0026-895X/93/040509-07$03.00/0

MOLECULAR PHARMACOLOGY, 43:509-515

Production and Characterization of Antibodies Specific for the Imidazoline Receptor Protein

H. WANG, S. REGUNATHAN, D. A. RUGGIERO, and D. J. REIS

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

Received December 28, 1992; Accepted February 2, 1993

SUMMARY

Polyclonal antibodies were raised in rabbits against a 70-kDa ligand-binding protein of the imidazoline receptor purified from solubilized bovine adrenal chromaffin cell membranes by ligand affinity chromatography. The antibodies labeled a single protein (≈70 kDa) in Western blots of bovine adrenal chromaffin cell membranes, inhibited 40% of specific [^{3}H]idazoxan binding to imidazoline receptors in chromaffin cell membranes, and specifically immunoprecipitated 75% of all imidazoline-binding activity of solubilized chromaffin cell membrane proteins. The antibodies specifically immunostained heterogeneous subsets of cultured bovine chromaffin cells. They stained subpopulations of chromaffin cells of rat adrenal medulla but not the cells of adrenal cortex. We conclude that the antibodies recognize with high specificity and selectivity a ≈70-kDa binding protein associated with or representing the imidazoline receptor that is expressed in mammalian species. Highly specific antibodies against the imidazoline receptor protein will permit mapping of the distribution of imidazoline receptors in brain and periphery and also may be useful as probes in cloning genes encoding the imidazoline receptors.

It is now recognized that clonidine, IDA, and their congeners bind not only to α_2-adrenergic receptors but also to a novel nonadrenergic binding site that recognizes the imidazoline ring of the parent molecule (1–5). This ligand binding site has been designated the I-receptor (4, 5), even though it is recognized that some agents that are not imidazolines, such as rilmenidine (an oxazoline) and guanabenz (a guanidium), also bind to the receptor with high affinity (4, 6).

The I-receptors, like many other receptors, also appear to be expressed in several subclasses that differ with respect to their relative affinities for ligands (7). One, the I-1-receptor, preferentially binds PAC, is purportedly localized in plasma membranes, and has a limited tissue distribution. In contrast, the I-2-receptor preferentially binds IDA, is mitochondrial, and is expressed in a number of organs, tissues, and cell types, including the cerebral cortex and chromaffin cells of the adrenal medulla.

The fact that agents that bind to I-receptors also bind to α_2-adrenergic receptors raises the following question: are the two receptors molecularly distinct? Although the I-receptor has yet to be purified and its structure established, there is strong inferential evidence that it is genetically different from the α_2-adrenergic receptor. Thus, the two receptors are independently expressed in different tissues (6, 8, 9) and subcellular compartments (10), they utilize different signal transduction mechanisms (4, 11), and transfection of cells with genes for two α_2-adrenergic receptor subtypes (12) fails to impart binding characteristics of the I-receptor to recipient cells.

Adrenal chromaffin cells are a good source for I-receptor isolation because they express only I-receptors and not α_2-adrenergic receptors (13). Recently we have isolated from detergent-solubilized bovine adrenal chromaffin cell membranes, using IDA-agarose and PAC-ReactiGel columns, a 70-kDa protein with ligand-binding characteristics of the native I-2-receptor. Thus, the affinities for the binding of [^{3}H]IDA to the purified binding protein ($K_d \approx 4$ nM) and the membrane I-receptor ($K_d \approx 5$ nM) were similar, as were the rank orders of potency of displacing ligands (cirazoline ≥ IDA > clonidine and amiloride ≫ rauwolscine and epinephrine) (14). Limon *et al.* (15) simultaneously and independently reported the isolation by other methods of an imidazoline/guanidinium-receptive site from rabbit kidney that is closely related to but not identical to the adrenal chromaffin cell I-receptor.

The isolation of the binding protein not only allows for determination of amino acid sequence but also provides an antigen for production of specific antibodies. Such antibodies, if they could be generated, would be useful in clonal selection and for mapping of receptor-associated protein in tissue by immunocytochemistry. In the present study we report the generation in rabbits of polyclonal antibodies against the affinity-

ABBREVIATIONS: IDA, idazoxan; PAC, *para*-aminoclonidine; IRP, imidazoline receptor protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PEG, polyethylene glycol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-[2-ethanesulfonic acid]; BSA, bovine serum albumin; I-receptor, imidazoline receptor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

purified IRP isolated from IDA- and PAC-linked columns. We demonstrate that these antibodies appear selective for the I-receptor, as demonstrated by Western blotting, immunoinhibition and immunoprecipitation of ligand-binding activity, and immunocytochemical localization of IRP.

Materials and Methods

Purification of IRP from bovine adrenal chromaffin cell membranes. The methods for isolation of bovine adrenal chromaffin cells, preparation of membranes, solubilization of membrane proteins, and purification by ligand affinity chromatography have been detailed in a recent publication from this laboratory (14) and are summarized here.

Bovine adrenal glands were obtained from a local slaughterhouse within 4 hr after death and were perfused with collagenase and DNase (Sigma Chemical Co.), and medullae were dissociated from cortices. The tissue was minced, digested with collagenase for 40 min at 37°, and filtered through a 105-μm wire sieve. The cell suspension was loaded onto a step gradient of 15% and 7.5% Renografin (Squibb) and centrifuged for 20 min at 10,000 × *g*. Chromaffin cells were collected from the interface of the gradients and washed twice with buffer (16). Cells were ruptured by sonication and cell membranes were isolated by centrifugation at 40,000 × *g* for 20 min. Membranes were washed two times with 50 mM Tris·HCl buffer, pH 7.4, with protease inhibitors and were then solubilized for 30 min at 4°, with agitation, in buffer containing 0.5% (w/v) CHAPS. The suspension was centrifuged at 100,000 × *g* for 30 min and the supernatant was then purified by affinity chromatography.

IDA- and PAC-coupled columns for ligand affinity chromatography were prepared as described previously (14) . The solubilized membrane proteins were divided equally and loaded onto the IDA-agarose and PAC-ReactiGel columns, which had been washed previously with solubilization buffer containing 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 30 mM KCl, 100 μM IDA, and 1 M KCl. Individual fractions from each elution were pooled, dialyzed extensively against 50 mM Tris·HCl, pH 7.4, containing 0.01% CHAPS, and concentrated in the dialysis membrane using 360-kDa polyvinylpyrrolidone (PVP-360; Sigma). These protein fractions were analyzed by [^{3}H] IDA binding assay and SDS-PAGE. IDA eluates from both IDA-agarose and PAC-ReactiGel had the highest [^{3}H]IDA-binding activity and a main 70-kDa protein band on SDS-PAGE. These IDA eluates from IDA-agarose and PAC-ReactiGel were used to immunize individual rabbits.

Production of polyclonal antibodies against the IRP. Polyclonal antibodies to the nondenatured 70-kDa IRP purified from IDA-agarose and PAC-ReactiGel were prepared by immunizing New Zealand white rabbits four times, subcutaneously, with 5–10 μg of protein each time. Immunizations were performed at 15-day intervals. The first immunization was done with the protein antigen emulsified in an equal volume of complete Freund's adjuvant; the next two immunizations were done with the protein antigen emulsified in an equal volume of incomplete Freund's adjuvant. After 1 month the rabbit received a boost of protein antigen emulsified in an equal volume of incomplete Freund's adjuvant. The rabbits were bled from the ears 8–10 days after immunization. Preimmune sera were collected before initiation of immunization. All sera were prepared from whole blood by clotting and low speed centrifugation and were stored in small aliquots at −70° until use.

PAGE and immunoblot staining. After SDS-PAGE, either the 10% SDS-polyacrylamide gels were stained with silver (17) or the proteins were electrotransferred onto Immobilon-P membranes (18). Immunochemical staining (Western blotting) of transferred proteins was performed as described previously (19). After electrotransfer of proteins, the membranes were first washed with PBS/0.05% Tween-20 and then incubated for 30 min at 37° with 2% (w/v) gelatin in PBS to block the remaining protein binding sites. The blocked membranes were washed twice at 37° and then incubated for 2 hr at room temperature with rabbit anti-IRP antiserum or preimmune rabbit serum (1/2000 dilution in PBS/0.05% Tween-20). After incubation, the membranes were washed three times and incubated for 2 hr at room temperature with alkaline phosphatase conjugates of goat anti-rabbit IgG antibodies (1/1000 dilution in PBS/0.05% Tween-20). After extensive washing of the membranes, the alkaline phosphatase reaction was developed with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium substrate (Kirkegaard and Perry Laboratories). The electrotransferred molecular weight marker proteins and chromaffin cell membrane proteins were localized by staining with colloidal gold Aurodye (Janssen Pharmaceutical), according to the specifications of the manufacturer.

Immunoinhibition of [^{3}H]IDA binding to membrane I-receptors. The methods of assesing binding of [^{3}H]IDA to chromaffin cell membranes have been described in detail elsewhere (13). In brief, chromaffin cell membranes (100 μg of protein/assay tube, in 50 mM Tris·HCl buffer, pH 7.4) were preincubated for 1 hr at 4° with rabbit anti-IRP antiserum (1/5, 1/10, and 1/100 dilutions) or with preimmune rabbit serum (1/5 and 1/10 dilutions), with gentle shaking. The mixture was then incubated for 30 min at 25° with [^{3}H]IDA (10 nM final concentration), with gentle shaking. Nonspecific binding was defined with 10 μM unlabeled cirazoline. Incubation was terminated by rapid vacuum filtration over Whatman GF/B filters (presoaked in 0.1% polyethylenimine) using a Brandel cell harvester. The filters were washed with 10 ml of ice-cold 50 mM Tris·HCl buffer, pH 7.4. 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.

Preparation of immunoglobulins from immune and preimmune sera. Individual serum samples were diluted 1/4 with PBS, and saturated ammonium sulfate was added to a final concentration of 40%. The mixture was incubated for 4 hr at 4° and then centrifuged at 3000 × *g* for 20 min at 4°. The pellet was resuspended in 50 mM sodium phosphate buffer, pH 7, and dialyzed extensively against the same buffer at 4°. The dialysate was applied to DEAE-Sephadex in the same buffer, and the protein content in the fractions was monitored at 280 nm with a UV spectrophotometer. The first peak obtained was the IgG and the fractions were pooled and concentrated. Assurance of purified IgG was obtained by SDS-PAGE analysis and Western blotting using alkaline phosphatase-conjugated goat anti-rabbit IgG.

Immunoprecipitation of solubilized IRP. Immunoextraction of solubilized IRP from bovine chromaffin cell membranes was performed with slight modification of procedures described previously (19). Bovine chromaffin cell membranes were solubilized with 0.5% CHAPS for 30 min and then centrifuged at 100,000 × *g* for 30 min at 4°. The solubilized proteins in the supernatant were precipitated for 15 min on ice with 30% (w/v) PEG in 50 mM Tris·HCl, pH 7.4, and were then centrifuged at 40,000 × *g* for 15 min to extract the proteins from the detergent solution. The pellet was resuspended in 650 μl of 50 mM Tris·HCl buffer, pH 7.4. Three 200-μl aliquots, each containing 600 μg of solubilized membrane proteins, were mixed with 100 μl (100 μg) of rabbit anti-IRP (IgG fraction), with 100 μl (100 μg) of preimmune rabbit IgG, or with 100 μl of 50 mM Tris·HCl, pH 7.4. The mixtures of membrane proteins and IgG or buffer were incubated for 1 hr at 4° and then added to 100 μl (bed volume) of washed Trisacryl-immobilized Protein A. The immobilized Protein A (Protein A-Trisacryl GF 2000; Pierce Chemical Co.) was prepared by washing the Trisacryl-Protein A matrix twice with 50 mM Tris·HCl buffer, pH 7.4, containing 1 mg/ml ovalbumin (added to minimize nonspecific protein absorption to the matrix). The tubes that contained membrane proteins plus Protein A-Trisacryl were incubated for 1 hr at 4°, with gentle mixing. Samples were then centrifuged at 40,000 × *g* for 20 min at 4°. Each supernatant was

carefully removed from the pellet, divided into six aliquots (100 μg of solubilized proteins/aliquot), and used in binding assays. Binding of [^{3}H]IDA to the membrane protein samples (after immunoextraction) was measured by the PEG-γ-globulin precipitation-vacuum filtration assay method (14, 20) with slight modifications. Total binding and nonspecific binding were measured in triplicate samples. The final concentration of [^{3}H]IDA was 10 nM and nonspecific binding was defined with 10 μM cirazoline. A final concentration of 0.5% γ-globulin was present in all assay tubes as a carrier protein. The membrane protein samples (after immunoextraction) were incubated with [^{3}H] IDA 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 with 5 ml of ice-cold 10% PEG in 50 mM Tris·HCl buffer, pH 7.4. The radioactivity of the filters was measured as described above.

Immunocytochemistry of chromaffin cell cultures. Monolayer primary cultures of chromaffin cells were prepared from bovine adrenal glands by the method of Wilson and Viveros (21), with some modifications (22). Chromaffin cells, isolated by gradient centrifugation as described above, were washed and resuspended in Dulbecco's modified Eagle medium/F12 (1:1) supplemented with 10 mM HEPES (pH 7.4), 10% heat-inactivated fetal calf serum, and antibiotics (penicillin and streptomycin). The cells were plated directly onto chamber slides (Nunc, Inc.) at 5 × 10^5 cells/chamber. Cultures were maintained in a humidified atmosphere (5% CO_2/95% room air) at 37°.

After 3–5 days in culture, the cells were washed with PBS and then fixed for 1 hr at room temperature in 4% paraformaldehyde. The cells were then washed twice with PBS. The cell membranes were permeabilized by exposure to 0.3% Triton X-100 in PBS for 15 min. Cells were then incubated for 30 min in the presence of blocking serum (1/30 dilution of normal goat serum), followed by an overnight incubation at 4° with the primary antibodies (rabbit anti-IRP antiserum or preimmune rabbit serum) at a 1/2000 dilution in PBS containing 1% normal goat serum. After removal of the primary antibody and washing, the cultures were then processed using a Vector Laboratories ABC kit with biotinylated goat anti-rabbit IgG and diaminobenzidine tetrahydrochloride/H_2O_2, according to the instructions of the manufacturer. The slides were dehydrated, treated with xylene, mounted with coverslips in Permount mounting medium (Fisher), and photographed using a Nikon light microscope.

Immunohistochemistry of rat adrenal glands. Six male Sprague-Dawley rats (300–350 g) were anesthetized with pentobarbital (100 mg/kg intraperitoneally) and perfused transcardially with heparinized normal saline, followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. The adrenal glands were removed, postfixed in the same fixative (4% paraformaldehyde) for 3 hr at 4°, and cryoprotected overnight, by immersion in 0.1 M PBS containing 10% sucrose. Serial frozen sections were cut on a sliding microtome at 30 μm, collected in PBS, and rinsed in TBS, pH 7.6, with 0.25% Triton X-100. Tissues were incubated in spot test wells and continuously agitated on a Thomas rotator table. All incubations were carried out at room temperature and were preceded by two 5-min washes with 0.1 M TBS. Nonspecific binding sites were blocked by preincubating sections for 60 min in 0.1 M TBS containing 1% BSA and 0.25% Triton X-100. Tissues were rinsed and incubated for 18 hr with antiserum raised against IRP, diluted (1/10,000) in 0.1 M TBS with 0.1% BSA and 0.25% Triton X-100. Tissues were immunoprocessed by the avidin-biotin horseradish peroxidase method (23) using a Vectastain Elite kit (Vector Laboratories). The immunoreaction product was visualized by incubating the sections for 6–10 min in a solution containing 22.5 mg of diaminobenzidine and 10 μl of 30% H_2O_2 in 100 ml of TBS. Sections were rinsed with TBS, mounted on gelatin-coated slides, dehydrated, cleared, and coverslipped. Tissues were photographed using a Nikon microscope and Kodak T-Max 100 film.

Protein determination. The protein concentration of individual samples was estimated by the Coomassie Blue protein assay (Pierce). BSA was used as the standard.

Results

Specificity of antibodies produced against IRP. IRP was purified from bovine adrenal chromaffin cell membranes by IDA or PAC affinity chromatography. The IDA eluates (from both IDA-agarose and PAC-ReactiGel) contained mainly a 70-kDa protein (IRP), as analyzed by silver staining of SDS-polyacrylamide gels (Fig. 1) and radioiodination (14). These proteins also showed the highest [^{3}H]IDA-binding activity (14).

Immunization of individual rabbits with proteins purified on IDA-agarose and PAC-ReactiGel, separately and in their native state, resulted in antibodies that reacted specifically with proteins purified by either IDA or PAC affinity chromatography. For example, antiserum raised against IRP purified on the IDA matrix reacted strongly with a 70-kDa protein in whole-cell membranes and with protein purified on the IDA column (Fig. 2, *lanes 2* and *4*). Moreover, antiserum against this protein also reacted with protein purified on the PAC affinity matrix (Fig. 2, *lane 6*). Results obtained with antiserum produced against protein purified on the PAC column were similar, in that it recognized IRP in whole-cell membranes and protein purified on IDA and PAC affinity columns (Fig. 2, *lanes 3*, *5*, and *7*). When chromaffin cell membranes or the purified IRP from the IDA or PAC affinity matrix was incubated with preimmune rabbit serum, there was no labeling of any protein bands. An example of preimmune serum staining of chromaffin cell membranes is shown in Fig. 2, *lane 8*. The observations that rabbit antisera raised against native IRP purified on either the IDA or PAC affinity matrix had a high degree of specificity and cross-reactivity in recognizing the two proteins indicated structural homology between these two proteins.

Inhibition of [^{3}H]IDA binding to membrane I-receptors. The presence of antibodies in the rabbit antiserum against the IRP was demonstrated by immunoinhibition of [^{3}H]IDA binding to adrenal chromaffin cell membranes. Cell membranes were preincubated with preimmune serum or anti-IRP antiserum, and then the [^{3}H]IDA-binding activity of these samples

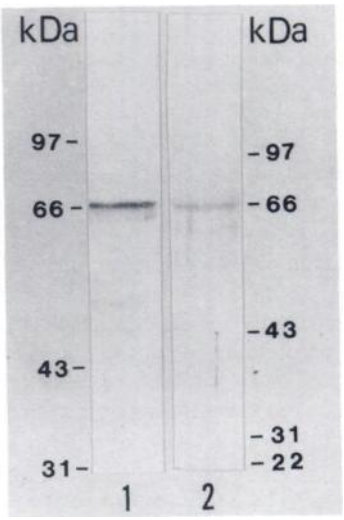

Fig. 1. SDS-PAGE (silver stain) of IRP purified by ligand affinity chromatography. *Lane 1*, proteins (1 μg) eluted with 100 μM IDA from the IDA-agarose column; *lane 2*, proteins (300 ng) eluted with 100 μM IDA from the PAC-ReactiGel column.

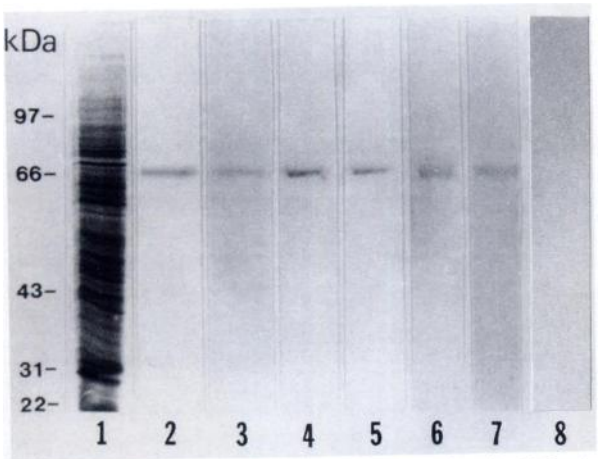

Fig. 2. Immunoreactivity of the rabbit antisera against bovine adrenal chromaffin cell membrane proteins and purified IRP. The membrane proteins (*lanes 1, 2, 3,* and *8*; 20 μg), IRP purified on the IDA column (*lanes 4* and *5*; 1 μg), and IRP purified on the PAC column (*lanes 6* and *7*; 1 μg) were subjected to SDS-PAGE and electrotransferred onto Immobilon-P membranes. *Lane 1* was stained with colloid gold Aurodye to visualize the transferred proteins. *Lanes 2, 4,* and *6* were incubated with a 1/2000 dilution of rabbit antibodies raised against IRP purified on the IDA column. *Lanes 3, 5,* and *7* were incubated with a 1/2000 dilution of rabbit antibodies raised against IRP purified on the PAC column. *Lane 8* was incubated with a 1/2000 dilution of preimmune rabbit serum. Immunoreactivity was visualized by incubating the washed membranes with anti-IgG alkaline phosphatase conjugate (1/1000 dilution), followed by reaction with the chromogenic phosphatase substrate.

TABLE 1

Inhibition of [^{3}H]IDA binding to chromaffin cell membranes by antibodies against the IRP

Serum dilution	Specific binding activity[a]	Inhibition of specific binding[b]
	fmol/mg of protein	%
None	360 ± 5	0
Preimmune		
1/5	346 ± 1	4
1/10	342 ± 7	5
Hyperimmune		
1/5	203 ± 1	44
1/10	264 ± 2	27
1/100	304 ± 5	16

[a] Ligand binding (10 nM [^{3}H]IDA) activity is the mean of six determinations for each sample. Nonspecific binding was 29% of total binding.
[b] The immunoinhibition experiments were performed as described in Materials and Methods.

was measured. Results in Table 1 show that antibodies to the IRP (purified from the IDA affinity matrix) partially inhibited the binding of [^{3}H]IDA to membrane I-receptors. The immunoinhibition appears specific for the membrane I-receptors for the following reasons. First, the immunoinhibition using anti-IRP antiserum at 1/100, 1/10, and 1/5 dilutions was concentration dependent, yielding 16%, 27%, and 44% inhibition, respectively. Second, the preimmune serum at 1/10 and 1/5 dilutions resulted in only 5% and 4% inhibition, respectively. Moreover, antisera raised against unrelated antigens (glial fibrillary acidic protein and induced nitric oxide synthase) at 1/10 and 1/5 dilutions produced 3–5% and 7–10% inhibition, respectively. The observed nonspecific inhibition may have been caused by serum proteins. Third, the antiserum failed to inhibit the binding of [^{3}H]IDA to α_2-adrenergic receptors in bovine cerebral cortex (data not shown). The inhibition by anti-IRP antiserum was partial, because a maximum of 44% inhibition was achieved. Some inhibition of [^{3}H]IDA binding to membrane proteins by the antiserum against the IRP suggests that some antibody molecules appear to recognize the I-receptor binding site or sites in its proximity and thus directly inhibit or block ligand binding. Because the antiserum is polyclonal, some of the antibodies are probably directed against regions of the receptor that are distinct from the ligand binding site and, therefore, would not interfere with ligand binding of the receptor. It has been reported that polyclonal antibodies raised against the insulin receptor (native or denatured) do not inhibit insulin binding but both antibodies immunoprecitate solubilized insulin receptor (24).

Extraction of [^{3}H]IDA-binding entities from solubilized membranes by immunoprecipitation. If the proteins recognized by the antibodies were responsible for [^{3}H]IDA binding in cell membranes, then immunoextraction of these proteins should produce a decrease in binding activity. To test this proposition, we used purified IgG from the rabbit antiserum against the IRP (purified from the IDA affinity matrix) and from preimmune rabbit serum. Solubilized bovine chromaffin cell membrane proteins were preincubated with either rabbit anti-IRP IgG, preimmune rabbit IgG, or 50 mM Tris·HCl buffer, pH 7.4. Immobilized Protein A was used as a matrix for the precipitation by centrifugation of the IRP-IgG complexes from solubilized membrane proteins. Because Protein A binds to rabbit IgG molecules strongly, it is expected that most IgGs, including IgG complexes with the IRP, would be extracted by this procedure. After such immunoprecipitation, the individual samples were examined for [^{3}H]IDA-binding activity. The percentage of activity remaining reflects the extracted [^{3}H]IDA-binding activity of each type of IgG. The results in Table 2 show that 83% of [^{3}H]IDA-binding activity was removed from solubilized chromaffin cell membrane proteins by treatment with rabbit anti-IRP IgG, whereas only 8% of the binding activity was removed by immunoextraction with preimmune rabbit IgG. Therefore, immunoextraction with immune IgG resulted in specific removal of 75% of IDA-binding entities from chromaffin cell membranes. These observations are indicative of selective immunoprecipitation of most solubilized [^{3}H]IDA-binding activity of chromaffin cell membranes by IgG from rabbits immunized with IRP in its native state.

Immunocytochemical labeling of bovine chromaffin cells by the anti-IRP antiserum. To examine the expression and distribution of the IRP, cultured bovine adrenal chromaffin cells were fixed, permeabilized with detergent, and immunostained with rabbit antibodies raised against the IRP (purified from the IDA affinity matrix) (see Materials and Methods). Examples of labeling with the rabbit anti-IRP antiserum are shown in Fig. 3, B and C. Some cells were stained intensely,

TABLE 2

Immunoextraction of the IRP from solubilized chromaffin cell membranes

Immunoextraction treatment[a]	Specific [^{3}H]IDA-binding activity[b]
	fmol/mg of protein
Buffer (no IgG)	380 (100%)
Preimmune IgG	350 (92%)
Hyperimmune IgG	65 (17%)

[a] The immunoextraction methods were those described in Materials and Methods.
[b] Ligand binding (10 nM [^{3}H]IDA) is the mean of triplicate determinations for each sample.

Fig. 3. Localization of IRP immunoreactivity in cultured bovine adrenal chromaffin cells. Chromaffin cells (5 days in culture) were processed for immunocytochemical localization of IRP using anti-IRP antiserum and Vectastain ABC kits (Vector Laboratories) (B and C). The plasma membrane of the cells was permeabilized with Triton X-100. Preimmune serum was used as control (A). Note the stained chromaffin cells (*arrows*) and unstained chromaffin cells and fibroblasts (*arrowheads*). *Bar*, 120 μm (A and B) or 60 μm (C).

some stained moderately or lightly, and some were not stained by the antibody, indicating that subsets of the chromaffin cells express IRP and that various levels of IRP are present in these chromaffin cells. Some chromaffin cells and background cells (likely fibroblasts), as well as the nuclei of the stained cells, were not labeled, supporting the finding that the immunostaining was specific. Because the cell membrane was permeabilized, the immunoreactivity included labeling of cell membrane surface and intracellular proteins. Preimmune rabbit serum failed to stain the cultured chromaffin cells (Fig. 3A). Polyclonal rabbit antibodies against IRP (purified from the PAC affinity matrix) immunostained the cultured chromaffin cells similarly (data not shown).

Immunohistochemical staining of rat adrenal gland using anti-IRP antiserum. After verifying the specificity of the rabbit polyclonal antibody raised against the IRP purified from bovine adrenal chromaffin cell membranes by IDA affinity chromatography, we examined the species cross-reactivity of this antibody by immunohistochemical staining of rat adrenal gland. IRP-like immunoreactivity was identified in chromaffin cell bodies of the adrenal medulla. Consistent distributional and density patterns were observed in the adrenal glands of six rats. As shown in Fig. 4, A and B, IRP-immunoreactive chromaffin cells in the adrenal medulla were characterized by moderate to high levels of immunoreaction product. These cells were organized in clusters associated with blood vessels. The adrenal cortex was unlabeled on tissues processed with either the IRP antiserum (Fig. 4A) or preimmune serum (data not shown). On control sections, light, heterogeneous, background staining of adrenal chromaffin cells with preimmune rabbit serum (Fig. 4C) was comparable to that observed in adrenal cortex, which does not express I-receptors. On alternate sections, the patches of darkly stained chromaffin cells by anti-IRP antiserum did not overlap with background staining by preimmune serum. Moreover, entirely different labeling patterns were observed in the adrenal medulla when serial sections were incubated with antisera against other antigens, such as L-glutamate or choline acetyltransferase (data not shown). These control experiments support the view that selective cell staining by anti-IRP antiserum is specific, rather than being an artifact caused by hyperimmunization. In addition to the specificity of the anti-IRP antiserum, its species cross-reactivity with rat IRP-like protein will enable us to immunohistochemically characterize the IRP in rats at the light microscopic and ultrastructual levels.

Discussion

Although there is abundant evidence to support the existence in brain and peripheral organs of I-receptors that differ from known transmitter/hormone receptors, proof of the uniqueness of these receptors requires that their structure and function be established. To this end we have recently isolated and purified a protein from bovine adrenal chromaffin cells that has all the ligand-binding characteristics of the native receptor in intact membranes (14). In this study, we have successfully produced polyclonal antibodies against the isolated IRP and have shown that these antibodies specifically recognize I-receptors *in vitro* and *in vivo*. By Western blotting, the antibodies raised against IRP isolated by either IDA or PAC affinity chromatography reacted strongly with the IRP that was used for immunization. Interestingly, antibodies raised against the IRP purified on the

Fig. 4. Photomicrographs of adrenal gland tissues immunoprocessed with an antiserum raised against IRP, using the avidin-biotin technique. A, Adrenal chromaffin cells contain IRP-like immunoreactivity (*curved arrow*), whereas the adrenal cortex is not labeled by anti-IRP antiserum. B, Higher power magnification of the same tissue stained by antiserum, revealing the characteristic immunoreaction product within discrete clusters of chromaffin cell bodies (*curved arrow*). Note the absence of immunoreactivity in other chromaffin cell groups. C, Control tissue of adrenal gland incubated with preimmune serum. *Bar*, 300 μm (A) or 120 μm (B and C). *V*, blood vessel.

IDA affinity matrix cross-reacted with the IRP purified on the PAC affinity matrix and, vice versa. This finding indicates that the two proteins share some structural similarities.

The anti-IRP antibodies specifically inhibited [^{3}H]IDA binding to bovine chromaffin cell membranes by 40%, suggesting that the epitopes recognized by the polyclonal antibodies may be associated with or in close proximity to ligand binding sites. The failure to achieve complete immunoinhibition might be explained by the polyclonal nature of this antibody and/or the lack of association of main immunogenic domains with ligand binding sites. An understanding of the structure of the IRP and of the topography of immunogenic domains in this protein will be required to clarify this issue.

Further evidence that the protein labeled by antibodies in the Western blot corresponded to [^{3}H]IDA binding sites in chromaffin cell membranes was obtained by immunoextraction experiments using solubilized bovine chromaffin cell membrane proteins. The reaction of these membrane proteins with immune IgG (against IRP purified on the IDA affinity matrix) followed by precipitation with Protein A-Trisacryl beads resulted in disappearance of 75% of binding in the supernatant. The observation indicates further that the 70-kDa protein recognized by the antibodies belongs to a major class of imidazoline-binding proteins or receptors in bovine chromaffin cell membranes.

The antibody to the IRP is also capable of immunostaining the native protein in bovine adrenal chromaffin cells in culture. Of interest was the fact that the intensity of staining was not uniform in all cells. Some chromaffin cells were stained heavily, whereas others contained moderate levels of immunoreactivity or were unlabeled. Moreover, the antibodies failed to stain a cell line derived from bovine adrenal cortex (SBAC; ATCC CRL 1796) (data not shown), in agreement with observations that membranes isolated from whole bovine adrenal cortex or cultured bovine adrenal cortical cells (25) fail to bind [^{3}H]IDA. The IRP antibodies also stained subpopulations of cells in the rat adrenal medulla, prepared by perfusion fixation, but did not stain cells in the adrenal cortex. The findings firstly suggest that the IRP shares similarities in cow and rat and hence is common to two species, secondly confirm that expression of the IRP is restricted to populations of cells in adrenal medulla and hence is tissue- and cell-specific, and finally indicate that anti-IRP antibodies raised against nondenatured protein reacted well with native or denatured molecule.

Although the antibodies used in this study are polyclonal, we strongly believe, for several reasons, that they are highly specific in recognizing I-receptors and not other proteins that may have been co-purified from adrenal medulla. First, the protein that was isolated and used for immunization had all the ligand-binding characteristics of an IRP and after radioiodination exhibited a single band, indicating little if any contamination by other proteins (14). Second, Western blot analysis demonstrated that, despite the presence of numerous membrane proteins on the filter, only a single protein with a molecular weight corresponding to that of the immunizing protein was labeled. Third, the antibodies, in addition to partially inhibiting [^{3}H]IDA binding to cell membranes, also extracted most of the binding entities from solubilized cell membranes, indicating that the antibodies were directed specifically against the IRP. Fourth, immunocytochemically the antibodies recognized only subpopulations of adrenal chromaffin cells *in vitro* and *in vivo*, indicating that they do not recognize proteins such as chromagrannins or tyrosine hydroxylase that may be distributed among all chromaffin cells. Fifth, the specific staining of subsets of chromaffin cells in adrenal medulla of cow and rat but not adrenal cortex indicates that the antibodies are specific for tissues expressing I-receptors but are not species specific. Sixth,

as we have demonstrated elsewhere the antibodies recognize the I-receptors of rat cortical astrocytes (26, 27), a tissue not expressing usual proteins of adrenal medulla. Finally, there is a striking overlap between the regional distribution of I-receptors demonstrated immunohistochemically in rat brain with this antibody (27) and that defined by ligand-binding autoradiography by Mallard *et al.* (28). Thus, we believe that our antibody identifies IRP but not other adrenal medullary proteins.

Based on the high degree of specificity of the anti-IRP antibodies that we have developed, we believe that these antibodies may be very useful probes in future investigations into the structure and function of the bovine I-receptors. Current work is in progress using these anti-IRP antibodies to map the distribution of I-receptors in brain and periphery, including their ultrastructural localization, to determine the structural basis of receptor function. We are also attempting to use these anti-IRP antibodies as a probe for isolation and cloning of the I-receptors.

Acknowledgments

The authors wish to thank Ms. Liubov Lyandvert for her technical assistance and Ms. Nancy Marmor for her expert editorial assistance.

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. 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).
7. Michel, M. C., and P. A. Insel. Are there multiple imidazoline binding sites? *Trends Pharmacol. Sci.* **10**:342–344 (1989).
8. 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).
9. 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).
10. 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).
11. 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).
12. 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).
13. 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).
14. Wang, H., S. Regunathan, M. P. Meeley, and D. J. Reis. Isolation and characterization of imidazoline receptor protein from bovine adrenal chromaffin cells. *Mol. Pharmacol.* **42**:792–801 (1992).
15. Limon, I., I. Coupry, S. M. Lanier, and A. Parini. Purification and characterization of mitochondrial imidazoline-guanidinium receptive site from rabbit kidney. *J. Biol. Chem.* **267**:21645–21649 (1992).
16. Wilson, S. P. Purification of adrenal chromaffin cells on Renografin gradients. *J. Neurosci. Methods* **19**:163–171 (1987).
17. Wray, W., T. Boulikas, V. P. Wray, and R. Hancock. Silver-staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118**:197–203 (1981).
18. Blake, M. S., K. H. Johnston, G. T. Russell-Jones, and E. C. Gotschlich. A rapid sensitive method for detection of alkaline phosphatase conjugated anti-antibody on Western blots. *Anal. Biochem.* **136**:175–179 (1984).
19. 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).
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. Wilson, S. P., and O. H. Viveros. Primary cultures of adrenal medullary chromaffin cells in a chemically defined medium. *Exp. Cell Res.* **133**:159–169 (1981).
22. Ross, M. E., M. J. Evinger, S. E. Hyman, J. M. Carroll, L. Mucke, M. Comb, D. J. Reis, T. H. Joh, and H. M. Goodman. Identification of a functional glucocorticoid response element in the phenylethanolamine *N*-methyltransferase promoter using fusion genes introduced into chromaffin cells in primary culture. *J. Neurosci.* **10**:520–530 (1990).
23. Hsu, S.-M., L. Raine, and H. Fanger. The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (peroxidase) procedures. *J. Histochem. Cytochem.* **29**:577–580 (1981).
24. Jacobs, S., and P. Cuatrecasas. Insulin receptors and insulin receptor antibodies: structure-function relationships. *Ciba Found. Symp.* **90**:82–90 (1982).
25. Regunathan, S., M. P. Meeley, and D. J. Reis. Expression of non-adrenergic imidazoline sites in chromaffin cells and mitochondrial membranes of bovine adrenal medulla. *Biochem. Pharmacol.*, in press.
26. Reis, D. J., S. Regunathan, H. Wang, D. L. Feinstein, and M. P. Meeley. Imidazoline receptors in the nervous system. *Fundam. Clin. Pharmacol.* **6** (Suppl. 1):23s-29s (1992).
27. Ruggiero, D. A., T. A. Milner, H. Wang, S. Regunathan, and D. J. Reis. Imidazoline receptor-associated binding protein in central nervous system. *Soc. Neurosci. Abstr.* **18**:541 (1992).
28. Mallard, N. J., A. L. Hudson, and D. J. Nutt. Characterization and autoradiographical localization of non-adrenoceptor idazoxan binding sites in rat brain. *Br. J. Pharmacol.* **106**:1019–1027 (1992).

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