptor (I_2-type) (Michel & Ernsberger, 1992). [^{3}H]-clonidine and its derivatives have been used to label I_1-imidazoline receptors, which display high affinity for imidazolidines (clonidine, moxonidine), medium affinity for imidazolines (idazoxan, cirazoline) and imidazoles (cimetidine and histamine) and low affinity for guanidines (guanabenz and aganodine) (Molderings *et al.*, 1993). On the other hand, [^{3}H]-idazoxan has been used to label I_2-imidazoline receptors, which display high affinity for imidazolines and guanidines, medium affinity for imidazolidines and low affinity for imidazoles (Miralles *et al.*, 1993a). I_2-imidazoline receptors have been further subclassified into the I_{2A}-subtype (high affinity for the guanidide amiloride) and the I_{2B}-subtype (low affinity for amiloride) (Diamant *et al.*, 1992; Miralles *et al.*, 1993a). Moreover, functional studies have revealed the existence of additional imidazoline receptors which cannot be grouped into any of the previous types (i.e. non I_1/I_2-type) (Chan *et al.*, 1994; Olmos *et al.*, 1994b; Molderings & Göthert, 1995). The amine agmatine (decarboxylated arginine) is an endogenous agonist at imidazoline receptors (Li *et al.*, 1994) and may act as a neurotransmitter or neuromodulator for some members of this receptor family (Li *et al.*, 1994; Piletz *et al.*, 1995).

Various imidazoline receptor proteins of 27–85 kDa have been visualised or purified from adrenal chromaffin cells (Wang *et al.*, 1992), rabbit and rat kidney (Limon *et al.*, 1992; Lanier *et al.*, 1995; Tesson *et al.*, 1995), rat and human liver and placenta (Lanier *et al.*, 1993; 1995; Raddatz *et al.*, 1995), rat and human brain (Escribá *et al.*, 1994b; 1995a; Greney *et al.*, 1994) and human platelets (García-Sevilla *et al.*, 1996).

[1] Author for correspondence.

These studies clearly indicated the existence of heterogeneity of imidazoline receptor proteins in agreement with previous pharmacological studies. However, the possible association of the various imidazoline receptor proteins with any known pharmacological type of imidazoline receptors is at present not precisely known. In this context, the present study was designed to assess whether chronic treatment with several imidazol(in)e drugs, selective for I_2- and/or I_1-imidazoline receptors or α_2-adrenoceptors, as well as with monoamine oxidase (MAO) inhibitors could modulate immunoreactive imidazoline receptor proteins in brain, and also to relate possible changes in the levels of immunoreactivity with parallel changes in the density of non-adrenoceptor [^{3}H]-idazoxan binding sites (I_2-imidazoline receptors). A preliminary account of part of this work was given at the 19th annual meeting of the Spanish Society of Pharmacology (Alemany *et al.*, 1995a).

Methods

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±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, idazoxan (10 mg kg^{-1}), cirazoline (1 mg kg^{-1}), LSL 60101 (10 mg kg^{-1}), efaroxan (10 mg kg^{-1}), RX821002 (10 mg kg^{-1}) and the non-imidazoline α_2-adrenoceptor antagonist yohimbine (10 mg kg^{-1}), as well as the MAO inhibitors clorgyline (10 mg kg^{-1}) or phenelzine (10 mg kg^{-1}). The rats were killed 24 h after the last injection. In another set of experiments, rats were treated with the peptide coupling agent EEDQ (1.6 mg kg^{-1}, dissolved in ethanol and then diluted with propylene glycol/water in a final volume ratio of 1:1:2). Rats were killed 6 h after a single EEDQ injection. Treatment with EEDQ has been shown to inactivate a portion of non-adrenoceptor [^{3}H]-idazoxan binding sites (20–45% inactivation) (Miralles *et al.*, 1993b).

Three series of treatments were performed each with the corresponding saline control group (idazoxan, cirazoline and LSL60101; efaroxan, RX821002 and yohimbine; clorgyline, phenelzine and EEDQ) (Tables 1–3). In most cases, the cerebral frontal cortex was used for quantitation of imidazoline receptor proteins and the parieto-occipital cortex of the same animal for quantitation of I_2-imidazoline receptors (see below). These experiments in rats were performed according to the guidelines of the University of the Balearic Islands.

Immunoblot detection and quantitation of imidazoline receptor proteins and α-tubulin

For the immunoblot experiments, 100–200 mg of rat brain frontal cortex was homogenised in 2 ml of ice-cold 50 mM Tris-HCl, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride, 5 mM iodoacetamide, pH 7.5, and centrifuged at 4°C and 800 × *g* for 10 min. The resulting pellet was discarded, and the supernatant was centrifuged at 40,000 × *g* for 10 min. The final pellet was resuspended in 250 µl of 40 mM Tris-HCl, 4% sodium dodecyl sulphate (SDS), pH 6.8 and incubated at 75°C for 5 min. After addition of 150 µl of electrophoresis loading buffer (62.5 mM Tris-HCl buffer, 3% SDS, 20% glycerol, 0.1% 2-mercaptoethanol, 0.005% bromophenol blue, pH 6.8) the samples were boiled and then submitted to polyacrylamide gel electrophoresis (sodium dodecyl sulphate-PAGE) in a 10% Laemmli gel. After electrophoretical transfer of the proteins onto nitrocellulose membranes, they were blocked at room temperature for 1 h with phosphate buffered saline (PBS composition (mM): NaCl 137, KCl 2.7, Na_2HPO_4 12, KH_2PO_4 1.38, pH 7.2) containing 10% nonfat dry milk and 0.1% Tween 20 (blocking solution). Anti-imidazoline receptor antiserum was then added in fresh blocking solution (1:5,000 dilution) and incubated for 14–16 h at 4°C. After two quick rinses with PBS, membranes were washed, for 10 min at room temperature, three times with PBS. The secondary antibody, horseradish peroxidase-linked donkey anti-rabbit IgG, was then added in fresh blocking solution, and incubated for 2 h at room temperature (1:5,000 dilution). Immunoreactivity was detected with the Enhanced Chemiluminescence (ECL) western blot detection system (Amersham) followed by exposure to autoradiography film. Omission of the primary antibody was used as a negative control; i.e. immunoreactivity was absent under these conditions. The integrated optical densities (IODs) of the bands were measured with the image analyser BioImage (Millipore) and the protein quantitation was performed as described previously (Escribá *et al.*, 1994a; 1995b). Briefly, duplicate unknown samples were evaluated by use of standard curves (i.e., total protein loaded versus IOD) consisting of at least four points of different protein content (usually 5 to 50 µg of total membrane protein per gel well) of brain cortex samples from saline-treated rats, along with the unknown samples, all loaded on the same gel (2 to 4 gels for each treated rat). The theoretical amount of protein (P_T) loaded in the gel well was obtained by interpolation of the IOD value from an unknown sample in the standard curve from control samples, and was compared with the real amount of protein (P_R) loaded on the gel. The percentage change in the content of imidazoline receptor protein with respect to the control was calculated as: % control = $(P_T/P_R) \times 100$, where this value was 100% for the control sample used as the standard. With the ECL method, the IOD value versus the protein concentration curve followed a sigmoidal behaviour. However, only IOD values within the linear range of the curve were considered for quantitation. Because of the lesser immunoreactivity of the 45- and 66-kDa imidazoline receptor proteins, some IOD values out of the linear range were not used (see number of experiments presented in Tables 1 and 3).

α-Tubulin, a cytoskeletal protein used as a loading control, was separated similarly as described above. The primary antibody used was a monoclonal anti-α-tubulin (clone DM 1A) diluted 1:2,000 in blocking solution, and incubated for 4 h at room temperature. The secondary antibody, horseradish peroxidase-linked sheep anti-mouse IgG, was incubated at 1:5,000 dilution for 2 h at room temperature. Detection and quantitation of α-tubulin were performed as above.

In some experiments, imidazoline receptor proteins and α_{2A}-adrenoceptors were separated and immunodetected in membranes from Sf9 (*Spodoptera frugiperda*) cells overexpressing α_{2A}-adrenoceptors to assess further the specificity of the polyclonal antibody used for the detection of imidazoline receptor proteins.

[^{3}H]-idazoxan binding assay and quantitation of I_2-imidazoline receptors

Cortical membranes (P_2 fraction) were prepared by established methods from the parieto-occipital cortex. Briefly, the tissue samples were homogenised in 5 ml of ice-cold Tris-sucrose buffer (5 mM Tris-HCl, 250 mM sucrose, 1 mM $MgCl_2$, pH 7.4). The homogenates were centrifuged at 4°C and 1,000 × *g* for 10 min. Pellets were discarded and the supernatants were then centrifuged at 4°C and 40,000 × *g* for 10 min. The resulting pellet was washed with 2 ml of fresh incubation buffer (50 mM Tris-HCl, 0.1% ascorbic acid, pH 7.5), recentrifuged twice and resuspended to a final protein content of 0.8–1.1 mg ml^{-1}. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Total [^{3}H]-idazoxan binding was measured in 1.1 ml-aliquots (50 mM Tris-HCl, 0.1% ascorbic acid, pH 7.5) of cortical membranes which were incubated with shaking for 30 min at 25°C. Binding of [^{3}H]-idazoxan to I_2-imidazoline receptors was always done in the presence of 10^{-6} M (−)-adrenaline to prevent the binding of the radioligand to α_2-adrenoceptors (Miralles *et al.*, 1993a). Nonspecific binding was determined in the presence of 10^{-4} M naphazoline. In the sa-

turation studies, cortical membranes were incubated with eight concentrations of [^{3}H]-idazoxan (6×10^{-10} M to 5×10^{-8} M) as above. After incubation and dilution, bound ligand was separated from free ligand by vacuum filtration through glass fibre filters which were counted for radioactivity by liquid scintillation spectrometry at 50% efficiency (Packard model 1900 TR). Analyses of individual saturation isotherms (K_d, dissociation constant; B_{max}, maximum density of binding sites) were performed by use of the EBDA-LIGAND programmes as previously described (Miralles *et al.*, 1993a).

Statistics

In immunoblot quantitation experiments, results after treatments are expressed as a percentage of values of saline-treated rats, as described above, and represent the mean ± s.e.mean. In binding experiments, comparisons were done with the binding parameters (K_d, B_{max}) obtained in saturation curves and are expressed as mean ± s.e.mean. One-way analysis of variance (ANOVA), followed by Scheffé's or Fisher's tests, was used for the statistical evaluations. Correlation coefficients were calculated by the method of least squares. The level of significance was chosen as $P = 0.05$.

Materials and drugs

The antiserum against imidazoline receptor proteins was a generous gift from Prof. D.J. Reis (Cornell University Medical College, New York, U.S.A.). The production and specificity of this antiserum for the detection of imidazoline receptor proteins have been described elsewhere (Wang *et al.*, 1993). Briefly, a polyclonal antibody was raised against an imidazoline receptor protein purified from bovine chromaffin cell membranes by ligand affinity chromatography (Wang *et al.*, 1992). The purified protein had a major protein component of 70 kDa and bound [^{3}H]-idazoxan with kinetics and a drug inhibition profile comparable to the native receptor and differed from α_2-adrenoceptors (Wang *et al.*, 1992). Polyclonal antibodies were raised in rabbits against this protein. On Western blots, the antibody labelled a protein band of ≈70 kDa prepared from chromaffin cell membranes. It also immunoextracted 75% of all imidazoline-binding activity associated with solubilized chromaffin cell membrane proteins, partially blocked binding of ligand to the receptor and, immunocytochemically, labelled imidazoline receptors *in vitro* and *in vivo* (e.g. see Wang *et al.*, 1993). In spite of this specificity (see also present results), the possibility that this antibody could cross-react with other uncharacterized proteins cannot be completely discarded.

The polyclonal antiserum against the human α_{2A}-adrenoceptor (3rd intracellular loop, peptide 262-276, NH_2-GGAEAEPLPTQLMGA-COOH) was produced and characterized in this laboratory. Briefly, the above peptide was coupled to keyhole limpet hemocyanine (Pierce, U.S.A.), and was used to immunize rabbits. The polyclonal antiserum produced recognised the peptide, as determined by enzyme linked immunosorbent assays (ELISA) and dot blot assays (data not shown). In human brain membranes, the antibody labelled a protein band of ≈65–70 kDa and a lesser band of ≈50 kDa. These Mr values corresponded to the mature (glycosylated) and the precursor (nonglycosylated) forms of α_{2A}-adrenoceptors, respectively (Kobilka *et al.*, 1987). Upon deglycosylation, only the ≈50 kDa band was observed (data not shown).

Anti-α-tubulin monoclonal antibody (clone DM 1A) was purchased from Sigma Chemical Co. (U.S.A.). Membranes from Sf9 (*Spodoptera frugiperda*) cells overexpressing the human α_{2A}-adrenoceptor were purchased from Research Biochemicals International (RBI, U.S.A.). Horseradish peroxidase-linked donkey anti-rabbit and sheep anti-mouse IgG were obtained from Amersham International plc (U.K.). [^{3}H]-idazoxan (specific activity 42–46 Ci mmol^{-1}, batches 44–45) was purchased from Amersham International plc (U.K.). Other drugs (and their sources) included: idazoxan HCl, LSL 60101 HCl [2-(2-benzofuranyl)imidazole] and RX821002 HCl (2-methoxy idazoxan) (synthesized by Dr F. Geijo and Dr F. Pla at S.A. LASA Laboratorios, Spain); cirazoline HCl (Synthélabo Recherche, France); clorgyline HCl, EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline), phenelzine sulphate and yohimbine HCl (Sigma Chemical Co., U.S.A.) and efaroxan HCl (RBI).

Results

Immunodetection of imidazoline receptor proteins and α_{2A}-adrenoceptors

Western blot analysis of the rat frontal cortex, by use of a specific anti-imidazoline receptor protein antiserum, showed the presence of three immunoreactive imidazoline receptor protein bands (Figure 1, lane 1). The apparent molecular masses of these peptides were ≈29/30, ≈45 and ≈66 kDa as previously described (Escribá *et al.*, 1994b). The relative immunoreactivity of these brain imidazoline receptor proteins with respect to total immunoreactivity was 65–75% for the 29/30-kDa protein; 15–25% for the 45-kDa protein and 5–10% for the 66-kDa protein (Figure 1, lane 1).

Because most of the drugs used for the chronic treatments also are α_2-adrenoceptor antagonists, a possible cross-reaction of the polyclonal anti-imidazoline receptor antibody with α_{2A}-adrenoceptors, the most abundant subtype in the rat cerebral cortex, was studied. In membranes from Sf9 (*Spodoptera frugiperda*) cells transfected with baculovirus to overexpress the human recombinant α_{2A}-adrenoceptor, the imidazoline receptor antiserum did not recognize the α_{2A}-adrenoceptor

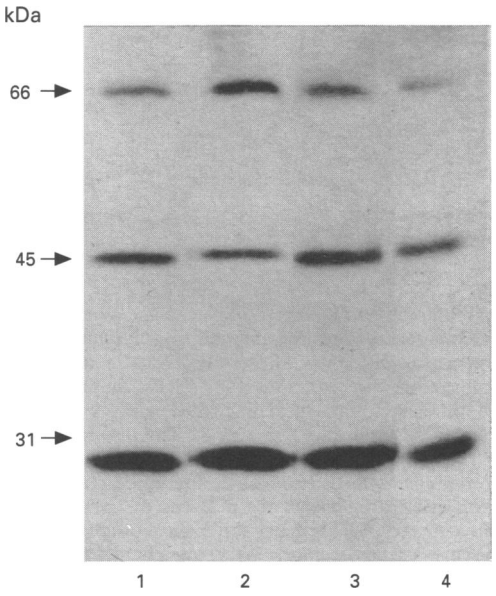

Figure 1 Representative immunoreactive bands with the antiserum against imidazoline receptor proteins in the rat brain after saline (lane 1) or chronic treatment (10 mg kg^{-1} i.p., every 12 h for 7 days) with idazoxan (lane 2), RX821002 (2-methoxy idazoxan) (lane 3) or phenelzine (lane 4). Samples from the frontal cortex were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes (immunoblotting), incubated with the specific primary and secondary antibodies, and visualized by the Enhanced Chemiluminescence (ECL) method. All samples were run in the same gel. The amount of total membrane protein loaded per gel and well was as follows (in μg): 34 (lane 1), 36 (lane 2), 42 (lane 3) and 33 (lane 4). Relative molecular masses (kDa) of marker proteins are given on the left. The apparent molecular masses of the imidazoline receptor proteins, calculated with these standard marker proteins, were ≈29/30-, ≈45- and ≈66-kDa. See Tables 1, 2 and 3 for changes in mean percentage values of imidazoline receptor protein immunoreactivities after the various treatments and other details.

(≈70 kDa glycosylated protein) which was readily detected with an antibody against this α_2-adrenoceptor subtype (Figure 2). These negative results discounted possible unspecific effects of drug treatment on brain immunoreactive imidazoline receptor proteins.

Effects of various drug treatments on imidazoline receptor protein immunoreactivities and I_2-imidazoline receptor density in the rat cerebral cortex

Chronic treatment (7 days) with the imidazol(in)e drugs idazoxan (10 mg kg^{-1}), cirazoline (1 mg kg^{-1}) and LSL 60101 (10 mg kg^{-1}) which possess high or moderate affinity and high selectivity for I_2-sites (Uhlén *et al.*, 1995; Alemany *et al.*, 1995b), differentially increased the immunoreactivity of specific imidazoline receptor proteins in the rat cerebral cortex (Table 1). Thus, the levels of the 29/30-kDa protein were significantly increased by idazoxan (22%, $P<0.05$) (Figure 1, lane 2) and LSL 60101 (23%, $P<0.01$); the levels of the 45-kDa protein were increased only by cirazoline (44%, $P<0.01$) and those of the 66-kDa protein only by idazoxan (50%, $P<0.001$) (Figure 1, lane 2). In the cerebral cortex of the same rats, chronic treatments with idazoxan, cirazoline and LSL 60101 also increased the density of I_2-imidazoline receptors (32–42%, $P<0.01$) without significantly changing the affinity (K_d) of [^{3}H]-idazoxan for these sites (Table 1).

Chronic treatment (7 days) with the imidazoline drugs efaroxan (10 mg kg^{-1}) and RX821002 (2-methoxy idazoxan, 10 mg kg^{-1}) which possess very low affinity for I_2-sites (Miralles *et al.*, 1993a; Alemany *et al.*, 1995b), or chronic treatment with the non-imidazoline α_2-adrenoceptor antagonist yohimbine (10 mg kg^{-1}), did not alter significantly either the immunoreactivity of imidazoline receptor proteins or the density of I_2-imidazoline receptors in the rat cerebral cortex (Table 2; Figure 1, lane 3).

Chronic treatment (7 days) with the MAO inhibitors clorgyline (10 mg kg^{-1}) and phenelzine (10 mg kg^{-1}) significantly decreased the immunoreactivities of the 29/30-kDa (17–24%, $P<0.001$), 45-kDa (19%, $P<0.05$) and 66-kDa (23–31%, $P<0.05$) imidazoline receptor proteins in the rat cerebral cortex (Table 3; Figure 1, lane 4). Similarly, chronic treatments with clorgyline and phenelzine consistently decreased the density of I_2-imidazoline receptors (45–57%, $P<0.001$) in the rat brain (Table 3). The alkylating agent EEDQ (1.6 mg kg^{-1}, 6 h) decreased the levels of the 29/30-kDa (27%, $P<0.001$), 45-kDa (20%, $P<0.01$) and 66-kDa (47%, $P<0.001$) imidazoline receptor proteins in parallel with the reduction induced in the density of I_2-imidazoline receptors (31%, $P<0.05$) in the rat cerebral cortex (Table 3).

None of these drug treatments modified significantly the immunoreactive levels of α-tubulin which was used as a negative control. These negative results discounted possible effects of unspecific variables on brain imidazoline receptor proteins.

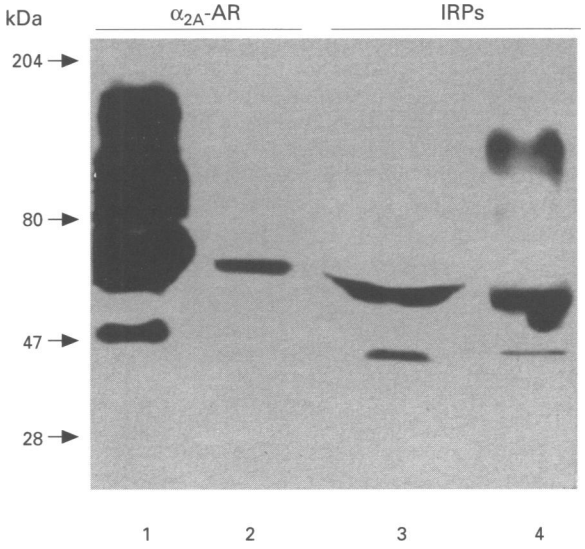

Figure 2 Immunoblot analysis of Sf9 (*Spodoptera frugiperda*) cell membranes overexpressing the human α_{2A}-adrenoceptor, with a specific anti-α_{2A}-adrenoceptor antiserum (α_{2A}-AR, lanes 1 and 2) or an anti-imidazoline receptor antiserum (IRPs, lanes 3 and 4). The amount of total membrane protein loaded per gel well was 600 ng (lanes 1 and 3) or 20 ng (lanes 2 and 4). The anti-α_{2A}-adrenoceptor antiserum recognized the ≈70-kDa adrenoceptor protein and the ≈50-kDa non-glycosylated form of this receptor. The anti-imidazoline receptor antiserum showed the presence of the ≈45- and ≈66-kDa imidazoline receptor proteins and it did not cross-react with the overexpressed α_{2A}-adrenoceptors. Other details as for Figure 1.

Relationship between drug-induced changes in immunoreactive imidazoline receptor proteins and I_2-imidazoline receptor density in the rat cerebral cortex

When the mean percentage changes in total immunoreactivity of imidazoline receptor proteins (i.e. the sum of the three bands shown in Figure 1, lane 1) were related to the mean percentage changes in the density of I_2-imidazoline receptors (i.e. B_{max} values for [^{3}H]-idazoxan binding) after the various drug treatments, a positive and significant correlation was found ($r=0.93$; $P<0.001$). This correlation suggested that the three imidazoline receptor proteins detected by immunoblot analysis might contribute to the modulation of I_2-imidazoline binding sites after drug treatment. Specifically, the modulation of immunoreactivity of the 29/30-kDa protein strongly correlated ($r=0.92$; $P<0.001$) with the modulation of I_2-sites density after the same drug treatments (Figure 3a). Similarly, the pharmacological modulation of immunoreactive 45-kDa protein also correlated ($r=0.69$; $P<0.05$) with the density of I_2-imidazoline receptors after the

Table 1 Effects of chronic treatments with I_2-selective imidazol(ine) drugs on imidazoline receptor protein immunoreactivities and I_2-imidazoline binding sites in the rat cerebral cortex

		Imidazoline receptor protein immunoreactivity						*[^{3}H]-idazoxan binding sites*		
	Dose	*29/30-kDa*		*45-kDa*		*66-kDa*		B_{max}	K_d	
Treatment	(mg kg^{-1})	(% of control)	n	(% of control)	n	(% of control)	n	(fmol mg^{-1} protein)	(nM)	n
Saline	–	98±2	15	96±4	12	101±3	10	53±2	13.2±0.8	14
Idazoxan	10	120±9*	10	84±11	8	151±13***	5	74±6**	16.9±2.1	7
Cirazoline	1	110±8	6	138±19**	6	108±17	3	70±3**	14.2±1.1	7
LSL60101	10	121±9**	9	120±10	5	100±11	7	75±4**	16.3±1.0	4

Each drug was administered i.p., every 12 h for 7 days. The rats were killed 24 h after the last injection. See the Methods section for experimental details. Each value represents the mean±s.e.mean of *n* experiments per group with one animal per experiment. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ as compared with saline-treated group (one-way ANOVA followed by Scheffé's or Fisher's test).

Table 2 Effects of chronic treatments with imidazoline and α_2-adrenoceptor drugs on imidazoline receptor protein immunoreactivities and I_2-imidazoline binding sites in the rat cerebral cortex

		Imidazoline receptor protein immunoreactivity						*[^{3}H]-idazoxan binding sites*		
	Dose	*29/30-kDa*		*45-kDa*		*66-kDa*		B_{max}	K_d	
Treatment	(mg kg^{-1})	(% of control)	n	(% of control)	n	(% of control)	n	(fmol mg^{-1} protein)	(nM)	n
Saline	–	98±1	13	99±2	10	100±1	7	64±5	16.2±1.9	11
Efaroxan	10	98±4	5	81±13	3	91±8	3	70±8	13.5±0.7	3
RX821002	10	95±8	6	96±14	6	100±17	6	61±6	13.8±2.2	5
Yohimbine	10	91±9	6	110±11	3	93±11	3	75±3	16.1±1.6	5

Each drug was administered i.p., every 12 h for 7 days. The rats were killed 24 h after the last injection. See the Methods section for experimental details. Each value represents the mean±s.e.mean of *n* experiments per group with one animal per experiment. One-way ANOVA did not detect any significant change in immunoreactive proteins or in the binding parameters for [^{3}H]-idazoxan.

Table 3 Effects of chronic treatments with monoamine oxidase inhibitors and the alkylating agent EEDQ on imidazoline receptor protein immunoreactivities and I_2-imidazoline binding sites in the rat cerebral cortex

		Imidazoline receptor protein immunoreactivity						*[^{3}H]-idazoxan binding sites*		
	Dose	*29/30-kDa*		*45-kDa*		*66-kDa*		B_{max}	K_d	
Treatment	(mg kg^{-1})	(% of control)	n	(% of control)	n	(% of control)	n	(fmol mg^{-1} protein)	(nM)	n
Saline	–	99±2	8	95±4	8	102±4	6	65±5	11.2±1.1	10
Clorgyline	10	82±4***	8	77±4*	7	79±7*	7	28±3***	12.5±1.8	6
Phenelzine	10	75±3***	4	77±10*	4	70±6**	4	36±2*	9.2±1.6	3
EEDQ	1.6	72±4***	6	76±4**	6	54±13***	3	45±3*	8.3±1.1	6

Each drug was administered i.p., every 12 h for 7 days. The rats were killed 24 h after the last injection. See the Methods section for experimental details. Each value represents the mean±s.e.mean of *n* experiments per group with one animal per experiment. B_{max} values for clorgyline and phenelzine treatments were taken from Olmos *et al.* (1993) and Alemany *et al.* (1995c), respectively. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ as compared with saline-treated group (one-way ANOVA followed by Scheffé's or Fishers's test).

various treatments (Figure 3b). Finally, the changes in immunoreactive 66-kDa protein correlated well ($r=0.75$, $P<0.01$) with the drug-induced changes in the density of I_2-imidazoline receptors (Figure 3c).

Discussion

Imidazoline receptors exist in multiple forms

Pharmacological, biochemical and functional studies have shown that imidazoline receptors are a new family of membrane proteins which have been grouped into various types and subtypes according to their ligand binding or functional preferences: I_1-, I_{2A}-, I_{2B}- and non-I_1/I_2-imidazoline receptors as well as possible identification with the enzyme MAO (Michel & Ernsberger, 1992; Reis *et al.*, 1995; Parini *et al.*, 1996). This evidence suggested a molecular diversity of imidazoline receptors and that each of these pharmacological types could correspond to more than one molecular entity (Lanier *et al.*, 1993; 1995; Wang *et al.*, 1993; Escribá *et al.*, 1994b; 1995a). The present results clearly indicate that the pharmacological I_2-type labelled by [^{3}H]-idazoxan in the rat cerebral cortex is heterogeneous in nature and can be related to various imidazoline receptor proteins that are not MAO.

Pharmacological modulation of the 29/30-kDa imidazoline receptor protein

The 29/30-kDa imidazoline receptor protein was first identified by immunodetection in various rat and human tissues, including the brain and liver (Escribá *et al.*, 1994b). Recently, a similar double band of 25/27-kDa has been identified by photoaffinity labelling with the imidazoline radioligand [^{125}I]-AZIPI in rat liver and kidney (Lanier *et al.*, 1995). The 29/30-kDa double band appears to correspond to two peptides differing in less than 1-kDa as observed in some immunoblots, which are not the result of degradation of higher Mr peptides and that are independent of the presence of the other imidazoline receptor proteins (Escribá *et al.*, 1994b; 1995a).

The best correlation between drug-induced changes in immunoreactive imidazoline receptor proteins and I_2-sites density was obtained for the 29/30-kDa peptide ($r=0.92$, Figure 3a). Since repeated treatment with antagonist drugs often results in up-regulation of receptors, the increased density of I_2-sites after chronic treatment with idazoxan, cirazoline and LSL 60101 (Table 1) may indicate that these drugs are antagonists at brain I_2-imidazoline receptors. At present, however, the diverse effects of these drugs and other imidazol(in)e drugs are difficult to define in the context of known agonistic/antagonistic effects at imidazoline receptors. In any case, idazoxan has been used as a specific antagonist to assess the function of I_1-imidazoline receptors *in vivo* (Tibiriça *et al.*, 1991; Allan *et al.*, 1996). It is of interest to note that, in contrast to idazoxan and LSL 60101, chronic treatment with cirazoline, a selective I_2-ligand (Uhlén *et al.*, 1995), did not increase the levels of the 29/30-kDa protein (Table 1). However, the correlation analysis clearly indicated that this protein is closely related to I_2-sites in the rat brain. In this context, the 29/30-kDa imidazoline receptor protein has been also associated with the I_2-imidazoline receptor identified with [^{3}H]-idazoxan in the human brain during the process of ageing (parallel increases) (García-Sevilla *et al.*, 1995) and in postmortem brains of suicide victims (Sastre *et al.*, 1995; Sastre & García-Sevilla, 1996) and of heroin addicts (Sastre *et al.*, 1996) (parallel decreases). Together these findings indicate that the 29/30-kDa protein detected by immunoblot is related to the I_2-imidazoline receptor labelled with [^{3}H]-idazoxan. In the rat and human brain, the I_2-imidazoline receptor belongs to the I_{2B}-subtype (low affinity for amiloride) (Miralles *et al.*, 1993a). Therefore, the brain 29/30-kDa imidazoline receptor protein could be related to the pharmacologically characterized I_{2B}-subtype.

Recently, it has been suggested that the I_2-sites identified with [^{3}H]-idazoxan are located on both MAO-A and MAO-B

isoenzymes (Tesson *et al.*, 1995). However, the identification of specific I_2-imidazoline receptors as proteins of 29/30-kDa in the rat brain (present results) and human brain (García-Sevilla *et al.*, 1995), as well as of 45-kDa in the rat brain (see below), do not support the conclusion that all I_2-sites are related to MAO-A/B (Tesson *et al.*, 1995), which have molecular masses of 63-kDa and 59-kDa, respectively (Bach *et al.*, 1988). These data further indicate the existence of various I_2-imidazoline receptors identified with [^{3}H]-idazoxan that are not related to MAO.

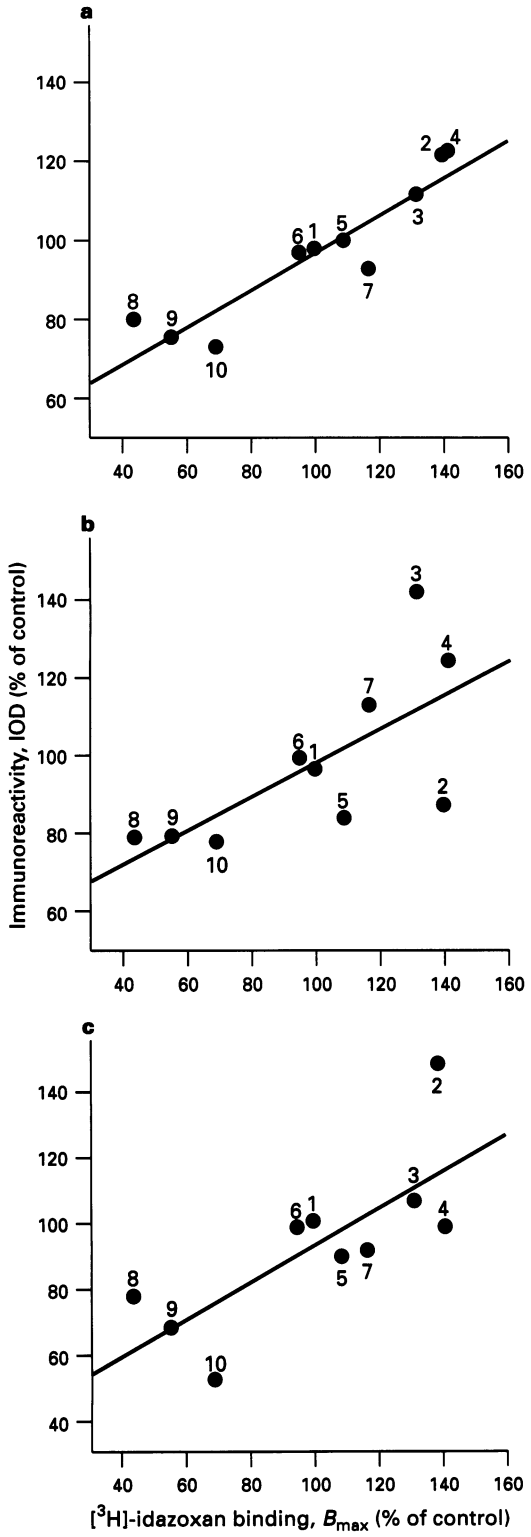

Figure 3 Correlations between the immunoreactivity of imidazoline receptor proteins (IOD, integrated optical densities) and the density of I_2-sites for the binding of [^{3}H]-idazoxan (B_{max} values) in the rat cerebral cortex after various chronic drug treatments: (1) saline; (2) idazoxan; (3) cirazoline; (4) LSL 60101; (5) efaroxan; (6) RX821002; (7) yohimbine; (8) clorgyline; (9) phenelzine; (10) EEDQ. See the Methods section for other details. The data were best described by the following expressions, (a) 29/30-kDa band: $y = 50.1 + 0.47x$ ($r = 0.92$, $P < 0.001$); (b) 45-kDa band: $y = 55.0 + 0.43x$ ($r = 0.69$, $P < 0.05$); (c) 66-kDa band: $y = 38.5 + 0.55x$ ($r = 0.75$, $P < 0.01$).

Pharmacological modulation of the 45-kDa imidazoline receptor protein

The immunodetection, isolation and molecular characterization of a 43/45-kDa imidazoline receptor protein from rat and human brains (Escribá *et al.*, 1994b; 1995a; Greney *et al.*, 1994) have been recently accomplished which demonstrates the unequivocal existence of these novel receptors. Moreover, the pharmacological profile of the solubilized 45-kDa imidazoline receptor protein from the rat brain appeared to correspond to an I_2-imidazoline-like receptor (Escribá *et al.*, 1995a).

The pharmacological modulation of immunoreactive 45-kDa protein also correlated with the modulation of I_2-sites density after the same drug treatments ($r = 0.69$, Figure 3b). These results indicate that this protein is also related to the I_2-imidazoline receptor, which is in agreement with the pharmacological profile of the solubilized 45-kDa imidazoline receptor protein from the rat brain (Escribá *et al.*, 1995a). Only chronic treatment with cirazoline up-regulated the levels of the 45-kDa protein (Table 1). Since chronic cirazoline did not alter the levels of either the 29/30-kDa or 66-kDa proteins, but resulted in up-regulation of I_2-sites density (Table 1), it appears clear that this selective I_2-ligand (Uhlén *et al.*, 1995) specifically interacts with the 45-kDa imidazoline receptor of the rat brain. This 45-kDa imidazoline receptor protein has been shown to be up-regulated in platelets of depressed patients and in post-mortem brains of suicide victims (García-Sevilla *et al.*, 1996) and down-regulated by chronic treatment with MAO inhibitor antidepressants (present results).

Pharmacological modulation of the 66-kDa imidazoline receptor protein

Various imidazoline receptor proteins with apparent molecular masses of 55–70-kDa have been visualised or purified from various cell types and tissues (Limon *et al.*, 1992; Wang *et al.*, 1992; Lanier *et al.*, 1993; 1995; Raddatz *et al.*, 1995). Some of these proteins and the rat brain 66-kDa imidazoline receptor protein identified here by immunodetection (Escribá *et al.*, 1994b; 1995a) might be structurally related.

The pharmacological modulation of immunoreactive 66-kDa imidazoline receptor protein also correlated ($r = 0.75$, Figure 3c) with drug-induced changes in the density of I_2-sites. These results indicate that the 66-kDa protein detected by immunoblot in the rat brain is related to I_2-imidazoline receptors identified with [^{3}H]-idazoxan. It has been recently shown that some imidazoli(di)ne drugs can inhibit basal MAO activity in a non-competitive manner (Carpéné *et al.*, 1995; Mackinnon *et al.*, 1995; Tesson *et al.*, 1995) and that the two MAO isoforms can be photolabelled with the imidazoline radioligand [^{125}I]-AZIPI (Raddatz *et al.*, 1995). These results led to the hypothesis that the I_2-site identified with [^{3}H]-idazoxan is a previously unknown MAO binding domain that allosterically modulates the enzyme activity (Tesson *et al.*, 1995; Parini *et al.*, 1996). The present results show that the levels of all the immunoreactive imidazoline receptor proteins (29/30-, 45- and 66-kDa) were decreased by chronic treatments with the irreversible MAO inhibitors clorgyline and phenelzine (Table 3). It could be argued, however, that the parallel decrease in [^{3}H]-idazoxan binding is mainly related to the decrease in the levels of the 66-kDa protein, the molecular mass of which resembles that of the MAO-A isoenzyme (63-kDa) (Bach *et al.*, 1988). The possibility that the 66-kDa imidazoline receptor protein of the rat brain is related to MAO is unlikely because the polyclonal antibody used for the detection of

imidazoline receptor proteins did not recognize a purified fraction of MAO from bovine plasma (Alemany *et al.*, 1995c) and in the rat liver, a tissue expressing high levels of both MAO isoforms, it only recognized the 29/30-kDa imidazoline receptor protein (Escribá *et al.*, 1994b). Moreover, it is difficult to explain how [^{3}H]-idazoxan could label an allosteric site on MAO at nanomolar concentrations when millimolar concentrations of imidazoline drugs are needed to inhibit MAO activity, when no correlation exists between drug affinity for I_2-sites and inhibition of MAO, and when idazoxan itself failed to inhibit basal MAO activity (Carpéné *et al.*, 1995; Mackinnon *et al.*, 1995). Together these findings indicate that the observed down-regulation of I_2-imidazoline receptors after chronic treatments with irreversible MAO inhibitors (Olmos *et al.*, 1993; Alemany *et al.*, 1995c) is related to decreased levels of various imidazoline receptor proteins (29/30- 45- and 66-kDa) and not to an irreversible binding of these drugs to these receptors.

This study was supported by DGICYT Grant PB94-0002-Mod C from the Ministerio de Educación y Ciencia, Spain. The anti-imidazoline receptor antiserum was a generous gift from Dr Donald J. Reis (Cornell University Medical College, New York). R.A. was supported by a fellowship from FIS (Fondo de Investigaciones Sanitarias). A.O. was supported by a fellowship from the Basque Government. J.A.G.-S. is a member of the Institut d'Estudis Catalans.

References

ALEMANY, R., ESCRIBÁ, P.V., SASTRE, M., OLMOS, G., OZAITA, A. & GARCÍA-SEVILLA, J.A. (1995a). Pharmacological modulation of immunoreactive 29/30, 45 and 66 kDa imidazoline receptor proteins in the rat brain. *Meth. Find. Exp. Clin. Pharmacol.*, **17,** Suppl. A, 70.

ALEMANY, R., OLMOS, G., ESCRIBÁ, P.V., MENARGUES, A., OBACH, R. & GARCÍA-SEVILLA, J.A. (1995b). LSL 60101, a selective ligand for imidazoline I_2 receptors, on glial fibrillary acidic protein concentration. *Eur. J. Pharmacol.*, **280,** 205–210.

ALEMANY, R., OLMOS, G. & GARCÍA-SEVILLA, J.A. (1995c). The effects of phenelzine and other monoamine oxidase inhibitor antidepressants on brain and liver I_2 imidazoline-preferring receptors. *Br. J. Pharmacol.*, **114,** 837–845.

ALLAN, D.R., PENNER, S.B. & SMYTH, D.D. (1996). Antagonism by idazoxan at low dose but not high dose, of the natriuretic action of moxonidine. *Br. J. Pharmacol.*, **117,** 29–34.

BACH, A.W., LAN, N.C., JOHNSON, P.L., ABELL, C.W., BEMBENEK, M.E., KWAN, S.W., SEEBURG, P.H. & SMITH, J.C. (1988). cDNA cloning of human liver monoamine oxidase A and B. Molecular basis of differences in enzymatic properties. *Proc. Natl. Acad. Sci. U.S.A.*, **85,** 4934–4938.

BOUSQUET, P., FELDMAN, J. & SCHWARTZ, J. (1984). Central cardiovascular effects of α-adrenergic drugs: differences between catecholamines and imidazolines. *J. Pharmacol. Exp. Ther.*, **230,** 232–242.

CARPÉNÉ, C., COLLON, P., REMAURY, A., CORDI, A., HUDSON, A., NUTT, D. & LAFONTAN, M. (1995). Inhibition of amine oxidase activity by derivatives that recognize imidazoline I_2 sites. *J. Pharmacol. Exp. Ther.*, **272,** 681–688.

CHAN, S.L.F., BROWN, C.A. & MORGAN, N.G. (1994). The imidazoline site involved in control of insulin secretion: characteristics that distinguish it from I_1- and I_2-sites. *Br. J. Pharmacol.*, **112,** 1065–1070.

DIAMANT, S., ELDAR-GEVA, T. & ATLAS, D. (1992). Imidazoline binding sites in human placenta: evidence for heterogeneity and a search for physiological function. *Br. J. Pharmacol.*, **106,** 101–108.

ESCRIBÁ, P.V., OZAITA, A., MIRALLES, A., REIS, D.J. & GARCÍA-SEVILLA, J.A. (1995a). Molecular characterization and isolation of a 45-kilodalton imidazoline receptor protein from the rat brain. *Mol. Brain Res.*, **32,** 187–196.

ESCRIBÁ, P.V., SASTRE, M. & GARCÍA-SEVILLA, J.A. (1994a). Increased density of guanine nucleotide-binding (G) proteins in the postmortem brain of heroin addicts. *Arch. Gen. Psychiatry*, **151,** 494–501.

ESCRIBÁ, P.V., SASTRE, M. & GARCÍA-SEVILLA, J.A. (1995b). Disruption of cellular signaling pathways by daunomycin through destabilization of non-lamellar membrane structures. *Proc. Natl. Acad. Sci. U.S.A.*, **92,** 7595–7599.

ESCRIBÁ, P.V., SASTRE, M., WANG, H., REGUNATHAN, S., REIS, D.J. & GARCÍA-SEVILLA, J.A. (1994b). Immunodetection of putative imidazoline receptor proteins in the human and rat brain and other tissues. *Neurosci. Lett.*, **178,** 81–84.

EVINGER, M.J., ERNSBERGER, P., REGUNATHAN, S. & REIS, D.J. (1995). Regulation of phenylethanolamine N-methyltransferase gene expression by imidazoline receptors in adrenal chromaffin cells. *J. Neurochem.*, **65,** 988–997.

GARCÍA-SEVILLA, J.A., ESCRIBÁ, P.V., SASTRE, M., WALZER, C., BUSQUETS, X., JAQUET, G., REIS, D.J. & GUIMÓN, J. (1996). Immunodetection and quantitation of imidazoline receptor proteins in platelets of patients with major depression and in brains of suicide victims. *Arch. Gen. Psychiatry*, (in press).

GARCÍA-SEVILLA, J.A., SASTRE, M. & ESCRIBÁ, P.V. (1995). Age-dependent increases of immunoreactive imidazoline receptors in the human brain: possible association of a 29/30 kDa protein with the I_2-imidazoline receptor identified by [^{3}H]-idazoxan. *Neurosci. Lett.*, **184,** 133–136.

GÖTHERT, M. & MOLDERINGS, G.J. (1991). Involvement of presynaptic imidazoline receptors in the α_2-adrenoceptor-independent inhibition of noradrenaline release by imidazoline derivatives. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **343,** 271–282.

GRENEY, H., BENNAI, F., MOLINES, A., BELCOURT, A., DONTENWILL, M. & BOUSQUET, P. (1994). Isolation of human cerebral imidazoline-specific binding protein. *Eur. J. Pharmacol.*, **265,** R1–R2.

KOBILKA, B.K., MATSUI, H., KOBILKA, T.S., YANG-FENG, T.L., FRANKE, U., CARON, M.G., LEFKOWITZ, R.J. & REGAN, J.W. (1987). Cloning, sequencing, and expression of the gene coding for the human platelet α_2-adrenergic receptor. *Science*, **238,** 650–656.

LANIER, S.M., IVKOVIC, B., SINGH, I., NEUMEYER, J.L. & BAKTHAVACHALAM, V. (1993). Visualization of multiple imidazoline/guanidinium-receptive sites. *J. Biol. Chem.*, **268,** 16047–16051.

LANIER, B., RADDATZ, R., BAKTHAVACHALAM, V., COUPRY, I., NEUMEYER, J.L. & LANIER, S.M. (1995). Structural and ligand recognition properties of imidazoline binding proteins in tissues of rat and rabbit. *Mol. Pharmacol.*, **48,** 703–710.

LI, G., REGUNATHAN, S., BARROW, C.J., ESHRAGHI, J., COOPER, R. & REIS, D.J. (1994). Agmatine: an endogenous clonidine-displacing substance in the brain. *Science*, **263,** 966–969.

LIMON, I., COUPRY, I., LANIER, S.M. & PARINI, A. (1992). Purification and characterization of mitochondrial imidazoline-guanidinium receptive site from rabbit kidney. *J. Biol. Chem.*, **267,** 21645–21649.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.C. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193,** 265–275.

MACKINNON, A.C., LINTON, C. & BROWN, C.M. (1995). Inhibition of monoamine oxidase and (I_2) imidazoline affinity in rat kidney. *Br. J. Pharmacol.*, **115,** 72P.

MICHEL, M.C. & ERNSBERGER, P. (1992). Keeping an eye on the I site: Imidazoline-preferring receptors. *Trends Pharmacol. Sci.*, **13,** 369–370.

MIRALLES, A., OLMOS, G., SASTRE, M., BARTUREN, F., MARTÍN, I. & GARCÍA-SEVILLA, J.A. (1993a). Discrimination and pharmacological characterization of I_2-imidazoline sites with [^{3}H]-idazoxan and alpha-2 adrenoceptors with [^{3}H]RX821002 (2-methoxy idazoxan) in the human and rat brains. *J. Pharmacol. Exp. Ther.*, **264,** 1187–1197.

MIRALLES, A., RIBAS, C., OLMOS, G. & GARCÍA-SEVILLA, J.A. (1993b). Differential effects of the alkylating agent N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline on brain α_2-adrenoceptors and I_2-imidazoline sites *in vitro* and *in vivo*. *J. Neurochem.*, **61,** 1602–1610.

MOLDERINGS, G.J. & GÖTHERT, M. (1995). Inhibitory presynaptic imidazoline receptors on sympathetic nerves in the rabbit aorta differ from I_1- and I_2-imidazoline binding sites. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **351,** 507–516.

MOLDERINGS, G.J., MOURA, D., FINK, K., BÖNISH, H. & GÖTHERT, M. (1993). Binding of [^{3}H]clonidine to I_1-imidazoline sites in bovine adrenal medullary membranes. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **348,** 70–76.

OLMOS, G., ALEMANY, R., ESCRIBÁ, P.V. & GARCÍA-SEVILLA, J.A. (1994a). The effects of chronic imidazoline drug treatment on glial fibrillary acidic protein concentrations in rat brain. *Br. J. Pharmacol.*, **111,** 997–1002.

OLMOS, G., GABILONDO, A.M., MIRALLES, A., ESCRIBÁ, P.V. & GARCÍA-SEVILLA, J.A. (1993). Chronic treatment with the monoamine oxidase inhibitors clorgyline and pargyline down-regulates non-adrenoceptor [^{3}H]-idazoxan binding sites in the rat brain. *Br. J. Pharmacol.*, **108,** 597–603.

OLMOS, G., KULKARNI, R.N., HAQUE, M. & MACDERMOT, J. (1994b). Imidazolines stimulate release of insulin from RIN-5AH cells independently from imidazoline I_1 and I_2 receptors. *Eur. J. Pharmacol.*, **262,** 41–48.

PARINI, A., GARGALIDIS MOUDANOS, C., PIZZINAT, N. & LANIER, S.M. (1996). The elusive family of imidazoline binding sites. *Trends Pharmacol. Sci.*, **17,** 13–16.

PILETZ, J.E., CHIKKALA, D.N. & ERNSBERGER, P. (1995). Comparison of the properties of agmatine and endogenous clonidine-displacing substance at imidazoline and alpha-2 adrenergic receptors. *J. Pharmacol. Exp. Ther.*, **272,** 581–587.

PINEDA, J., UGEDO, L. & GARCÍA-SEVILLA, J.A. (1993). Stimulatory effects of clonidine, cirazoline and rilmenidine on locus coeruleus noradrenergic neurones: possible involvement of imidazoline-preferring receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **348,** 134–140.

RADDATZ, R., PARINI, A. & LANIER, S.M. (1995). Imidazoline/guanidinium binding domains on monoamine oxidases. Relationship to subtypes of imidazoline-binding proteins and tissue-specific interaction of imidazoline ligands with monoamine oxidase B. *J. Biol. Chem.*, **270,** 27961–27968.

REGUNATHAN, S., FEINSTEIN, D.L. & REIS, D.J. (1993). Expression of non-adrenergic imidazoline sites in rat cerebral cortical astrocytes. *J. Neurosci. Res.*, **34,** 681–688.

REIS, D.J., BOUSQUET, P. & PARINI, A. ed. (1995). The Imidazoline Receptor: Pharmacology, Functions, Ligands, and Relevance to Biology and Medicine. In *Ann. New York Acad. Sci.*, **Vol. 763.**

SASTRE, M., ESCRIBÁ, P.V., REIS, D.J. & GARCÍA-SEVILLA, J.A. (1995). Decreased number and immunoreactivity of I_2-imidazoline receptors in the frontal cortex of suicide victims. *Ann. New York Acad. Sci.*, **763,** 520–522.

SASTRE, M. & GARCÍA-SEVILLA, J.A. (1996). Densities of I_2-imidazoline receptors, α_2-adrenoceptors and monoamine oxidase B in brains of suicide victims. *Neurochem. Inter.*, (in press).

SASTRE, M., VENTAYOL, P. & GARCÍA-SEVILLA, J.A. (1996). Decreased density of I_2-imidazoline receptors in the postmortem brains of heroin addicts. *NeuroReport*, **7,** 509–512.

SCHULZ, A. & HASSELBLAT, A. (1989). Dual action of clonidine on insulin release: supression, but stimulation when α_2-adrenoceptors are blocked. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **340,** 712–714.

TESSON, F., LIMON-BOULEZ, I., URBAN, P., PUYPE, M., VANDEKERCKHOVE, J., COUPRY, I., POMPON, D. & PARINI, A. (1995). Localization of I_2-imidazoline binding sites on monoamine oxidases. *J. Biol. Chem.*, **270,** 9856–9861.

TIBIRIÇA, E., FELDMAN, J., MERMET, C., GONON, F. & BOUSQUET, P. (1991). An imidazoline-specific mechanism for the hypotensive effect of clonidine: a study with yohimbine and idazoxan. *J. Pharmacol. Exp. Ther.*, **256,** 606–613.

UHLÉN, S., MUCENIECE, R., RANGEL, N., TIGER, G. & WIKBERG, J.E.S. (1995). Comparison of the binding activities of some drugs on α_{2A}, α_{2B}, and α_{2C}-adrenoceptors and non-adrenergic imidazoline sites in the guinea pig. *Pharmacol. Toxicol.*, **76,** 353–364.

WANG, H., REGUNATHAN, S., MEELEY, M.P. & REIS, D.J. (1992). Isolation and characterization of imidazoline receptor protein from bovine adrenal chromaffin cells. *Mol. Pharmacol.*, **42,** 792–801.

WANG, H., REGUNATHAN, S., RUGGIERO, D.A. & REIS, D.J. (1993). Production and characterization of antibodies specific for the imidazoline receptor protein. *Mol. Pharmacol.*, **43,** 509–515.

(Received March 8, 1996
Revised May 8, 1996
Accepted May 9, 1996)