0026-895X/92/050792-10$03.00/0

MOLECULAR PHARMACOLOGY, 42:792-801

Isolation and Characterization of Imidazoline Receptor Protein from Bovine Adrenal Chromaffin Cells

H. WANG, S. REGUNATHAN, M. P. MEELEY, and D. J. REIS

Division of Neurobiology, Department of Neurology and Neuroscience, Cornell University Medical College, New York, New York 10021

Received March 5, 1992; Accepted August 31, 1992

SUMMARY

We sought to isolate and partially purify proteins corresponding to the binding element of the imidazoline receptor (IR) from adrenal chromaffin cell membranes. These cells express IRs of the I-2 subclass and not α_2-adrenergic receptors. Proteins were solubilized in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate-containing buffer and were assayed by binding of [^{3}H]idazoxan, an imidazoline radioligand. Two ligand affinity resins, *p*-aminoclonidine-Trisacryl GF-2000 (PAC-ReactiGel) and idazoxan-PharmaLink agarose (IDA-agarose), were synthesized. These allowed purification by single-step affinity chromatography of a major receptor binding protein component of 70 kDa, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and [^{3}H]idazoxan binding assay. The purified imidazoline-binding proteins from IDA-agarose and PAC-ReactiGel had similar affinities for the radioligand [^{3}H]idazoxan (K_d = 3.7 and 4.9 nM, respectively) and a displacement profile, showing sensitivity to imidazoline agents (cirazoline > clonidine) and insensitivity to catecholamines and adrenergic agents (epinephrine ≈ rauwolscine), that was similar to that of the intact membrane receptor. The imidazoline-binding protein did not bind to concanavalin A, suggesting that it may not be glycosylated or that the sugar moieties present are not recognized by this lectin. The results indicate that IR and α_2 receptor proteins may be biochemically distinct and that IDA-agarose and PAC-ReactiGel columns are useful for purification of sufficient quantities of imidazoline-binding proteins to allow for structural and functional studies of the IR.

Although traditionally the drugs clonidine, idazoxan, and related agents have been believed to act exclusively at α_2-adrenergic receptors (1, 2), there is now evidence that they also bind to a novel nonadrenergic receptor, the IR (3–5). That IRs and α_2-adrenergic receptors may be molecularly distinct has been suggested by the findings that they are independently expressed in different tissues (6–8), brain areas (9, 10), and subcellular compartments (11) and may utilize different signal transduction mechanisms (4, 12). Moreover, transfection of fibroblasts with the mRNA for α_2-adrenergic receptors does not confer binding characteristics of IRs to the transfected cells (13).

However, the structure of IRs has not been established, a prerequisite for unequivocally establishing that IRs and α_2-adrenergic receptors are distinctive molecular entities. Parini *et al.* (8) solubilized and physically separated IRs from α_2-adrenergic receptors of rabbit kidney by affinity column chromatography using heparin or wheat-germ lectin as ligands. Whereas the α_2-adrenergic receptor was retained on the columns (suggesting that it is a glycosylated protein), the IR was not, nor was it further purified.

It has been shown that most tissues express both IRs and α_2-adrenergic receptors and all ligands that bind to IRs also bind to α_2-adrenergic receptors (6–8, 14, 15). Purification of the IRs would be facilitated by identification of either a selective ligand for affinity chromatography and/or a tissue source that expressed only the IRs. We have recently discovered that adrenal chromaffin cells express only IRs and not α_2-adrenergic receptors.[1] This fact introduces the possibility that the IR might be isolated from chromaffin cells by affinity chromatography using imidazoline agents (e.g., idazoxan or PAC) as ligands.

We report here the development of two such columns, namely a PAC-Trisacryl GF-2000 (PAC-ReactiGel) affinity matrix with low nonspecific binding and an idazoxan-PharmaLink agarose (IDA-agarose) affinity matrix onto which the relatively unreactive idazoxan structure was coupled to the gel matrix through unconventional methods. Using these matrices, we achieved partial purification of imidazoline-binding proteins

[1] S. Regunathan, M. P. Meeley, and D. J. Reis. Bovine adrenal chromaffin cells express imidazoline, but not α_2-adrenergic, receptors. Submitted for publication.

ABBREVIATIONS: IR, imidazoline receptor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; EGTA, ethylene glycol bis β-(aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PEG, polyethylene glycol; IDA-agarose, idazoxan-agarose; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DADPA, diaminodipropylamine; MES, α-(*N*-morpholino)ethanesulfonic acid; PAC, *p*-aminoclonidine.

from detergent-solubilized chromaffin cell membranes using single-step affinity chromatography.

Experimental Procedures

Materials

Bovine adrenal glands were obtained from a local slaughterhouse. Pepstatin A, benzamide, benzamidine hydrochloride, ε-aminocaproic acid, phenylmethylsulfonyl fluoride, EGTA, CHAPS, polyvinylpyrrolidone (PVP-360), epinephrine, and amiloride were obtained from Sigma Chemical Co. Rauwolscine, clonidine, PAC, and idazoxan were obtained from Research Biochemicals, Inc. Cirazoline was a gift from Synthelabo (Paris, France). Carbonyl diimidazole-Trisacryl GF-2000 (ReactiGel) and DADPA-agarose (PharmaLink gel) were from Pierce Chemical. [^{3}H]Idazoxan was from Amersham. Na^{125}I was from New England Nuclear. Chloramine T was from Kodak. Sephadex G-25 was from Pharmacia.

Synthesis of the PAC and Idazoxan Affinity Matrices

PAC matrix. The primary amino group of PAC was coupled to carbonyl diimidazole-derivatized Trisacryl GF-2000 (ReactiGel; Pierce Chemical) by the method suggested by the manufacturer. The principle of the PAC and ReactiGel reaction is depicted in Fig. 1A. In brief, the gel, in acetone, was washed with distilled water and transferred to a solution of 10–20 mM PAC in 0.1 M sodium carbonate buffer, pH 9.5, and the coupling reaction was carried out for 30 hr at 4° with gentle mixing. The PAC solution was then drained out of the gel bed and nonreactive sites on the gel were blocked by reaction with 1 M Tris·HCl, pH 8, for 4 hr at 4°. The gel was subsequently washed with 1 M KCl in 50 mM Tris·HCl, pH 7.4, poured into a small column, and equilibrated with 50 mM Tris·HCl buffer, pH 7.4.

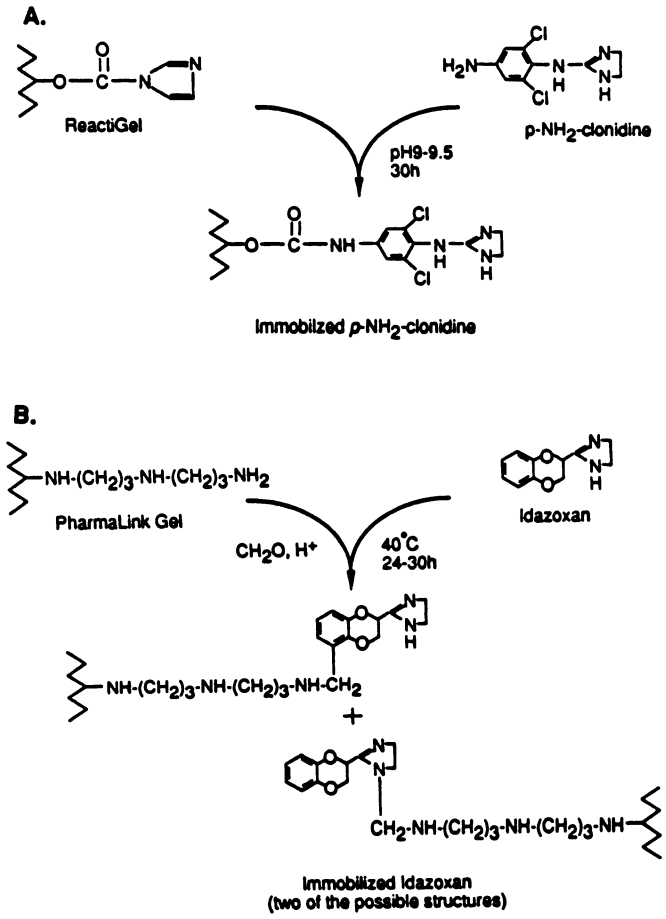

Fig. 1. Schematic diagrams of the synthesis of the PAC-Trisacryl GF-2000 (ReactiGel) affinity matrix (A) and IDA-PharmaLink agarose affinity matrix (B) and the putative structure of the final product(s).

The efficiency of coupling of PAC to the gel was determined by including [^{3}H]PAC as tracer in the cross-linking reaction. Nearly 10% of [^{3}H]PAC added as tracer was incorporated onto the gel. Using this 10% coupling efficiency and the amount of unlabeled PAC added, the degree of PAC substitution was, therefore, estimated to be 0.5–1 mg of PAC/ml of gel. In addition, the successful coupling of PAC to the gel was confirmed by retention of anti-PAC antibodies.

Idazoxan matrix. Because idazoxan does not contain a highly reactive functional group, immobilization onto a matrix is difficult. However, a recently developed method (PharmaLink immobilization kit; Pierce Chemical) that utilizes the principles of the Mannich reaction allows immobilization via condensation of an active hydrogen of the ligand with formaldehyde and coupling to DADPA-derivatized agarose. The proposed principle of the reaction between idazoxan and agarose resin is depicted in Fig. 1B.

The coupling of idazoxan to DADPA-derivatized agarose was carried out as suggested by the manufacturer. The gel, in 0.1 M MES, pH 4.7, containing 0.02% sodium azide, was washed with coupling buffer (0.1 M MES) and incubated with 10–20 mM idazoxan in coupling buffer and 200 μl of the coupling reagent (37% formaldehyde). Coupling was carried out for 24–30 hr at 40° with gentle shaking. The gel bed was then washed with 25 bed volumes of wash buffer (0.1 M Tris·HCl, pH 8). The gel was subsequently washed with 1 M KCl in 50 mM Tris·HCl, pH 7.4, poured into a small column, and equilibrated with 50 mM Tris·HCl buffer, pH 7.4.

The successful coupling of idazoxan to the gel was determined by including [^{3}H]idazoxan as tracer in the cross-linking reaction. Nearly 4% of [^{3}H]idazoxan added as tracer was incorporated to the gel. Using this 4% coupling efficiency and the amount of unlabeled idazoxan added, the degree of idazoxan substitution was, therefore, estimated to be 0.2–0.4 mg of idazoxan/ml of gel.

Preparation of Solubilized Chromaffin Cell Membrane Proteins

Chromaffin cells were isolated from bovine adrenal medulla as previously described (16), except that protease inhibitors were added at each step of the procedure. These were included in the 50 mM Tris·HCl, pH 7.4, buffer, which consisted of 0.3 mM phenylmethylsulfonyl fluoride, 0.1 mM EGTA, 10 mM ε-aminocaproic acid, 0.8 M pepstatin A, 0.1 mM benzamide, and 0.1 mM benzamidine hydrochloride.

Chromaffin cells were ruptured by sonication (3 × 10 sec, using a sonifier cell disruptor at setting 4) and homogenization with a Potter-Elvejham Teflon homogenizer. The cell membranes were isolated by centrifugation at 40,000 × *g* for 20 min and were washed two times in 50 mM Tris·HCl buffer, pH 7.4, that contained all of the protease inhibitors. The membrane pellet was then solubilized, by gentle hand homogenization, in 50 mM Tris·HCl buffer, pH 7.4, that contained all of the protease inhibitors (see above) and a final concentration of 0.5% (w/v) CHAPS. The suspension was shaken at 4° for 30 min and centrifuged at 100,000 × *g* for 30 min. The supernatant was subsequently purified by affinity chromatography.

Affinity Chromatography of the Solubilized Imidazoline-Binding Proteins

For affinity chromatography, a previously described procedure (17, 18) was utilized with some modifications. All buffers were 50 mM Tris·HCl, pH 7.4, with protease inhibitors. The solubilized chromaffin cell membrane protein solution was equally divided (approximately 30 mg) and loaded in a 25-ml volume onto IDA-agarose (2-ml bed volume) and PAC-ReactiGel columns (5-ml bed volume), which had been previously washed with the solubilization buffer that contained 0.5% CHAPS. After loading of the solubilized proteins, the column was washed first with solubilization buffer (0.5% CHAPS) and then with running buffer (0.05% CHAPS) until absorbance at 280 nm returned to base line. This

was followed by sequential elution with the 0.05% CHAPS/Tris·HCl buffer, pH 7.4, that contained an elution agent of choice (30 mM KCl, 30 mM NaCl, 100 μM rauwolscine, or 1 mM epinephrine), then 100 μM idazoxan or 100 μM cirazoline, and, finally, 1 M KCl. All steps were performed at 4°, with the same 5–10 ml/hr flow rate. Individual fractions from each elution were pooled (25–30 ml total), dialyzed extensively (4 liters, three changes) against 50 mM Tris·HCl buffer, pH 7.4, containing 0.01% CHAPS, and concentrated in the dialysis membrane, using PVP-360 (molecular weight of 360,000; Sigma), to a final volume of 300–500 μl for subsequent radioligand binding assays and SDS-PAGE analysis.

Protein Determination

Protein was estimated in particulate and detergent-solubilized preparations by the Coomassie Blue protein assay (Pierce Chemical). Bovine serum albumin was used as the standard. Assays were carried out in microtiter plates in order to measure the small amounts of protein in isolated protein preparations. Absorbance of microtiter plate wells was read at 570 nM using an enzyme-linked immunosorbent assay reader.

[^{3}H]Idazoxan Binding Assay

[^{3}H]Idazoxan binding to untreated chromaffin cell membranes was measured in 50 mM Tris·HCl buffer, pH 7.4, as previously described (19). The final concentration of [^{3}H]idazoxan was 10 nM and the nonspecific binding was defined with 10 μM unlabeled idazoxan or cirazoline. The amount of membrane protein was 100–200 μg/assay tube. Membranes were incubated with [^{3}H]idazoxan for 30 min at 25°, with gentle shaking. The incubation was terminated by filtration through Whatman GF/B filters (presoaked in 0.1% polyethylenimine), followed by two washes with 5 ml of ice-cold 50 mM Tris·HCl buffer, pH 7.4, using a Brandel cell harvester. The radioactivity trapped by the filters was measured in 5 ml of CytoScint fluid (Beckman) and counted in a Beckman liquid scintillation counter at approximately 45% efficiency.

Binding of [^{3}H]idazoxan to isolated proteins from affinity column fractions was measured by the PEG-γ-globulin precipitation-vacuum filtration assay method (8, 17, 20), with some modifications. Nonspecific binding was defined as described above. The amount of protein used in the assay was 100–200 μg for solubilized proteins, and 50–100 ng for isolated proteins, per assay tube. A final concentration of 0.5% γ-globulin was present in all assay tubes as a carrier protein. Total protein was incubated with [^{3}H]idazoxan in the absence or presence of various drugs at 25° for 30 min and then at 4° for 30 min, followed by addition of an equal volume of ice-cold 20% PEG in 50 mM Tris·HCl, pH 7.4, and incubation at 4° for 30 min. The PEG-protein precipitate was then rapidly filtered through Whatman GF/B filters (presoaked in 0.1% polyethylenimine) and rinsed once with ice-cold 10% PEG in 50 mM Tris·HCl buffer, pH 7.4. The radioactivity on the filters was counted as described above.

Competition studies were carried out with 10^{-9} to 10^{-4} M concentrations of drugs selected to assess displacement of [^{3}H]idazoxan binding (5 nM) to the isolated proteins. Assays were typically conducted in triplicate, and occasionally in duplicate. The data from saturation binding studies were analyzed by a nonlinear least squares fit to the generalized Scatchard equation using a computer-based optimization procedure for curve fitting (21). The data from studies of displacement of [^{3}H]idazoxan by other ligands were also analyzed by nonlinear least squares fitting procedures (22).

SDS-PAGE and Concanavalin A-Biotin Labeling

Isolated protein samples were analyzed on 10% SDS-polyacrylyamide gels. Either gels were stained with silver nitrate or the proteins were electrotransferred onto nitrocellulose sheets (23). Subsequent staining for glycoproteins that interact with concanavalin A-biotin conjugates was performed as described previously (18). After electrotransfer of proteins, the nitrocellulose filters were first washed with PBS that contained 0.05% Tween-20 and then blocked with PBS/0.5% Tween-20 for 30 min at room temperature. The filters were then rinsed with PBS/0.05% Tween-20 and incubated with concanavalin A-biotin complex (1/700 dilution in PBS/0.05% Tween-20) for 2 hr at room temperature. After washing three times, the filters were incubated with alkaline phosphatase-avidin conjugate (1/1000 dilution in PBS/0.05% Tween-20) for 2 hr at room temperature. The filters were washed extensively, and the alkaline phosphatase reaction was carried out in 0.1 M Tris·HCl buffer, pH 9.8, that contained $MgCl_2$, 5-bromo-4-chloro-3-indolyl phosphate, and nitroblue tetrazolium (blue reaction product). The electrotransferred marker proteins for molecular weight determination and the purified proteins on nitrocellulose filters were localized by staining with colloidal gold Aurodye (Janssen Pharmaceutical), according to the specifications of the manufacturer.

Radioiodination of Purified Proteins and Autoradiography

Radioiodination of proteins for detection by autoradiography was performed using the chloramine T-metabisulfite procedure (24). In brief, 1 μg (in 100 μl) of idazoxan eluates from PAC-ReactiGel or IDA-agarose was incubated with 5 μl of ^{125}I (0.5 mCi) and 20 μl of chloramine T (2 mg/ml in 50 mM sodium phosphate buffer, pH 7.4) for 5 min at room temperature. The reaction was terminated by the addition of 50 μl of 50 mM sodium phosphate buffer (pH 7.4) containing 2 mg/ml NaI and 2 mg/ml sodium metabisulfite. The 175-μl total reaction mixture was then passed through a 1-ml Sephadex G25 column to separate radioiodinated proteins from free ^{125}I. The column had been previously equilibrated with 50 mM sodium phosphate buffer containing 0.05% CHAPS, 0.5 mg/ml NaI, and 0.5 mg/ml sodium metabisulfite. Fractions (≈100 μl) were collected and an aliquot (10 μl) was used to measure the radioactivity in proteins after trichloroacetic acid precipitation. The radioiodinated protein fractions were pooled (≈270 μl), divided equally into two aliquots, and precipitated with trichloroacetic acid. They were then resuspended in SDS sample buffer with or without a reducing agent (5%, v/v, β-mercaptoethanol). The samples were heated for 5 min at 100° and analyzed on a 10% SDS-polyacrylamide gel. After electrophoresis, the gel was fixed for 1 hr in a solution of 25% methanol and 10% glacial acetic acid, stained with 0.1% (w/v) Coomassie Blue to identify molecular weight standards, and destained in the fixing solution for 4 hr. The gel was dried onto filter paper under vacuum. The dried gel was then exposed for 10 days to Kodak X-Omat AR film at −70°, using DuPont Cronex Lightning Plus intensifying screens.

Results

Purification of Imidazoline-Binding Proteins from Bovine Adrenal Chromaffin Cell Membranes by Affinity Chromatography

Affinity chromatography was used for purification of the IR from membranes of bovine adrenal chromaffin cells. These cells express IRs but not α_2-adrenergic receptors (19).[1] The membrane proteins were first solubilized by the detergent CHAPS, which has also been used to successfully solubilize IRs from membranes of guinea pig cerebral cortex; the solubilized receptors retained nearly the same binding properties as those of the membrane-bound receptor (25). IDA-agarose and PAC-ReactiGel affinity matrices were used to further isolate the solubilized imidazoline-binding proteins.

Use of an IDA-agarose column. The pattern of chromatographic separation of solubilized chromaffin cell membrane proteins by IDA-agarose is shown in Fig. 2. After sample loading and extensive washing of the column with the sample loading buffer (0.5% CHAPS buffer) and the sample running buffer (0.05% CHAPS buffer), the column was subsequently washed with a buffer of choice (containing 30 mM KCl or 30 mM NaCl) to remove proteins nonspecifically interacting with

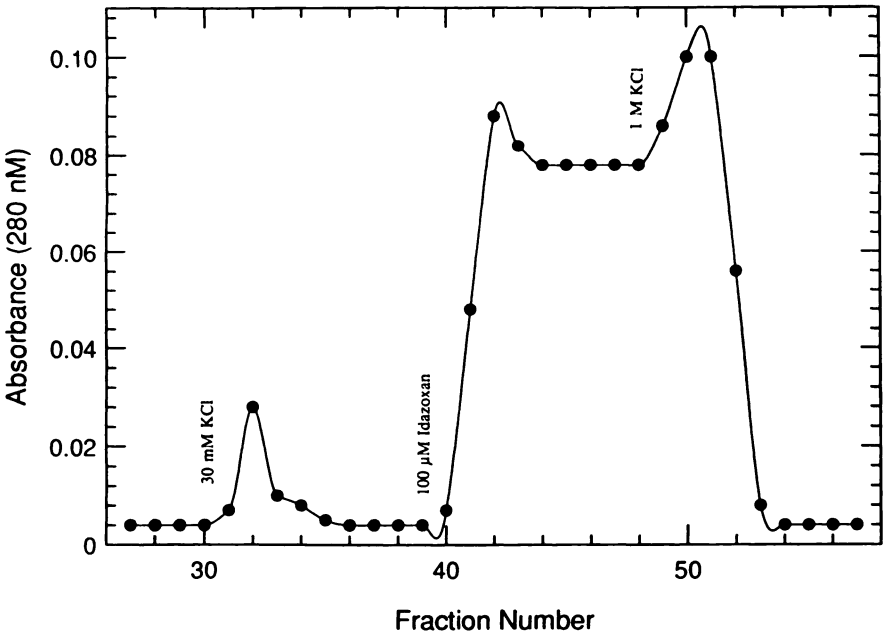

Fig. 2. Chromatography of solubilized bovine chromaffin cell membrane proteins on an IDA-agarose column. The stepwise elution of proteins with 30 mM KCl, 100 μM idazoxan, and 1 M KCl is shown. The absorbance value is not returned to base line before addition of 1 M KCl because the idazoxan solution itself absorbs at 280 nm. The initial loading and washing with 0.5% CHAPS and with 0.05% CHAPS in buffer are not included.

the affinity column. In some preparations, the 30 mM salt prewash was omitted and the column was washed instead with buffers containing 100 μM rauwolscine or 1 mM epinephrine to remove any contamination by small amounts of α_2-adrenergic receptors. The proteins retained on the column were then eluted with 100 μM idazoxan-containing buffer and 1M KCl.

The 30 mM KCl prewash eluted a few proteins detectable by SDS-PAGE. These included a predominant protein of 70 kDa and several other bands (Fig. 3A, *lane 1*). After this step the column was eluted with 100 μM idazoxan, which yielded only two distinct protein bands, a major one of 70 kDa and a minor one of 55 kDa (Fig. 3A, *lane 2*). The final elution with 1 M KCl yielded many more proteins (Fig. 3A, *lane 3*), obscuring the 70- and/or 55-kDa proteins, if present. The additional proteins are most likely contaminating proteins that nonspecifically adsorbed to the IDA-agarose affinity matrix.

To determine whether a low (30 mM) concentration of another salt would also result in elution of the 70-kDa protein, the column was prewashed with 30 mM NaCl followed by elution with 100 μM idazoxan. Few proteins were eluted by the prewash with NaCl and no protein bands were enriched (Fig. 3B, *lane 1*). Subsequent elution with 100 μM idazoxan resulted in the appearance of the 70- and 55-kDa protein bands (Fig. 3B, *lane 2*). A final elution with 1 M KCl yielded proteins of various sizes (Fig. 3B, *lane 3*).

In other preparations, 100 μM cirazoline was substituted for 100 μM idazoxan as an eluting agent of the IDA-agarose affinity matrix. Cirazoline, like idazoxan, mainly eluted a protein of 70 kDa (data not shown). In contrast, prewashing of the columns with 100 μM rauwolscine, a selective α_2-adrenergic antagonist, failed to elute any proteins, although subsequent elution with

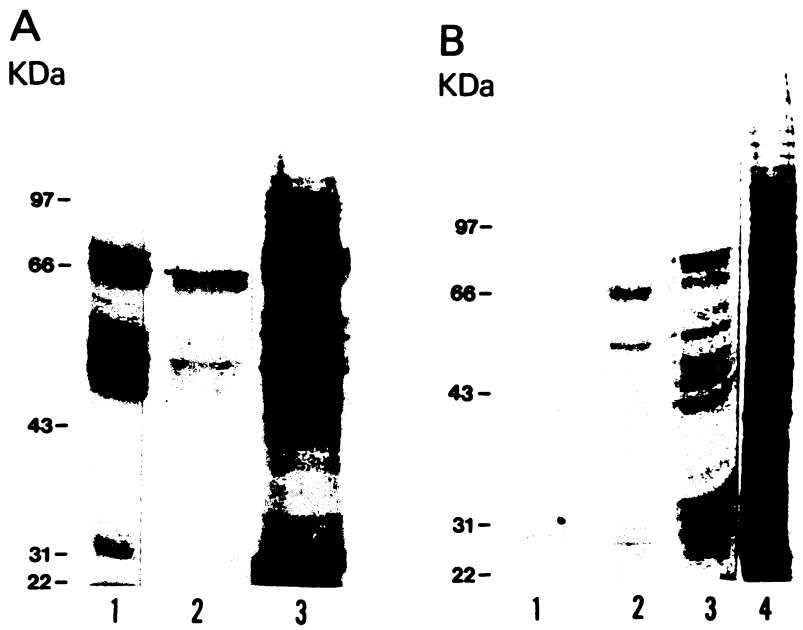

Fig. 3. Silver stain after SDS-PAGE of one fifth of the proteins contained in each elution from IDA-agarose column. Fractions were eluted with 30 mM KCl, 100 μM idazoxan, and 1 M KCl [A, *lane 1* (≈1.6 μg, *lane 2* (≈1 μg), and *lane 3* (≈3 μg), respectively] or with 30 mM NaCl, 100 μM idazoxan, and 1 M KCl [B, *lane 1* (<10 ng), *lane 2* (≈0.8 μg), and *lane 3* (≈2 μg), respectively]. B, *Lane 4*, a fraction of total solubilized chromaffin cell membrane proteins (20 μg).

100 μM idazoxan again yielded the 70-kDa protein (data not shown). These findings indicate that the proteins retained on the IDA-column are related to IRs, not α_2-adrenergic receptors.

Use of a PAC-ReactiGel column. When PAC-ReactiGel was used, prewashing of the column with 30 mM KCl (Fig. 4A, *lane 1*), 30 mM NaCl (data not shown), or 1 mM epinephrine (Fig. 4B, *lane 1*) eluted negligible amounts of protein. Elution with the 100 μM idazoxan buffer, on the other hand, yielded a 70-kDa protein (Fig. 4A, *lane 2*) or, in some cases, several proteins (62-kDa, 55-kDa, and 20-kDa proteins) (Fig. 4B, *lane 2*). Application of a 1 mM idazoxan-containing buffer after elution with 100 μM idazoxan sometimes yielded another protein of 62 kDa (Fig. 4B, *lane 3*). Elution of the PAC-ReactiGel column with 1 M KCl showed an increase in the 70-kDa protein obtained (Fig. 4A, *lane 3*) or, in other experiments, 62- and 30-kDa proteins (Fig. 4B, *lane 4*).

Further characterization of imidazoline-binding proteins by radioiodination. In most preparations, by silver stain analysis we observed mainly a 70-kDa protein in the idazoxan eluates from PAC-ReactiGel and IDA-agarose. However, in some preparations a minor 55-kDa protein was copurified with the 70-kDa protein. Because radioiodination is another sensitive method to detect protein purity, we performed radioiodination in an effort to examine protein purity and obtain information about the IR subunit composition. Aliquots of idazoxan eluates from PAC-ReactiGel and IDA-agarose were used for radioiodination. These radioiodinated protein preparations were equally divided and analyzed in SDS-containing gels, both in the presence and in the absence of a reducing agent (β-mercaptoethanol). As shown in Fig. 5, the only detectable material in the radioiodinated idazoxan eluates from both PAC-ReactiGel and IDA-agarose migrated with a molecular mass of 70 kDa in the reduced and nonreduced state. These results suggested that the idazoxan eluates from both PAC-ReactiGel and IDA-agarose contained the 70-kDa component in nearly homogeneous form. The similar mobilities observed under both reduced and nonreduced conditions indicate that the protein chains were not linked by disulfide bonds. The radioiodinated protein gel analysis data, along with the observation of specific [^{3}H]idazoxan-binding activity of the nonradioiodinated protein aliquots (data not shown), suggest that the idazoxan eluates from both PAC-ReactiGel and IDA-agarose are nearly homogeneous preparations and the 70-kDa protein is responsible for binding.

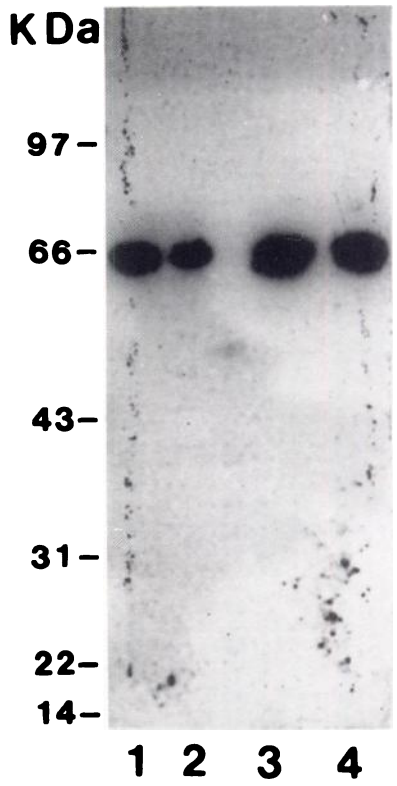

Fig. 5. Autoradiography of radioiodinated binding proteins. One microgram of the idazoxan eluates from PAC-ReactiGel and IDA-agarose was radioiodinated as described in Experimental Procedures. These two radioiodinated protein samples were divided equally and then denatured with SDS in the presence or absence of the reducing agent (β-mercaptoethanol) before electrophoresis. *Lanes 1* and *2*, idazoxan eluate from PAC-ReactiGel in the reduced and nonreduced state, respectively; *lanes 3* and *4*, idazoxan eluate from IDA-agarose in the reduced and nonreduced state, respectively.

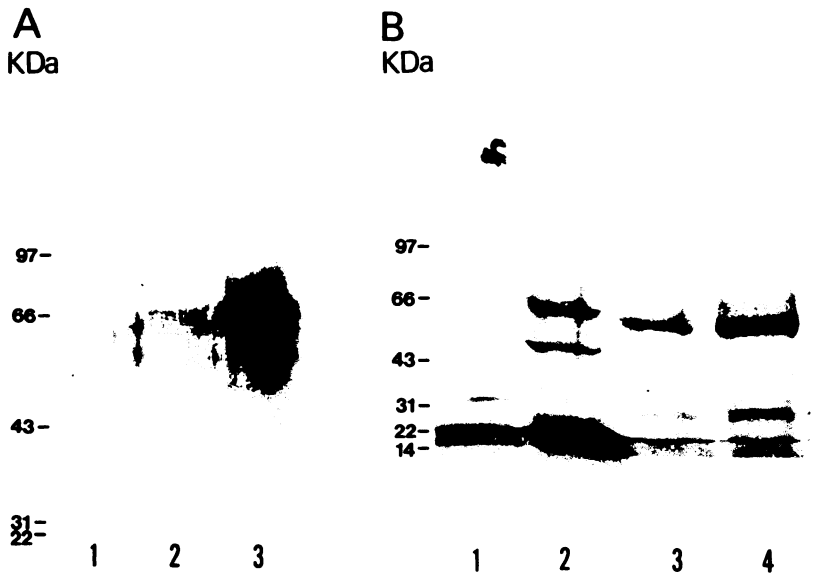

Fig. 4. Silver-stained patterns after SDS-PAGE of one fifth of the proteins contained in each elution from PAC-ReactiGel. Proteins were eluted with 30 mM KCl, 100 μM idazoxan, and 1 M KCl [A, *lane 1* (<10 ng), *lane 2* (≈300 ng), and *lane 3* (≈1 μg), respectively] or with 1 mM epinephrine, 100 μM idazoxan, 1 mM idazoxan, and 1 M KCl [B, *lane 1* (<50 ng), *lane 2* (≈500 ng), *lane 3* (≈200 ng), and *lane 4* (≈1 μg), respectively]. Double-silver staining of the gel was performed in order to enhance visualization of the small amount of proteins present.

Comparison of recoveries of imidazoline-binding proteins from IDA-agarose and PAC-ReactiGel columns. Partial purification of putative solubilized receptor proteins by IDA-agarose or PAC-ReactiGel affinity chromatography resulted in the enrichment of a 70-kDa protein in the fractions eluted with idazoxan (*lanes 2* of Figs. 3A, 3B, 4A, and 4B), compared with the total proteins present in whole membranes (Fig. 3B, *lane 4*). Tables 1 and 2 show the specific binding of [^{3}H]idazoxan, the degree of purification, and the yield of recovered imidazoline-binding proteins from the IDA-agarose and PAC-ReactiGel columns, respectively.

The imidazoline-binding protein in the solubilized fraction was enriched in comparison with intact membranes by a factor of 1.3. With IDA-agarose affinity chromatography (Table 1), the fractions eluted with 30 mM KCl, 100 μM idazoxan, and 1 M KCl exhibited [^{3}H]idazoxan-binding activity that was increased, respectively, by 358-, 700-, and 175-fold. Together, the total recovery represented approximately 18% of all ligand-binding activity in intact chromaffin cell membranes.

With PAC-ReactiGel affinity chromatography (Table 2), [^{3}H]idazoxan-binding activity in the fractions eluted by 100 μM idazoxan was enriched by a factor of 750, whereas the binding activity associated with the 1 M KCl-eluted fractions was enriched 417-fold. A recovery of approximately 7% of all ligand binding activity in the membranes was achieved in these two fractions. Thus, although both columns were effective in retaining imidazoline-binding proteins, IDA-agarose affinity chromatography produced greater yield than PAC-ReactiGel.

Similar radioligand binding experiments were performed to measure the specific [^{3}H]PAC binding to the isolated protein preparations under identical conditions [i.e., the same amount of protein (100 ng) and the same ^{3}H-labeled ligand concentration (10 nM)]. There was no apparent specific binding of [^{3}H]PAC to the isolated proteins at the 10 nM radioligand concentration tested (data not shown).

Ligand-Binding Characteristics of Partially Purified Imidazoline-Binding Proteins

Additional radioligand binding experiments were performed to determine whether the imidazoline-binding proteins eluted from ligand affinity columns shared comparable kinetics and ligand affinity profiles with the native receptor of adrenal chromaffin cells. Binding was studied on idazoxan-eluted fractions using a PEG-γ-globulin-rapid filtration assay (see Experimental Procedures) (8, 17, 20).

The binding of [^{3}H]idazoxan to IDA, and PAC affinity-purified proteins was of high affinity and saturable (Figs. 6 and 7, respectively). Scatchard analysis showed a linear relationship (Figs. 6 and 7, *insets*), indicating binding of [^{3}H]idazoxan to a single site. The B_{max} of purified imidazoline-binding proteins recovered from the IDA-resin and PAC-resin was 465 ± 5 pmol/mg of protein and 580 ± 20 pmol/mg of protein, respectively. Nonspecific binding increased with increasing [^{3}H]idazoxan concentration and was 16% at 5 nM [^{3}H]idazoxan (K_d concentration) and still <30% at 20 nM [^{3}H]idazoxan in both cases (data not shown). The dissociation constants (K_d) for imidazoline-binding proteins purified from either IDA- or PAC-resins were 3.7 ± 0.2 and 4.9 ± 0.2 nM, respectively. These were similar to the K_d (5 nM) of the IR of chromaffin cell membranes.[1]

To determine whether the isolated proteins exhibit drug affinities similar to those of the native subclass of IR of chromaffin cell membranes, we studied the displacement of [^{3}H]idazoxan binding to protein eluted from the IDA-agarose column by a panel of ligands, including cirazoline, idazoxan, clonidine, amiloride, epinephrine, and rauwolscine. Fig. 8 shows that cirazoline and idazoxan were the most potent in inhibiting the binding of [^{3}H]idazoxan to purified imidazoline-binding proteins (K_i, 2.3 ± 0.3 and 3.1 ± 0.5 nM, respectively), followed by clonidine and amiloride (K_i, 72 ± 5 and 600 ± 50 nM, respectively). Epinephrine and rauwolscine exhibited very weak displacing effects (K_i > 1 mM). Idazoxan and cirazoline displaced 90% of the total specific binding at a concentration of 10 μM, whereas 100 μM epinephrine or rauwolscine inhibited only approximately 20% of the [^{3}H]idazoxan binding activity. Thus, ligand potency data from purified protein and membrane preparations were similar, with one exception, i.e., whereas amiloride was more potent than clonidine in inhibiting binding of [^{3}H]idazoxan in the intact chromaffin cell membrane,[1] clonidine was a more potent inhibitor in the purified protein preparation (Fig. 8). These findings indicate that the pharmacological profiles of the isolated proteins are consistent with those of IRs, especially of the I-2 subclass, and not α_2-adrenergic receptors.

Due to lower yields, proteins eluted from the PAC-ReactiGel column were tested for displacement by only cirazoline and rauwolscine. Results showed that 10 μM cirazoline displaced 90% of the specific [^{3}H]idazoxan binding, whereas 100 μM rauwolscine displaced only 20% (data not shown). Although these data suggest that both IDA-agarose and PAC-ReactiGel columns retain similar proteins, determination of whether they are indeed the same depends on future sequencing analysis.

Together, these observations indicate that the detergent-solubilized and affinity-purified imidazoline-binding proteins retain their high affinity for [^{3}H]idazoxan after extraction and are closely related to the native IR subclass of chromaffin cells, the I-2 receptor.

TABLE 1

Purification of imidazoline-binding proteins from chromaffin cells by IDA-agarose affinity matrix

Fraction	Total protein[a]	Specific binding activity[b]	Total binding activity	Yield	Fold purification
	mg	*pmol/mg of protein*	*pmol*	*% of total activity*	
Membrane	50	0.6	30	100	1
Solubilized protein	30	0.8	24	80	1.3
30 mM KCl eluate	0.008	215	1.72	6	358
100 μM Idazoxan eluate	0.005	420	2.1	7	700
1 M KCl eluate	0.015	105	1.58	5	175

[a] The values are from a representative purification experiment of seven purification experiments.
[b] Ligand binding (10 nM [^{3}H]idazoxan) activity is the mean of triplicate determinations obtained for each fraction from the representative purification experiment.

TABLE 2

Purification of imidazoline-binding proteins from chromaffin cells by PAC-ReactiGel affinity matrix

Fraction	Total protein[a]	Specific binding activity[b]	Total binding activity	Yield	Fold purification
	mg	*pmol/mg of protein*	*pmol*	*% of total activity*	
Membrane	50	0.6	30	100	1
Solubilized protein	30	0.8	24	80	1.3
100 μM Idazoxan eluate	0.002	450	0.9	3	750
1 M KCl eluate	0.005	250	1.25	4	417

[a] The values are from a representative purification experiment of four purification experiments.
[b] Ligand binding (10 nM [^{3}H]idazoxan) activity is the mean of triplicate determinations obtained for each fraction from the representative purification experiment.

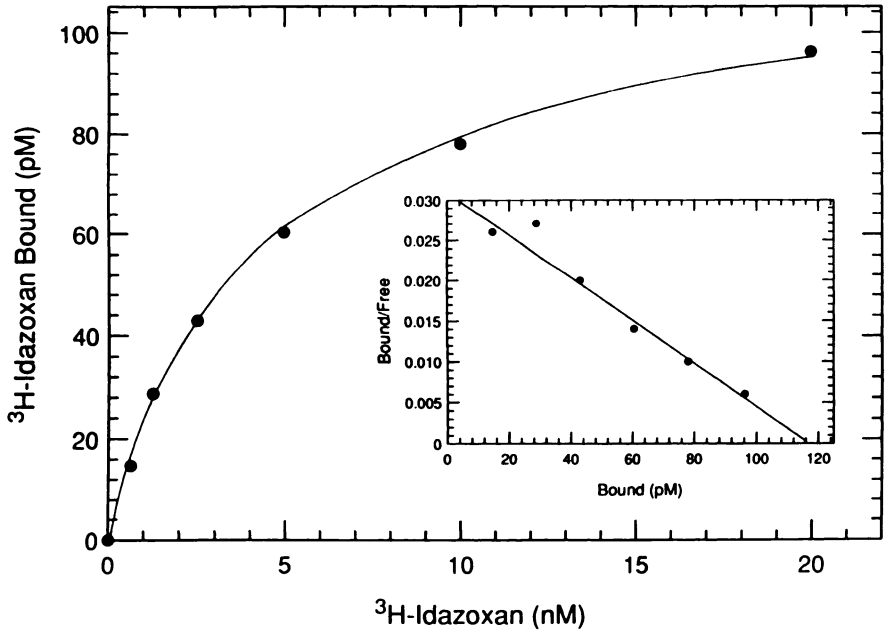

Fig. 6. Saturation curve of specific [^{3}H]idazoxan binding (0.625–20 nM) to purified imidazoline-binding proteins from IDA-agarose affinity matrix. *Inset*, Scatchard transform of the binding data. Estimates of K_d and B_{max} were 3.7 ± 0.2 nM and 465 ± 5 pmol/mg of protein, respectively. Each data point is the mean of six determinations from three experiments. The correlation coefficient for a single-site fit of the data was 0.97 (see Experimental Procedures for details).

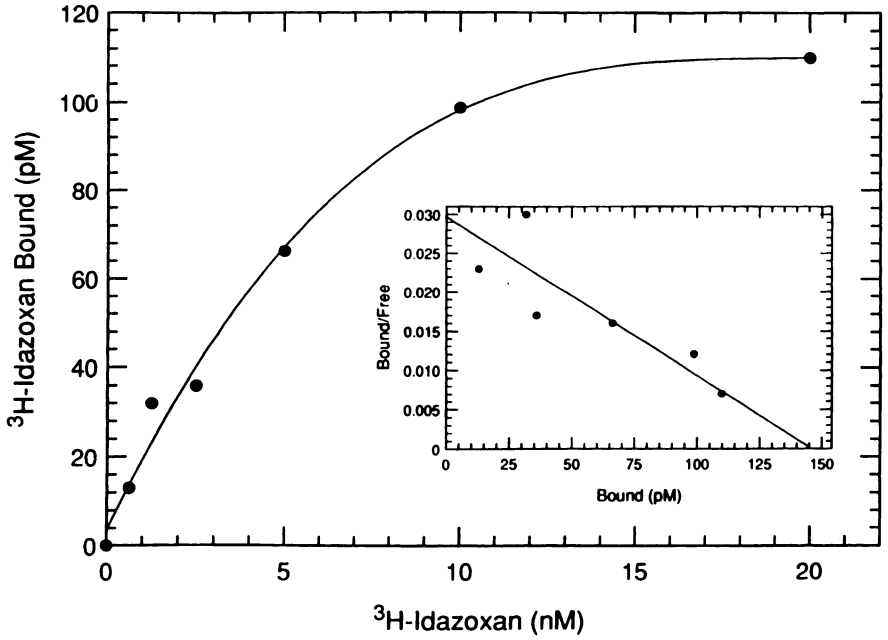

Fig. 7. Saturation binding curve of specific [^{3}H] idazoxan (0.625–20 nM) binding to isolated imidazoline-binding proteins from PAC-ReactiGel affinity matrix. *Inset*, Scatchard transform of the binding data. Estimates of K_d and B_{max} were 4.9 ± 0.2 nM and 580 ± 20 pmol/mg of protein, respectively. Each data point is the mean of six determinations from two experiments. The correlation coefficient for a single-site fit of the data was 0.84 (see Experimental Procedures for details).

Interactions of Purified Imidazoline-Binding Proteins with Concanavalin A-Biotin

It has been proposed by Parini *et al.* (8) that, unlike the α_2-adrenergic receptor, the IR protein is not a glycoprotein containing *N*-acetylglucosaminyl residues. This conclusion was based on the fact that the IR was not retained on a wheat germ lectin-agarose affinity matrix. We, therefore, utilized biotin-conjugated concanavalin A and avidin-alkaline phosphatase to determine whether the imidazoline-binding protein in bovine

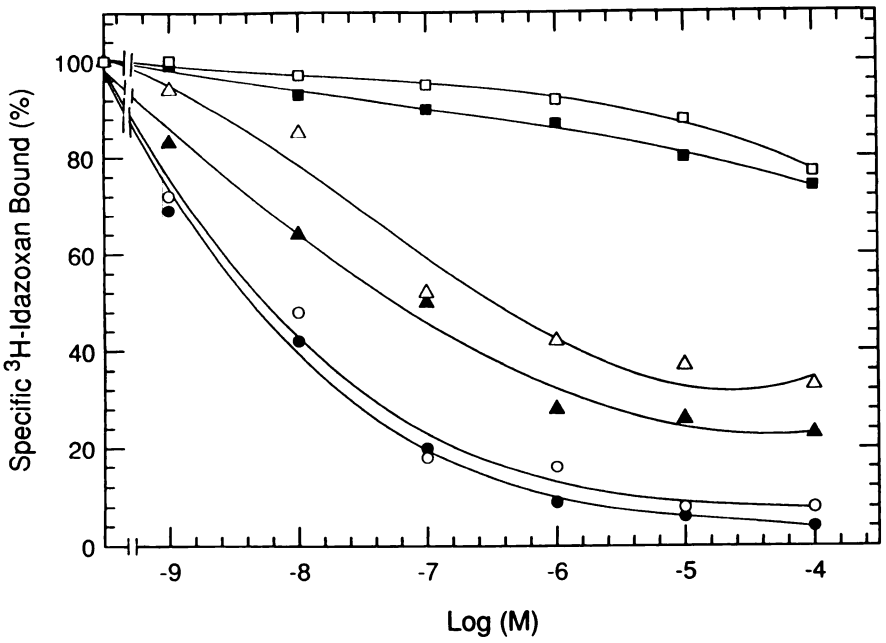

Fig. 8. Inhibition of [^{3}H]idazoxan binding to isolated imidazoline-binding proteins from IDA-agarose affinity matrix by various competing ligands. The imidazoline-binding proteins were incubated with a fixed concentration of [^{3}H]idazoxan (5 nM) in the presence of various concentrations (10^{-9} to 10^{-4} M) of various competing ligands, i.e., cirazoline (●) (2.3 ± 0.3 nM), idazoxan (○) (3.1 ± 0.5 nM), clonidine (▲) (72 ± 5 nM), amiloride (△) (600 ± 50 nM), rauwolscine (■) (>1 mM), and epinephrine (□) (>1 mM). Estimates of K_i values for the aforementioned competing ligands are indicated in parentheses. All data points are the average of six determinations at each ligand concentration, from two experiments.

adrenal chromaffin cell membranes might be a different type of glycoprotein that contains α-D-mannopyranosyl, α-D-glucopyranosyl, and sterically related residues (26).

Isolated proteins eluted from either the IDA-resin or the PAC-resin with idazoxan were electrotransferred onto nitrocellulose. Various amounts (10 μg, 1 μg, 100 ng, and 10 ng) of a known glycoprotein, chicken egg albumin (45 kDa), were used as a positive control for assay sensitivity. Results showed that, whereas the concanavalin A-biotin-avidin-alkaline phosphatase reaction gave positive staining with chicken egg albumin as low as 10 ng (Fig. 9, *lane 1–4*), 1 μg of the idazoxan-eluted imidazoline-binding proteins from either the IDA-resin or the PAC-resin was not stained (Fig. 9, *lanes 5* and *6*). The effectiveness of the transfer of purified imidazoline-binding proteins onto the nitrocellulose filter was demonstrated by the positive colloid gold (Aurodye) staining of equal amounts (1 μg) of protein electrophoresed in adjacent lanes of the SDS-PAGE gel and transferred at the same time (Fig. 9, *lanes 7* and *8*). In addition, complete transfer of Coomassie Blue-prestained molecular weight protein standards (2 μg each of six proteins, of 106, 80, 50, 33, 28, and 19 kDa) provided visual evidence of transfer efficiency under our conditions. The failure of concanavalin A-biotin to label imidazoline-binding proteins suggested that either they were not glycosylated in a pattern recognizable by concanavalin A (mannose and glucose residues) or their sugar moieties were lost during isolation and handling. These data extend previously reported results (8).

Discussion

In the present study we sought to isolate imidazoline-binding proteins from chromaffin cells of the bovine adrenal medulla. Chromaffin cells were selected as the tissue source because they express only IRs and not α_2-adrenergic receptors (19).[1] We reasoned that imidazoline-binding proteins from these cells could be isolated by affinity chromatography using idazoxan and/or PAC as immobilized ligands because both agents bind to α_2-adrenergic receptors and IRs; however, in the absence of the former, only imidazoline-binding proteins would be retained on the columns.

Partial purification of an imidazoline-binding protein was

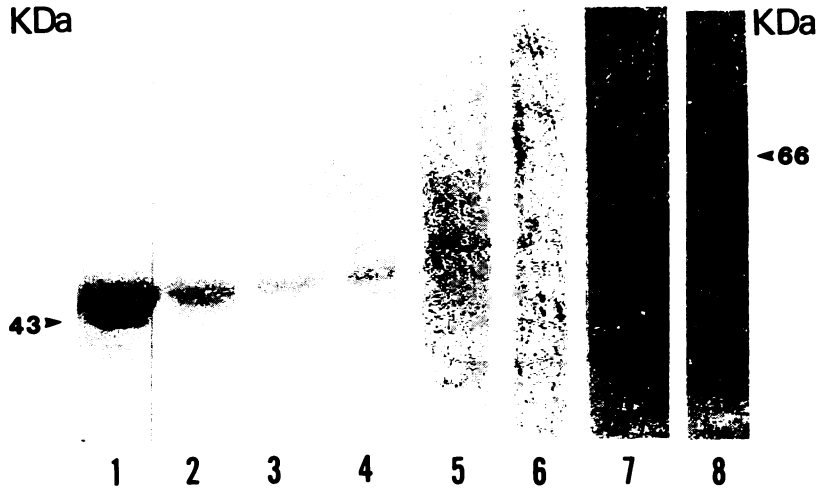

Fig. 9. Studies of concanavalin A-biotin reaction with purified imidazoline-binding proteins from IDA-agarose and PAC-ReactiGel affinity matrices. *Lanes 1, 2, 3,* and *4*, chicken egg albumin at 10 μg, 1 μg, 100 ng, and 10 ng; *lanes 5* and *7*, 1 μg of purified protein from IDA-agarose; *lanes 6* and *8*, 1 μg of purified proteins from PAC-ReactiGel. After electrotransfer of the proteins onto a nitrocellulose filter, the filter strips (*lanes 1–6*) were allowed to react first with concanavalin A-biotin complex and then with avidin-alkaline phosphatase complex. Development of the phosphatase reaction was performed as described in Experimental Procedures. Effective transfer of the purified proteins was established by parallel staining of strips from *lanes 7* and *8* with the colloid gold Aurodye, according to the instructions of the manufacturer.

achieved by solubilization of the receptors of bovine adrenal chromaffin cells with CHAPS, by synthesis of PAC-ReactiGel and IDA-agarose affinity matrices to bind the soluble IR protein(s), and by elution of these proteins from the column with idazoxan. A relatively short purification time (10–16 hr), as well as the use of protease inhibitors throughout the isolation process, minimized proteolytic degradation and receptor inactivation. The eluted protein(s) retained the ligand-binding properties of the receptor in intact membranes. By means of this single purification step, we were able to achieve a 700-fold purification of a [^{3}H]idazoxan-binding protein.

The most highly purified fractions of imidazoline-binding protein were obtained from fractions eluted with idazoxan. In most preparations, these elutions were highly enriched in protein with a molecular mass of 70 kDa (electrophoresed under reducing conditions), as characterized by radioiodination and silver stain analysis. Radioiodination analysis of idazoxan eluates detected only a 70-kDa protein band and this protein bound specifically to [^{3}H]idazoxan, suggesting that the 70-kDa protein is responsible for binding. For silver stain analysis, ≈1 μg of idazoxan eluate was applied to the gel and a main 70-kDa protein band and a minor 55-kDa protein band appeared. Based on the staining of bovine serum albumin, the sensitivity of our silver stain procedure is ≈5 ng of protein. Thus, a single contaminant representing ≈0.5% of the preparation theoretically would be detectable. Whether the 55-kDa protein that copurified with the 70-kDa protein in some preparations is a degradative product of the 70-kDa protein remains to be determined. Compared with the staining appearance of various amounts of bovine serum albumin standards, the imidazoline-binding proteins were not stained well by silver and the staining sensitivity could be improved by a second silver stain process. This kind of silver stain pattern is often observed with acidic proteins and metal-containing proteins (Bio-Rad Silver Stain Bulletin 1089).

The relatively high degree of purification of the 70-kDa protein appears attributable to high affinity binding to the PAC or IDA affinity matrices, which allows retention during extensive washes of the columns with buffer. On the other hand, the binding proteins were not retained on control matrices that were devoid of the ligands (i.e., IDA-free Tris-NH_2-blocked agarose or PAC-free Tris-NH_2-blocked ReactiGel) (data not shown). Electrophoresis of the idazoxan-eluted proteins under nonreducing conditions showed a protein with molecular mass similar to that obtained under reducing conditions (i.e., 70 kDa). Thus, it appears that the idazoxan-eluted receptor binding proteins (70 kDa) were not linked by disulfide bonds.

The B_{max} of the imidazoline-binding proteins purified from IDA-agarose affinity chromatography was 465 pmol of [^{3}H] idazoxan binding/mg of protein. The theoretical specific binding activity of a homogeneous preparation of imidazoline-binding proteins of 70 kDa would represent ≈14 nmol of binding/mg of protein, assuming one binding site/molecule. The 30-fold discrepancy probably results from (a) receptor binding protein, upon release from the membrane environment by detergent solubilization, not binding to a ligand efficiently due to minor conformational change; (b) partial loss of binding activity during the purification procedure due to protein inactivation ([^{3}H] idazoxan-binding decreased with increasing storage time at 4°, using solubilized and purified protein preparations; data not shown); or (c) partial loss of binding activity during the PEG-vacuum filtration assay due to dissociation of bound ligands from binding proteins, incomplete precipitation of the ligand-protein complex by PEG, or incomplete trap of the PEG-precipitated ligand-protein complex by the filter.

Although PAC has been shown to exhibit lower affinity than idazoxan for membrane IRs (K_i, 200 nM), as well as isolated imidazoline-binding proteins (K_i, 72 nM), the large amount of PAC attached to the column matrix (0.5–1 mg/ml, estimated using [^{3}H]PAC as tracer) should be able to retain some imidazoline-binding proteins. We observed elution of greater amounts of imidazoline-binding proteins, as well as contaminating proteins, from the IDA-agarose matrix than from the PAC-ReactiGel affinity matrix. Conceivably, this may be due to a lower affinity of the receptor protein for PAC than for IDA. Moreover, elution of receptor proteins from IDA-agarose, but not from the PAC-ReactiGel, by low concentrations of K^+ may be due to a somewhat reduced affinity of idazoxan for receptor proteins in its coupled state. Increased contaminating proteins from IDA-agarose are presumed to be due to greater nonspecific binding to the agarose than to the acrylamide matrix.

The comparability of the ligand-binding properties of the 70-kDa protein eluted from the IDA-agarose column to those of membranes prepared from intact chromaffin cells[1] strongly suggests, for several reasons, that the isolated protein represents an imidazoline receptor binding site. First, the affinities for [^{3}H]idazoxan of the purified proteins (K_d ≈ 4 nM) are similar to those of membrane IRs from chromaffin cells (K_d, 5 nM),[1] from cerebral cortex of guinea pig (K_d, 3.6 nM) (25), and from human kidney (K_d, 2 nM) (27). Second, the displacement analysis indicates that proteins eluted from the IDA column have a high affinity for the imidazolines cirazoline and idazoxan, moderate affinity for clonidine and amiloride, and no affinity for the catecholamine epinephrine and the selective α_2-adrenergic antagonist rauwolscine. Thus, the rank order of potency for inhibition of [^{3}H]idazoxan binding to the 70-kDa protein was cirazoline ≥ idazoxan > clonidine > amiloride ≫ rauwolscine ≈ epinephrine. In general, this is in agreement with adrenal medullary mitochondria membrane binding data[1] and characteristic of binding to the so-called I-2 type of IR. A noticeable difference is that amiloride (K_i, 45 nM) showed higher affinity than clonidine (K_i, 200 nM) for mitochondria membrane IRs,[1] whereas the reverse was true (K_i of 72 nM for clonidine and 600 nM for amiloride) with regard to the purified binding protein from crude chromaffin cell membranes. This discrepancy may be due to either a slight conformational change in the IR induced by detergent solubilization or the fact that purified protein was obtained from crude membrane containing both mitochondrial and plasma membrane IRs.

Although both idazoxan and PAC bind to α_2-adrenergic receptors as well as IRs in brain (9, 10), kidney (8, 27), and other tissues (7, 13), we have demonstrated that in adrenal chromaffin cell membranes these agents bind only to IRs.[1] We have interpreted these observations as indicating that chromaffin cells fail to express the α_2-adrenergic receptor. The findings of this study strongly support this contention. For example, not only did the soluble binding proteins isolated from the affinity columns fail to bind selective adrenergic ligands, but a prewash of either column with 1 mM epinephrine or 100 μM rauwolscine failed to elute any detectable proteins. This is in contrast to the efficacy of 100 μM idazoxan in eluting the

70-kDa imidazoline binding protein. Moreover, the ability of 30 mM KCl but not NaCl to elute imidazoline-binding proteins is consistent with the evidence that the binding of [^{3}H]idazoxan to IRs in membranes is inhibited by K^+, but not Na^+, whereas the reverse is true for the α_2-adrenergic receptor (15, 27–29). Finally, Parini *et al.* (8) recently demonstrated that only α_2-adrenergic receptors, not IRs, from solubilized renal membranes were retained on a wheat germ lectin affinity matrix, suggesting that the IR is not a glycoprotein, at least not via *N*-linked glycosylation. Our data show that there is no detectable interaction between concanavalin A-biotin and purified imidazoline-binding proteins. This observation indicates either that IRs of chromaffin cells, like those of kidney, are not concanavalin A-detectable glycoprotein(s) or that relevant sugar residues were lost during the purification process. Thus preliminarily, this evidence suggests that IRs and α_2-adrenergic receptors are distinct.

The data presented here, therefore, confirm that bovine chromaffin cells express only IRs but not α_2-adrenergic receptors (19)[1] and provide preliminary evidence that IRs and α_2-adrenergic receptors differ. In addition, these receptors have topographically different distributions in brain (9, 10) and kidney (8, 27, 30), utilize different signal transduction mechanisms (4, 12), and have unique ligand-binding profiles (31). Such findings are consistent with the view that these are distinct molecular and genetic entities. The identification of an advantageous tissue source, the development of efficient ligand affinity columns, and the purification of the binding proteins should be of great utility in the further characterization of IRs. Recently, rabbit antisera raised against the isolated 70-kDa protein have been found to label specifically a 70-kDa protein out of numerous membrane proteins, by Western blot analysis, and to inhibit the binding of [^{3}H]idazoxan to chromaffin cell membranes. Antisera also immunoextracted [^{3}H]idazoxan-binding activity from a solubilized chromaffin cell membrane preparation.[2] These observations support the data presented in this report showing that the isolated 70-kDa protein is a ligand-binding entity of the IR.

References

1. Unnerstall, J. R., T. Kopajtic, and M. J. Kuhar. Distribution of α_2-agonist binding sites in the rat and human central nervous system: analysis of functional, anatomic correlates of the pharmacologic effects of clonidine and related adrenergic agents. *Brain Res. Rev.* **7**:69–101 (1984).
2. Timmermans, P. B. M. W. M., A. Schoop, H. Kwa, and P. A. Van Zwieten. Characterization of α-adrenoceptors participating in the central hypotensive and sedative effects of clonidine using yohimbine, rauwolscine, and corvnanthine. *Eur. J. Pharmacol.* **70**:7–15 (1981).
3. Wikberg, J. E. S. High affinity binding of idazoxan to a non-catecholaminergic binding site in the central nervous system: description of a putative idazoxan-receptor. *Pharmacol. Toxicol.* **64**:152–155 (1989).
4. Lehmann, J., E. Koenig-Berard, and P. Vitou. The imidazoline-preferring receptor. *Life Sci.* **45**:1609–1615 (1989).
5. Atlas, D. Clonidine-displacing substance (CDS) and its putative imidazoline receptor. *Biochem. Pharmacol.* **41**:1541–1549 (1991).
6. Ernsberger, P., R. Giuliano, R. N. Willette, A. R. Granata, and D. J. Reis. Hypotensive action of clonidine analogues correlates with binding affinity at imidazole and not *alpha*-2-adrenergic receptor in the rostral ventrolateral medulla. *J. Hypertens.* **6**:S554–S557 (1988).
7. Zonneschein, R., S. Diamant, and D. Atlas. Imidazoline receptors in rat liver cells: a novel receptor or a subtype of α_2-adrenoceptors? *Eur. J. Pharmacol.* **190**:203–215 (1990).
8. Parini, A., I. Coupry, R. M. Graham, I. Uzielli, D. Atlas, and S. M. Lanier. Characterization of an imidazoline/guanidinium receptive site distinct from the α_2-adrenergic receptor. *J. Biol. Chem.* **264**:11874–11878 (1989).
9. Boyajian, C. L., and F. M. Leslie. Pharmacological evidence for *alpha*-2-adrenoceptor heterogeneity: differential binding properties of [^{3}H]rauwolscine and [^{3}H]idazoxan in rat brain. *J. Pharmacol. Exp. Ther.* **241**:1092–1098 (1987).
10. Kamisaki, Y., T. Ishikawa, Y. Takao, H. Omodani, N. Kuno, and T. Itoh. Binding of [^{3}H]*p*-aminoclonidine to two sites, α_2-adrenoceptors and imidazoline binding sites: distribution of imidazoline binding sites in rat brain. *Brain Res.* **514**:15–21 (1990).
11. Tesson, F., C. Prip-Buus, A. Lemoine, J.-P. Pegorier, and A. Parini. Subcellular distribution of imidazoline-guanidinium receptive sites in human and rabbit liver. *J. Biol. Chem.* **266**:155–160 (1991).
12. Regunathan, S., M. J. Evinger, M. P. Meeley, and D. J. Reis. Effects of clonidine and other imidazole-receptor binding agents on second messenger systems and calcium influx in bovine adrenal chromaffin cells. *Biochem. Pharmacol.* **42**:2011–2018 (1991).
13. Michel, M. C., J. W. Regan, M. A. Gerhardt, R. R. Neubig, P. A. Insel, and H. J. Motulsky. Nonadrenergic [^{3}H]idazoxan binding sites are physically distinct from α_2-adrenergic receptors. *Mol. Pharmacol.* **37**:65–68 (1990).
14. Ernsberger, P., M. P. Meeley, J. J. Mann, and D. J. Reis. Clonidine binds to imidazole binding sites as well as α_2-adrenoceptors in the ventrolateral medulla. *Eur. J. Pharmacol.* **134**:1–13 (1987).
15. Michel, M. C., O.-E. Brodde, B. Schnepel, J. Behrendt, R. Tschada, H. J. Motulsky, and P. A. Insel. [^{3}H]Idazoxan and some other α_2-adrenergic drugs also bind with high affinity to a nonadrenergic site. *Mol. Pharmacol.* **35**:324–330 (1989).
16. Wilson, S. P. Purification of adrenal chromaffin cells on Renografin gradients. *J. Neurosci. Methods* **19**:163–171 (1987).
17. Chen, J.-W., M. D. Cunningham, N. Galton, and E. K. Michaelis. Immune labeling and purification of a 71-kDa glutamate-binding protein from brain synaptic membranes. *J. Biol. Chem.* **263**:417–426 (1988).
18. Wang, H., K. N. Kumar, and E. K. Michaelis. Isolation of glutamate-binding proteins from rat and bovine brain synaptic membranes and immunochemical characterization. *Neuroscience* **46**:793–806 (1992).
19. Regunathan, S., M. P. Meeley, and D. J. Reis. Clonidine-displacing substance from bovine brain binds to imidazoline receptors and releases catecholamines in adrenal chromaffin cells. *Mol. Pharmacol.* **40**:884–888 (1991).
20. Bruns, R. F., K. Lawson-Wendling, and T. A. Pugsley. A rapid filtration assay for soluble receptors using polyethylenimine-treated filters. *Anal. Biochem.* **132**:74–81 (1983).
21. McPherson, G. A. A practical computer-based approach to the analysis of radioligand binding experiments. *Comput. Prog. Biomed.* **17**:107–116 (1983).
22. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239 (1980).
23. Wray, W., T. Boulikas, V. P. Wray, and R. Hancock. Silver-staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118**:197–203 (1981).
24. Repaske, M. G., J. M. Nunnari, and L. E. Limbird. Purification of the α_2-adrenergic receptor from porcine brain using a yohimbine-agarose affinity matrix. *J. Biol. Chem.* **262**:12381–12386 (1987).
25. Wikberg, J. E. S., and S. Uhlen. Further characterization of the guinea pig cerebral cortex idazoxan receptor: solubilization, distinction from the imidazole site, and determination of cirazoline as an idazoxan receptor-selective drug. *J. Neurochem.* **55**:192–203 (1990).
26. Hendricks, L. C., C. A. Gabel, K. Suh, and M. G. Farquhar. A 58-kDa resident protein of the *cis* Golgi cisterna is not terminally glycosylated. *J. Biol. Chem.* **266**:17559–17565 (1991).
27. Lachaud-Pettiti, V., R.-A. Podevin, Y. Chretien, and A. Parini. Imidazoline-guanidinium and α_2-adrenergic binding sites in basolateral membranes from human kidney. *Eur. J. Pharmacol.* **206**:23–31 (1991).
28. Nunnari, J. M., M. G. Repaske, S. Brandon, E. J. Cragoe, Jr., and L. E. Limbird. Regulation of porcine brain α_2-adrenergic receptors by Na^+, H^+ and inhibitors of Na^+/H^+ exchange. *J. Biol. Chem.* **262**:12387–12392 (1987).
29. Yablonsky, F., and J. P. Dausse. Non-adrenergic binding sites for the "α_2-antagonist" [^{3}H]idazoxan in the rabbit urethral smooth muscle. *Biochem. Pharmacol.* **41**:701–707 (1991).
30. Ernsberger, P., G. Feinland, M. P. Meeley, and D. J. Reis. Characterization and visualization of clonidine-sensitive imidazole sites in rat kidney which recognize clonidine displacing substance. *Am. J. Hypertens.* **3**:90–97 (1990).
31. Michel, M. C., and P. A. Insel. Are there multiple imidazoline binding sites? *Trends Pharmacol. Sci.* **10**:342–344 (1988).

[2] H. Wang, S. Regunathan, and D. J. Reis. Production and characterization of antibodies specific for the inidazoline-binding proteins. Manuscript in preparation.

Send reprint requests to: Hong Wang, Ph.D., Division of Neurobiology, Cornell University Medical College, 411 East 69th Street, New York, NY 10021.