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Co-detection by Two Imidazoline Receptor Protein Antisera of a Novel 85 Kilodalton Protein

Tina R. Ivanov, He Zhu,* Soundararajan Regunathan,† Donald J. Reis,† Monique Dontenwill,‡ Catherine Vonthron,‡ Pascal Bousquet‡ and John E. Piletz*§‖¶*

*Division of Neurobiology and Behavior Research, Department of Psychiatry, and Departments of §Pharmacology & Toxicologyand ‖Physiology & Biophysics, University of Mississippi Medical Center, Jackson, MS; †Division of Neurobiology, Department of Neurology and Neurosciences, Cornell University Medical College, New York, NY 10021; and ‡Laboratorie de Neurobiologie et Pharmacologie Cardiovasculaire, Faculte de Medecine, CNRS, 67085 Strasbourg, France

ABSTRACT. Imidazoline receptors (I-receptors) are considered as potential therapeutic targets for a spectrum of stress-induced illnesses. Yet, I-receptors remain poorly defined at the molecular level. In this study, candidate imidazoline receptor proteins were compared using two imidazoline receptor-selective antisera of diverse origins. One antiserum was derived from affinity-purified imidazoline-binding protein. The second antiserum was produced as an anti-idiotypic antiserum, from purified IgG selective for imidazolines. Despite such diverse origins, both antisera co-identified an 85 kDa band on western blots from a variety of tissues. The integrity of the 85 kDa band was dependent on protection by eight different protease inhibitors. Other proteolytic breakdown products (obtained after homogenization with only one protease inhibitor) were comparable in size to previously reported smaller immunoreactive bands. The full-size 85 kDa band was also enriched in plasma membrane fractions and abundant in rat PC12 cells and brain regions known to be abundant in I_1 binding sites. Furthermore, the immunodensity of the 85 kDa band, against anti-idiotypic antiserum, was linearly correlated with reported I_1 site radioligand B_{max} values ($r^2 = 0.8736$, $P = 0.0002$) across nine rat tissues. Therefore, a possible candidate for the full-length imidazoline receptor(s) appears to be an 85 kDa protein. BIOCHEM PHARMACOL 55;5:649–655, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. imidazoline receptors; antibody; rat brain; PC12 cells; human cortex; monoamine oxidase; protease inhibitors

Pharmacological studies with clonidine and idazoxan have indicated that in addition to binding to α_2-adrenoceptors, imidazoline compounds can modulate a number of non-adrenergic effects through distinctive imidazoline receptors [1–3]. Imidazoline receptors were first surmised to exist based on their non-adrenergic, antihypertensive properties when clonidine and related compounds were micro-injected into cat brainstem nuclei [1–3]. Central imidazoline receptors have been studied for their possible utility in treating hypertension and metabolic syndrome [1, 2, 4]. Metabolic syndrome, or syndrome X, is associated with psychosocial stress, increased caloric intake, raised sympathetic outflow, and reduced glucose utilization [4].

Two imidazoline receptor subtypes, designated I_1** and I_2, have been classified according to their differential binding affinities, separable subcellular localizations, and regional brain distributions [2, 3, 5]. I_1 receptors exist in plasma membranes [3, 5], and they appear to couple to phosphatidylcholine-selective phospholipase C [6]. Monoamine oxidases A and B, from mitochondrial membranes, possess I_2 binding sites within a non-catalytic domain of the proteins [7]. Other non-adrenergic, I_2-like imidazoline binding sites have also been visualized in plasma membranes using photaffinity labeling [8]. Decarboxylated arginine, agmatine, has been suggested to be one possible endogenous neurotransmitter/ligand for imidazoline receptors [9].

Unfortunately, efforts to understand the molecular nature of imidazoline receptors have lagged behind their pharmacology. A 67 kDa protein was isolated from bovine adrenal chromaffin cells as the first imidazoline receptor protein candidate [10]. A 67 kDa protein was purified over two affinity chromatography columns linked to either idazoxan (preferential I_2 ligand) or *p*-aminoclonidine (preferential I_1 ligand) [10]. Because a 67 kDa protein was isolated from either idazoxan or *p*-aminoclonidine affinity columns, it was implied that both I_1 and I_2 proteins might be 67 kDa in size, at least in bovine adrenal chromaffin cells [10]. A polyclonal antiserum raised against that protein fraction was designated the IRBP antiserum [11]. IRBP

¶ Corresponding author: Dr. John E. Piletz, Department of Psychiatry, Rm. G128, University of Mississippi Medical Center, 2500 North State Str., Jackson, MS 39216-4505. Tel. (601) 984-5898; FAX (601) 984-5899.

** *Abbreviations:* ECL, enhanced chemiluminescence; I_1, imidazoline$_1$ subtype; I_2, imidazoline$_2$ subtype; IRBP, imidazoline receptor-binding protein; MAO, monoamine oxidase; and PMSF, phenylmethylsulfonyl fluoride.

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antiserum was shown to immunodetect not only endogenous bovine adrenal chromaffin cell's 67 kDa protein [11], but also other proteins, the largest of which was 86 kDa in size, in rat adrenal medulla [12]. IRBP antiserum inhibits [^{3}H]idazoxan binding to I_2 binding sites, but not the binding of the adrenergic ligand to their receptors [11]. Thus, a 67 kDa candidate imidazoline receptor protein possesses properties distinctive from those of other known $\approx$ 60 kDa proteins that bind imidazolines, i.e. MAO and α_2-adrenoceptors [7, 10, 13, 14].

An anti-idiotypic antiserum was also raised by Dontenwill and colleagues [15–17] using purified polyclonal antibodies selective for idazoxan, as a tool to isolate imidazoline receptors. This anti-idiotypic antiserum was also shown to inhibit brainstem binding of [^{3}H]clonidine and [^{3}H]idazoxan to their respective imidazoline receptor subtypes, but not of [^{3}H]rauwolscine (α_2-selective ligand) to α_2-adrenoceptors [15, 16]. A 43 kDa protein was labeled by this anti-idiotypic antiserum on western blots from human brainstem membranes [16–18]. Escriba *et al.* [19] also identified a similarly sized protein from idazoxan-Pharma-Link affinity chromatography of solubilized brain membranes. Solubilized 43 kDa protein binds both [^{3}H]idazoxan and [^{3}H]clonidine under a mask of 10 μM epinephrine, ruling out α_2-adrenoceptor binding sites [19]. Thus, Dontenwill's anti-idiotypic antiserum also appeared to interact with both I_1 and I_2 proteins, but not α_2-adrenoceptors [15–17].

Various protease inhibitors were used by different laboratories in earlier studies of the anti-imidazoline receptor antisera [11, 13–17]. Now, a novel mixture of eight protease inhibitors is described that encompasses all of the *critical* protease inhibitors from previous studies. Using this mixture of eight protease inhibitors to prepare membranes, both the IRBP and anti-idiotypic antisera co-identified a major 85 kDa band from all brain regions examined. Smaller bands of 33 kDa [20], 43–45 kDa [16, 19], and 67 kDa [11], similar to those reported earlier [11, 16, 19, 20], were found in the absence of this mixture, as possible proteolytic breakdown products. Based on the plasma membrane enrichment, and the tissue regionalization pattern similar to that for I_1 binding sites, this 85 kDa protein might be a new candidate for the full length I_1-imidazoline receptor protein.

MATERIALS AND METHODS

Cells and tissues were obtained as follows: Rat adrenal pheochromocytoma PC12 cells were cultured as previously described [21]. Human platelets were obtained from the Mississippi Blood Services, as previously described [5]. Human brain tissue was obtained courtesy of Dr. Craig Stockmeier and Dr. Gregory Ordway (Case Western Reserve University). Adult male Sprague–Dawley rats were obtained from Harlan Sprague–Dawley Inc. Rats that had habituated to the laboratory environment were decapitated quickly and tissues were removed rapidly to ice. Rat brain regions were dissected according to the method of Glowinski and Iversen [22].

Tissues and pelleted cells were homogenized on ice in 50 mM Tris pH 7.5, containing 0.25 M sucrose and 2 mM $MgCl_2$ buffer, plus eight key protease inhibitors (fresh 1 mM PMSF, 10 mM ϵ-amino-*n*-caproic acid, 0.1 mM phenanthroline, 10 μg/mL pepstatin A, 5 mM iodoacetamide, 10 μg/mL trypsin-chymotrypsin inhibitor, 10 μg/mL leupeptin, and 1.67 μg/mL calpain inhibitor I). All reagents were purchased from the Sigma Chemical Co. except for calpain inhibitor which was obtained from Boehringer Mannheim. This mixture of eight protease inhibitors is capable of targeting aspartic acid, cysteine, and serine proteases. They were chosen as a minimum because five additional protease inhibitors, including a metalloprotease inhibitor, used by Wang *et al.* [11] and Escriba *et al.* [12], did not enhance the density of the 85 kDa band. Homogenates were spun at 900 × *g* to pellet unlysed cells and nuclei. The supernatants were removed and stored on ice, and the pellets were re-homogenized in the same buffer and respun. Supernatant was pooled and spun at 20,000 × *g* for 20 min to obtain a pelleted internal membrane fraction. The supernatant was spun subsequently at 104,000 × *g* for 120 min to obtain a pelleted plasma membrane fraction. The membrane fractions were solubilized immediately in ice-cold 40 mM Tris–HCl buffer (pH 6.8) plus 4% SDS [12], and stored at −80°. An aliquot of each fraction was quantitated for total protein using a Lowry–Biuret reagent kit from Sigma.

Samples were denatured and electrophoresed through two 13.7% polyacrylamide gels (SDS–PAGE) according to the method of Laemmli [23]. A batch preparation of platelet total membranes was also electrophoresed into three lanes (5–25 μg) on each gel to obtain a standard curve for densitometry. Batched platelets were used since they provide a source of I_1 binding sites [5]. One of the gels was stained with Coomassie blue dye to verify the uniformity of total protein applied to each lane (i.e. 20 μg protein/lane). The second gel was electrotransferred onto nitrocellulose (Hybond ECL, Amersham). After electrotransfer, the nitrocellulose membranes were blocked for 1 hr with TBST/10% milk (Tris-buffered saline, pH 7.5, plus 0.1% Tween-20), briefly rinsed in TBST and incubated for 2 hr with either IRBP or anti-idiotypic antiserum (1:3000 dilution in TBST/10% milk) at room temperature. Blots were washed and incubated for 1 hr with horseradish peroxidase conjugated anti-rabbit IgG antiserum (1:3000 dilution in TBST/10% milk). Immunoreactive bands were detected with Amersham's ECL detection system followed by exposure to film (Amersham ECL Hyperfilm) for 1–3 min. After the films were developed, the nitrocellulose membranes were stripped according to the method described for Hybond ECL nitrocellulose membranes (Amersham) and reprobed with either IRBP or anti-idiotypic antiserum, whichever had not been used previously. The blots were stripped between each antiserum, and reprobed with the appropriate preimmune antiserum (1:3000 dil.) to

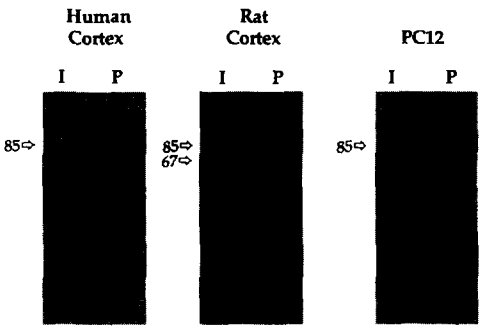

FIG. 1. Detection of 85 kDa protein enriched in plasma membrane fractions with an IRBP antiserum. Internal (I) and plasma (P) membrane proteins from human and rat cortex, and PC12 cells, were prepared in the presence of eight protease inhibitors. Electrophoresis was performed through an SDS/13.7% polyacrylamide gel, electrotransferred to a nitrocellulose membrane overnight, and probed with the IRBP antiserum (1:3000 dilution). Immunoreactive bands were detected by ECL. No bands were detected with pre-immune serum under identical conditions. Each lane contained 20 μg of proteins, determined by the Lowry assay. Arrows and numbers (relative molecular mass [M_r; in kilodaltons]) to the left of each panel identify the location and size of the immunoreactive bands.

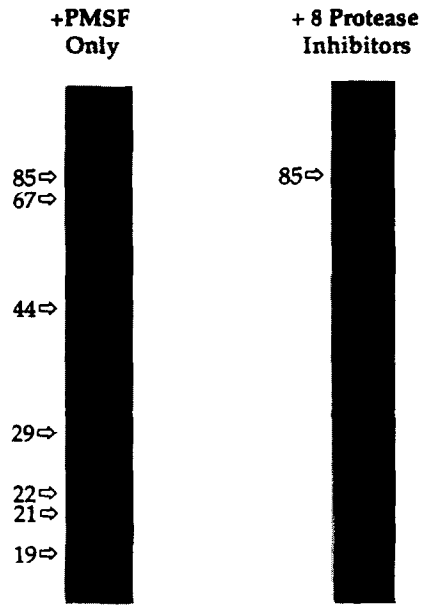

FIG. 2. Importance of protease inhibitors for the detection of the 85 kDa immunoreactive band in rat hippocampus homogenates. Rat hippocampal homogenates were prepared in buffer containing either 1 mM PMSF or a combination of eight protease inhibitors (see text). Homogenates were electrophoresed through a denaturing 13.7% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with the IRBP antiserum (1:3000 dilution). Immunoreactive bands were detected by ECL. Each lane contained 15 μg of protein homogenate. Arrows and numbers (relative molecular mass [M_r; in kilodaltons]) to the left of each panel identify the location and size of immunoreactive bands. A representative blot is shown.

ensure that the putative imidazoline receptor bands were specific. (No bands were observed with preimmune antisera.) Other blots were air-dried and subjected to Ponceau S staining for total proteins in order to be certain that the electrotransfer was complete compared with the duplicate Coomassie blue-stained gels. Densitometric readings of the bands were obtained using a Microcomputer Controlled Imaging Device (MCID; Imaging Research Inc.). An 8-point standard curve for the ECL detection system had a linear regression slope of 1.091 and $r^2 = 0.9782$ over the range of samples that were studied. Each experiment was repeated at least three times.

Correlational analyses were attempted between the distributions reported across tissues for radioligand binding to I-receptors [3, 24–27] and the distributions of the main bands (i.e. 85 kDa bands from either antiserum) to provide evidence for, or against, the hypothesis that the 85 kDa band might be an imidazoline binding protein. Due to our limited supply of antisera, it was not feasible to directly immunoprecipitate the 85 kDa band along with the radioligand binding sites (such experiments typically require 1:100 antiserum dilutions, or less).

RESULTS

As shown in Fig. 1 for IRBP antiserum, when PC12 cells or cortical tissue membranes were prepared in homogenization buffer containing the eight protease inhibitors, a prominent 85 kDa immunoreactive band was detected. This band was enriched markedly in plasma membrane fractions from different sources (designated P fractions in Fig. 1). With rat cortical membranes, a weaker, lower molecular weight band was also detected near 30 kDa. Less intense 67 kDa (rat only) and 85 kDa bands were also observed in internal membrane fractions (designated the I fractions) from human and rat cortex. The cytosol fractions (i.e. 104,000 × g supernatants) displayed low immunoreactivity (i.e. PC12), or equivalent immunoreactivity (i.e. rat and human cortex), as compared with the internal membrane fractions. No other sized bands were observed in the cytosols (data not shown). Thus, the 85 kDa band predominanted in plasma membranes.

The 85 kDa immunoreactive band was of higher molecular weight than most other bands previously reported [11, 12, 24] using the same antiserum. This suggested that the lower molecular weight bands might represent breakdown products. To test this possibility, we allowed the 85 kDa protein to experience incomplete protease protection. Rat hippocampal homogenates were prepared in buffer containing either 1 mM PMSF or the mixture of eight protease inhibitors (see "Materials and Methods"), and were analyzed by western blots using IRBP antiserum. (PMSF at 1 mM is the sole protease inhibitor used commonly in published I_1 and α_2-adrenoceptor radioligand binding assays.) The hippocampus was chosen because it is a region of rat brain enriched in IR_1 binding sites [25–27] and in the 85 kDa band. Representative results from this comparison of protease inhibitors are shown in Fig. 2. When the hip-

pocampus was homogenized in the presence of 1 mM PMSF alone, the 85 kDa band was not observed. Instead, immunoreactive bands were visualized at 67, 44, 29, 22, 21, and 19 kDa. In contrast, when all eight protease inhibitors were added to the homogenization buffer, a prominent 85 kDa band was observed (Fig. 2, right lane). This suggests that some previously reported immunoreactive bands with the IRBP antiserum may be due to the breakdown of an 85 kDa protein. The addition of four other protease inhibitors (1 mM EDTA, 1 mM EGTA, 0.1 mM benzamide, 0.1 mM benzamidine-HCl, and 10 μg/mL antipain) [as in Refs. 11, 12, and 24] to this group of eight protease inhibitors neither increased the immunodensity of the 85 kDa band, nor resulted in the detection of any bands larger than 85 kDa.

A previous report [12] has revealed quantitative differences in IRBP immunoreactive bands across rat brain regions. Therefore, we wanted to determine if a similar distribution would be evident for the 85 kDa band. Plasma membranes were prepared from freshly dissected rat brain regions in the presence of the eight key protease inhibitors. Western blots with IRBP antiserum (Fig. 3, top panel) indicated that the 85 kDa protein was present in all rat brain regions examined and was differentially distributed. The highest immunodensities were in the hippocampus, hypothalamus, cortex, and cerebellum, followed by the striatum and midbrain, with a lower level of the brainstem. A weaker 30 kDa band was also observed in all brain regions and quantitatively appeared to parallel the 85 kDa protein (Fig. 3, top panel). However, the 85 and 30 kDa bands were not seen in human platelet membranes. In human platelet membranes, bands of 95 kDa (weakest) and 33 kDa (major) were evident (Fig. 3, top panel, right lanes). Rat liver, kidney, and spleen were also analyzed by the same procedure with IRBP antiserum. In liver, kidney, and spleen, only trace amounts of an 85 kDa protein could be detected, with 67, 33, and 22 kDa bands being present in low amounts (data not shown for these peripheral tissues).

If the 85 kDa band detected by IRBP antiserum is an IRBP, it might be immunodetectable with a second imidazoline receptor specific antiserum. Dontenwill and coworkers [15] produced their anti-idiotypic antiserum that immunodetects a human 43 kDa imidazoline-binding protein with high affinity for clonidine [15–17]. Therefore, we sought to determine the cross-reactivity of the 85 kDa protein with Dontenwill's anti-idiotypic antiserum by stripping immunoblots free of IRBP antibodies and then reprobing them with the anti-idiotypic antiserum. Prior to reprobing with the second antiserum, stripped blots were processed omitting primary antibodies to ensure complete removal of the previous primary antibody. Reprobing with the anti-idiotypic antiserum revealed an 85 kDa band in all rat brain regions that was also distributed differentially (Fig. 3, lower panel). The distribution of the 85 kDa band in rat brain regions with the anti-idiotypic antiserum was similar to that observed with the IRBP antiserum. Rat PC12 and human cortex immunoblots were also reprobed with the anti-idiotypic antiserum, and they also demonstrated a similar 85 kDa band with the anti-idiotypic antiserum (data not shown).

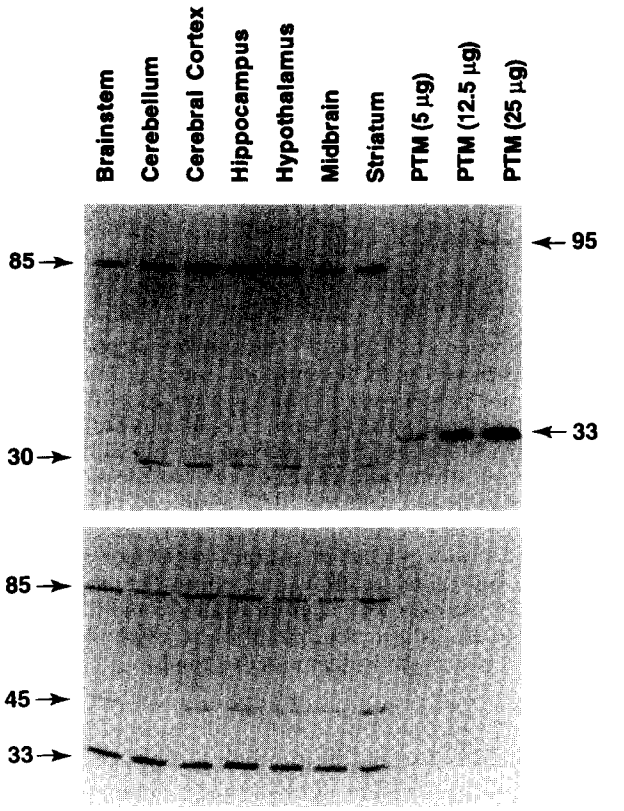

FIG. 3. Detection of 85 kDa protein in plasma membrane fractions from rat brain regions with antisera against imidazoline receptors. Plasma membrane fractions were prepared from rat brain regions in the presence of eight protease inhibitors. Proteins were fractionated by SDS–PAGE and transferred to a nitrocellulose membrane. The *top panel* was probed first with IRBP antiserum (1:3000). Immunoreactive bands were detected on film by ECL. The blot was then stripped free of the IRBP antibody by washing in 10 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris, pH 6.7, for 2 × 15 min at 70°, rinsed extensively with Tris-buffered saline, pH 7.5, plus 0.1% Tween-20, and checked for complete removal of the primary antibody. The *bottom panel* shows the results of the second probing of this blot with the anti-idiotypic antibody (1:3000 dilution). Lanes 1–7 contained 20 μg of plasma membrane proteins from different rat brain regions. Platelet total membrane (PTM) lanes correspond to human platelet total membrane proteins (5, 12.5, and 25 μg/lane), which were used as a standard curve. Arrows and numbers (relative molecular mass [M_r; in kilodaltons]) to the left (rat brain) and right (human platelet) of each panel identify the location and size of immunoreactive bands.

It should be noted that unique bands having relative M_r of 33 and 45 kDa (weaker) were also observed across rat brain regions using the anti-idiotypic antiserum, which were not seen with IRBP antiserum (Fig. 3). Another distinction was that the anti-idiotypic antiserum did not recognize the human platelet 33 kDa band (Fig. 3, lower panel) even though it strongly detected a similar 33 kDa band in rat brain. Future studies will be required to determine if this is due to a species difference.

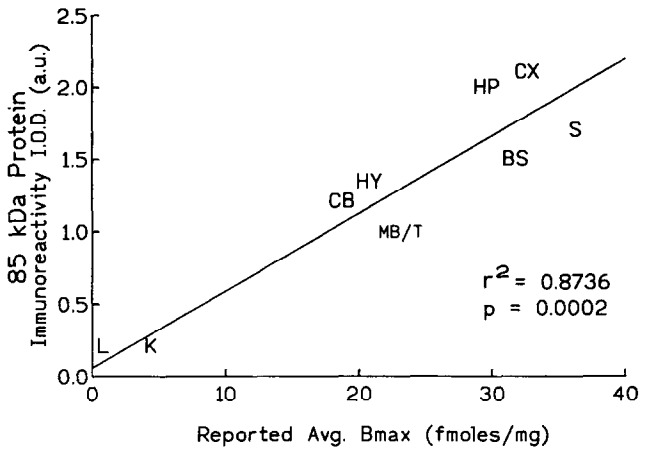

FIG. 4. Correlation of anti-idiotypic immunoreactivity and reported I_1-imidazoline binding site densities in tissues. Immunoreactivities were quantified from the western blots in Fig. 3 (lower panel) using densitometry. Values are expressed as Integrated Optical Density (I.O.D.) arbitrary units (a.u.), and the line was drawn by linear regression analysis. Radioligand B_{max} values were averaged from studies using human, rat, and cow tissues (the three best studied animal sources), as follows: K = kidney [27, 28], CB = cerebellum [25–27], HY = hypothalamus [26, 27], MB/T = midbrain and thalamus [25–27], HP = hippocampus [25–27], BS = brainstem [3, 25–27], CX = cortex [3, 25–27], S = striatum [25–27], and L = liver [Paul Ernsberger, Case Western Reserve University, personal communication regarding Ref. 3; cited with permission]. Human platelet and rat spleen immunoreactivity values were not included in this correlational analysis because of non-cross-reactivity of the two antisera with platelet membranes and because no literature values were available for the spleen I_1 B_{max} value.

Densitometric analyses and comparisons of the 85 kDa bands revealed a close correlation between immunodensity and published B_{max} values for the I_1-binding site (Fig. 4) [3, 25–28]. For this correlation, all available values on I_1 B_{max} tissue distribution data were pooled and averaged from the literature (references noted in the legend to Fig. 4). This linear correspondence observed with the I_1 B_{max} values was particularly strong for the 85 kDa immunoreactive band detected with the anti-idiotypic antiserum versus published B_{max} values for the I_1 binding sites ($r^2 = 0.8736$ and $P = 0.0002$; two-tailed analysis) (Fig. 4). A similar correlation for the 85 kDa band detected with the IRBP antiserum was in the same direction, although it reached only marginal statistical significance ($r^2 = 0.4416$ and $P = 0.051$; two-tailed analysis, data not shown). By contrast, attempts to correlate the 85 kDa immunodensity with published B_{max} values for the I_2 binding site were non-significant ($P = 0.5280$ and 0.5217) for either the anti-idiotypic or IRBP antiserum, respectively. These results were, therefore, consistent with the hypothesis that the 85 kDa protein might contain an I_1 binding site. In summary, an 85 kDa immunoreactive protein was found to possess the subcellular and tissue distribution pattern appropriate for an I_1-imidazoline receptor protein subtype.

DISCUSSION

Only two antisera have been reported previously [11, 15] to recognize an imidazoline receptor protein. Both of these antisera have now been shown to co-detect an 85 kDa band on western blots of rat and human tissues (Figs. 1–3). Escriba *et al.* [12] identified an 86 kDa protein from rat adrenal medulla with IRBP antiserum, but it was a minor band under those conditions (fewer protease inhibitors). Thus, the fact that this band was co-detected by both antisera—plus the fact that this 85 kDa band exhibits the subcellular and regionalization properties expected of an imidazoline receptor—makes this a likely imidazoline receptor protein candidate.

Previous indirect evidence had supported the possibility that both the IRBP antiserum and the anti-idiotypic antiserum might cross-react with identical I_1 and I_2 receptor proteins [10, 15, 16, 29]. First, IRBP antiserum was prepared from protein(s) purified through *p*-aminoclonidine (I_1-sensitive ligand) or idazoxan (I_2-sensitive ligand) affinity chromatography columns [10, 11]. This suggested that the immunogen (i.e. the 67 kDa band purified from the columns) used to prepare the IRBP antiserum might actually have contained a mixture of closely related I_1 and I_2 receptor proteins. Second, the anti-idiotypic antiserum of Bousquet and coworkers [15–18] was shown to inhibit both 70% of [^{3}H]clonidine binding (I_1-sensitive binding) and 50% of [^{3}H]idazoxan binding (I_2-sensitive binding) to non-adrenergic binding sites. Third, a 29–30 kDa doublet protein that is immunoreactive with IRBP antiserum was shown to correlate with I_2 binding site densities in brain regions, and with regulation by I_2 selective ligands in rat and human tissues [12, 13, 19, 20]. On the other hand, a platelet 33 kDa band and brain 43–45 kDa immunoreactive bands were suggested to be I_1-like [13, 16, 20]. Finally, IRBP antiserum labels neurons (suggested to be I_1 and I_2 containing cells) as well as glia (suggested to be I_2 containing cells) by immunohistochemistry [29]. Thus, both of these antisera probably detect both I_1 and I_2 proteins.

Although the IRBP and anti-idiotypic antisera had been described in separate reports [i.e. 14, 15, 29], methodological differences hindered their comparison. One complication had been the use of different protease inhibitors by different laboratories. To correct this, we compiled all of the protease inhibitors from previous investigators; in addition, calpain inhibitor-I was added to this list because Ernsberger *et al.* [30] reported that calpain inhibitor-I was necessary for I_1 radioligand binding to membranes. This list was eventually narrowed to eight key protease inhibitors essential for the detection of the 85 kDa protein (listed under "Materials and Methods"). Our data suggest that previously identified 33, 43–45, and 67 kDa immunoreactive proteins might be proteolytic fragments of an 85 kDa protein (Fig. 2). On the other hand, the platelet 33 kDa protein might be a separate protein because in platelets there is no evidence of an 85 kDa precursor protein under protection by our protease inhibitor mixture.

The 85 kDa immunoreactive band was enriched within plasma membrane fractions (Fig. 1). By comparison, previous particulate fractions prepared by Escriba *et al.* [12, 13] were likely to have been depleted of plasma membranes. In those studies, membranes were pelleted at only 20,000 × *g* [12], or in a later study at 40,000 × *g* [13], for 10 min, which is roughly equivalent to a mitochondrial membrane preparation [31]. In our study, the 85 kDa immunoreactive protein was found to be of low abundance within 20,000 × *g* (20 min) internal membrane fractions, but was enriched markedly in the pellets from those supernatants (104,000 × *g* for 120 min) (Fig. 1). Subcellular fractions, obtained by a procedure similar to ours in PC12 cells, were reported [3] to be enriched in plasma membranes and I_1 sites.

To our knowledge, the previous literature contains only one other report [8] of a plasma membrane-enriched imidazoline receptor protein candidate. Lanier *et al.* [8] used a photo affinity imidazoline radioligand to detect a smear of proteins of ≈ 25 kDa in plasma membranes from liver and kidney. That result is similar to the lower molecular weight immunoreactive bands that we observed with only one protease inhibitor (Fig. 2).

The present evidence indicates that the two imidazoline receptor selective antisera may not recognize MAO proteins. The 85 kDa band (a) was not enriched in mitochondria but in plasma membrane fractions, and (b) has a molecular size on SDS gels bigger than the 61 kDa size of MAO. Alemany *et al.* [14] have mentioned similar findings. They indicated that IRBP antiserum failed to recognize a purified fraction (up to 0.7 μg) of MAO from bovine plasma, and that I_2 binding proteins and the two isoforms of MAO displayed different isoelectric points [14]. Thus, although I_2 binding sites appear to be encoded within MAO [7], the 85 kDa protein seems clearly distinct.

In summary, four lines of evidence suggest that the 85 kDa protein may be an I_1 protein. First, a similar or identical 85 kDa band was detected on western blots using either of two purported imidazoline receptor antisera (Fig. 3). Second, the 85 kDa protein was enriched within plasma membrane fractions similar to that reported for I_1 receptors [3, 5], as compared with I_2 sites which are localized to intracellular membranes [7, 8]. Third, the 85 kDa protein was abundant within PC12 plasma membranes, a cell-type and membrane fraction used to study I_1 sites because they lack α_2-adrenoceptors [3]. Fourth, the tissue distribution of the 85 kDa protein, as detected by anti-idiotypic antiserum, corresponds with published B_{max} values for I_1 binding sites (Fig. 4). In preliminary studies, we have also observed that a human megakaryoblastoma MEG-01 cell line also expresses an 85 kDa IRBP-immunoreactive protein that is up-regulated by treatments with imidazolines, and related compounds, in a manner consistent with a non-adrenergic, imidazoline binding site.* Thus, we believe an 85 kDa protein is likely to possess an imidazoline binding site. The further steps of purifying, sequencing, and cloning this 85 kDa protein will be necessary to verify its candidacy as an imidazoline receptor protein.

* Ivanov T, Feng YZ, Wang H, Regunathan S, Reis DJ, Chikkala D, Gupta P and Piletz J, unpublished results.

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