Characterization of an Imidazoline/Guanidinium Receptive Site Distinct from the $\alpha_2$-Adrenergic Receptor*

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$\alpha_2$-Adrenergic receptors recognize a number of molecules with diverse chemical structures, including the yohimbine diastereomers yohimbine and rauwolscine, catecholamines, guanidinium analogs, and imidazolines, such as clonidine. The affinity of the receptor protein for some of these ligands can vary by 100-fold among various tissues and species, suggesting a heterogeneous class of binding sites. Certain cellular effects elicited by the compounds possessing an imidazoline or guanidinium moiety may actually be mediated by a membrane receptor distinct from the $\alpha_2$-adrenergic receptor. To determine whether this imidazoline/guanidinium receptive site (IGRS) and the $\alpha_2$-adrenergic receptor represent distinct proteins, we solubilized and partially characterized the two binding sites in rabbit kidney. This tissue expresses both $\alpha_2$-adrenergic receptors and high affinity imidazoline/guanidinium binding sites, the latter which are rauwolscine-insensitive but can be identified with the benzodioxan $^3$H]idazoxan. The IGRS and $\alpha_2$-adrenergic receptor in rabbit kidney exhibit distinct ligand recognition properties, which are maintained after solubilization and partial purification. In addition, the two receptors can be physically separated by heparin-agarose or lectin affinity chromatography indicating that the two binding sites are distinct entities. $^3$H]Idazoxan binding is trypsin-sensitive, indicating that the IGRS is a protein rather than a lipid component of the plasma membrane. $^3$H]Idazoxan binding is not inhibited by endogenous agonists for known neurotransmitter receptors. However, the IGRS does recognize clonidine-displacing substance, a small non-catechol compound isolated from calf brain, suggesting the existence of a previously uncharacterized hormonal/neurotransmitter receptor system.

Clonidine, guanabenz, and structurally related imidazolines

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guanidinium compounds elicit a wide variety of responses in both the central nervous system and peripheral tissues (1-4). Both functional and radioligand binding studies indicate that many of the cellular effects of these compounds are due to activation of $\alpha_2$-adrenergic receptors, membrane proteins involved in sympathetic neurotransmission. In some tissues, however, high affinity binding of imidazoline derivatives is insensitive to both rauwolscine, a selective $\alpha_2$-adrenergic receptor antagonist, and to the catecholamines epinephrine and norepinephrine (5, 6). In addition, the membrane receptor that mediates the effector cell response to these imidazoline/guanidinium compounds in certain tissues does not exhibit the pharmacologic profile expected of an $\alpha_2$-adrenergic receptor (7-12). These observations suggest the existence of a specific and functional imidazoline/guanidinium receptive site (IGRS).

We recently identified such a binding site in rabbit renal cortex utilizing $^3$H]Idazoxan, a benzodioxan possessing an imidazoline substituent. In the present study we describe the solubilization and partial purification of the IGRS and characterize its ligand recognition properties after physical separation from the $\alpha_2$-adrenergic receptor. Although the IGRS does not recognize endogenous ligands for known membrane-receptor proteins, IGRS does recognize clonidine-displacing substance, a small non-catechol, non-protein compound isolated from bovine brain.

EXPERIMENTAL PROCEDURES

Materials—[$^3$H]Idazoxan (60 Ci/mmol) and [$^3$H]rauwolscine (80 Ci/mmol) were obtained from Amersham International and Du Pont-New England Nuclear, respectively. Wheat germ lectin-agarose was from Vector Laboratories (Burlingame, CA). Trypsin (Type XIII), heparin-agarose (Type II), histamine, serotonin, dopamine, (-)epinephrine, yohimbine, N-acyetylglucosamine, guanabenz, and amiloride were obtained from Sigma. Digitonin (Lot No. 1144) was purchased from Gallard-Schlesinger Industries, Inc. (Carle Place, NY). Prazosin and UK14304 were a gift from Pfizer (Groton, CT). Cirazoline was a gift from Synthelabo (Paris, France), (±)-idazoxan and (−/-)-idazoxan were obtained from Servier Laboratories (Neuilly sur-Seine, France). All other materials were obtained as described previously (6, 13).

Preparation of Membranes—Male New Zealand White rabbits, weighing 1.5-2.5 kg, were anesthetized with pentobarbital. The kidneys were placed in ice-cold buffer containing 250 mM sucrose, 0.1 mM phenylmethysulfonyl fluoride, and 2 mM Tris-Hepes, pH 7.4, at 4°C. The renal cortex was disrupted with a Dounce homogenizer and then with a Teflon-glass homogenizer (1,200 rpm). After filtration

1 The abbreviations used are: IGRS, imidazoline/guanidinium receptive site; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenebis(oxyethylendinitril)tetraacetic acid; HPLC, high performance liquid chromatography.
through two layers of cheesecloth mesh, homogenized material was centrifuged at 450 × g for 10 min (Sorvall RC5B, type H-4), resulting in a supernatant containing 28,000 × g for 30 min to obtain the membrane pellet, which was washed twice and resuspended by homogenization in 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, and 5 mM Tris-HCl, pH 7.4. Membrane preparations were frozen in liquid nitrogen and stored at -80 °C. This crude membrane preparation was utilized as a source of solubilized receptor protein.

A purified membrane preparation of the basolateral surface of the renal proximal tubule was utilized to anotomically localize the IGRS and the α2-adrenergic receptor and to define the ligand recognition properties of the two binding sites. Purified basolateral membranes were prepared by density gradient centrifugation as described previously (6, 14). The specific activity of Na+/K+-ATPase in the purified membrane preparation (1.56 ± 0.4 μmol/mg protein/min) was increased 15-fold above the enzyme activity observed in crude membranes (0.1 ± 0.2 μmol/mg protein/min). Membrane protein was quantitated with a Bio-Rad protein assay kit. The density of membrane binding sites for [3H]rauwolscine (Kd = 11.5 ± 1.5 nM) and [3H]idazoxan (Kd = 1.45 ± 0.1 nM) in purified basolateral membranes was 155 ± 28 and 566 ± 118 fmol/mg membrane protein, respectively (n = 6).

**Binding Studies**—Radioligand binding assays in membranes were performed in a total volume of 50 μl with 150 μg of membrane protein. Membranes, competing ligand or buffer, and radioligand ([3H]idazoxan, 4 nM; [3H]rauwolscine, 10 nM) were incubated with shaking at 20 °C for 45 min and bound radioligand separated from free ligand as described previously (6, 14).

For solubilized receptor binding assays, increasing concentrations of competing ligand were incubated in a final volume of 500 μl containing 400 μl of solubilized receptor, 50 μl of radioligand, and 50 μl of buffer (or competing ligand) for 2 h at 4 °C (15). Incubation for longer time periods indicated that a 2-h incubation was sufficient to attain equilibrium of the binding interaction. Bound ligand was separated by precipitation with bovine γ-globulin/polyethylene glycol followed by vacuum filtration.

The filters from membrane and soluble binding assays were placed in 5 ml of Optifluor scintillation fluid and counted in a liquid scintillation spectrometer (LKB, model 1209 Rackbeta) with a counting efficiency of 58%. For the IGRS, nonspecific binding of [3H]idazoxan was determined in the presence of 1 μM cirazoline and represented 15–20% of total binding at a radioligand concentration of 4 nM in both membrane and solubilized preparations. The same amount of nonspecific binding was obtained with 10 μM tolazoline and 1 μM guanabenz. For the α2-adrenergic receptor, nonspecific binding of [3H]rauwolscine was determined in the presence of 10 μM phenolamine. Identical levels of nonspecific binding were obtained in the presence of 10 μM rauwolscine. IC50 values were defined as the concentration of competing ligand required to inhibit 50% of bound [3H]idazoxan (15). Kd values were defined with a nonlinear, iterative, computer fitting procedure and expressed as the mean ± S.E. (16). Saturation binding isotherms were analyzed by the method of Scatchard (17). Binding studies with [3H]idazoxan were performed in the presence of rauwolscine to prevent binding of the radioligand to α2-adrenergic receptors.

**Solubilization of Membranes and Chromatographic Techniques**—Crude membranes were solubilized at 4 °C for 15 min in a buffer containing 1% (w/v) digitonin, 2 mM EGTA, 100 mM NaCl, and 50 mM Tris-HCl, pH 7.6, at a detergent-to-protein ratio of 3:1 (w/v). The soluble fraction was then isolated by centrifugation at 100,000 × g for 1 h as described previously (13).

The heparin-agarose resin (1.14 × 7 cm, ~7 ml) was equilibrated with 0.5% digitonin, 2 mM EGTA, 100 mM NaCl, and 50 mM Tris-HCl, pH 7.6, at 4 °C. Three column volumes of solubilized material were then added to the column at a flow rate of 1.5 column volumes h⁻¹. The column was then washed with 50 column volumes (5 column volumes h⁻¹) of 0.2% digitonin, 2 mM EGTA, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.6, after which the absorbance of the flow-through was 0 at 280 nm. Proteins retained on the column were eluted with washing containing 700 mM NaCl at a flow rate of 2 column volumes h⁻¹ (13).

For wheat germ lectin-agarose chromatography, maenasium chloride was added to 10 mM in the solubilized material. One ml of lectin resin was initially equilibrated with 20 column volumes of 0.2% digitonin, 100 mM NaCl, and 50 mM Tris-HCl, pH 7.6 (Buffer A), followed by 5 column volumes of the same buffer containing 10 mM MgCl₂. The solubilized receptor preparation was then loaded onto the resin by gravity flow, and the resin was subsequently washed with 10 column volumes of Buffer A containing 10 mM MgCl₂ followed by 5 column volumes of Buffer A containing no MgCl₂. Bound glycoproteins were eluted by batch technique with 3 column volumes of the second wash buffer containing 300 mM N-acetylgalucosamine (13).

**Isolation of the Clonidine-displacing Substance**—The clonidine-displacing substance was purified from calf brain as described previously (18). Briefly, slices of calf brain were homogenized with a Brinkman Polytron (setting 9, 25 s) in 3 volumes (w/v) of 10 mM Tris-HCl, pH 7.4, at 4 °C. The supernatant obtained from centrifugation (100,000 × g, 30 min, 4 °C) of the homogenate was boiled for 15 min and aggregated material pelleted by centrifugation at 100,000 × g (15 min, 4 °C). The supernatant was lyophilized and the resulting material extracted with 20 volumes (v/v) of methanol. The methanolic extract was filtered and then concentrated by rotoevaporation. Approximately 3 g of lyophilized material was obtained from 500 g (wet weight after removal of cerebellum) of calf brain tissue. Approximately 3,000 units of clonidine-displacing substance were obtained by methanol extraction of 3 g of lyophilized material as determined in 20 different preparations.

The clonidine-displacing substance was quantitated as described previously (18). One unit of activity is defined as the amount needed to displace 50% of specifically bound [3H]clonidine (2 nM) in rat brain membrane preparations. Nonspecific binding of [3H]clonidine was determined in the presence of 10 μM norepinephrine.

The clonidine-displacing substance found in the methanolic extract was further purified by ion-exchange chromatography, zone electrophoresis and performance liquid chromatography (HPLC) (18). Approximately 30% of the activity found in the methanol extract was recovered after the final HPLC step. Biologically active fractions obtained after HPLC were ninhydrin-negative, fluorescamine-negative, and yielded no free amino acid after acid hydrolysis.

**RESULTS AND DISCUSSION**

[3H]Idazoxan exhibits high affinity and selectivity for the α2-adrenergic receptor (19) in several tissues, but in purified basolateral membranes from rabbit kidney cortex only 20–30% of its binding is inhibited by epinephrine or by rauwolscine, a selective α2-adrenergic receptor antagonist (6). However, 80% of [3H]idazoxan binding in this tissue is inhibited by high potency by α-adrenergic receptor ligands possessing an imidazoline or guanidinium moiety. To determine the ligand recognition properties of the IGRS, we performed competition binding studies with [3H]idazoxan after saturating α2-adrenergic receptor binding sites with 10 μM rauwolscine.

Although both the α2-adrenergic receptor and the IGRS recognized the imidazoline/guanidinium compounds, the IGRS recognized them with a rank order of affinity different from that exhibited by the α2-adrenergic receptor (Table I). [3H]Idazoxan binding was not inhibited by epinephrine, serotonin, or dopamine at competing ligand concentrations of 10 μM. The specificity of ligand recognition by IGRS is also demonstrated by the stereoselectivity of idazoxan binding. In competition binding studies with [3H]idazoxan, the racemic modification ((±)idazoxan) exhibited an affinity 10-fold higher than the (−)-stereoisomer (Table I). [3H]Idazoxan binding is not inhibited by the selective α1-adrenergic receptor agonist methoxamine and phenylephrine or by the H2-receptor antagonist cimetidine. Prazosin, a selective α1-adrenergic receptor antagonist, exhibited 10,000-fold lower affinity at IGRS (Kd = 1.014 ± 283 nM) than that observed at α1-adrenergic receptors (1). Therefore, IGRS exhibited ligand recognition properties distinct from those of adrenergic receptors and other known hormone receptors.

We further characterized the IGRS and to determine whether the α2-adrenergic receptor and the IGRS are distinct membrane proteins, we solubilized and partially purified both binding sites from crude rabbit renal cortex membranes. α2 Adrenergic receptors and IGRS were identified with [3H] rauwolscine and [3H]idazoxan, respectively. [3H]Idazoxan
were recovered in the solubilized material. Competition binding of [3H]idazoxan with a high salt buffer as described for the adrenergic action, resulted in the separation of [3H]idazoxan and [3H]affinity matrix, which primarily binds proteins by ionic interaction from the heparin-agarose affinity matrix. [3H]Rauwolscine binding could be eluted from the heparin-agarose column. All of which readily adsorb to a heparin-agarose affinity matrix at a flow rate of 1.5 column volumes h⁻¹. The eluted fraction was inhibited by adrenergic receptor agonists and antagonists with a rank order of potency consistent binding could not be dissociated from the membrane with high salt (600 mM KCl, 10 mM EDTA) washes suggesting that IGRS is an intrinsic membrane protein. Utilizing the nonionic detergent digitonin, approximately 30% of the IGRS and 20% of the α₂-adrenergic receptor found in membrane preparations were recovered in the solubilized material. Competition binding studies indicated that the binding of [3H]idazoxan was inhibited by various compounds with the same rank order of potency (cirazoline (Kᵢ = 2.6 nM) > idazoxan (Kᵢ = 4 nM) > guanabenz (Kᵢ = 14 nM) > amiloride (Kᵢ = 80 nM) > tolozolmine (Kᵢ = 110 nM) > UK14304 (Kᵢ = 1,400 nM)) as observed in membrane preparations (Table I) (6).

In initial studies to characterize the structural properties of the IGRS, we found that the IGRS and the α₂-adrenergic receptor could be physically separated on the basis of differences in charge and extent of protein glycosylation. Passage of a solubilized membrane preparation over a heparin-agarose affinity matrix, which primarily binds proteins by ionic interaction, resulted in the separation of [3H]idazoxan and [3H]rauwolscine binding (Fig. 1A). The α₂-adrenergic receptor was retained by the heparin-agarose resin, but [3H]idazoxan binding to IGRS appeared in the fall-through fraction. The IGRS was enriched 5-fold by this affinity step. The inability of IGRS to interact with the heparin-agarose affinity matrix contrasts with our previous experience with not only the α₂-adrenergic receptor, but also other adrenergic receptor subtypes (α₁, β₁, β₂), all of which readily adsorb to a heparin-agarose resin (13, 20). [3H]Idazoxan binding in the fall-through fraction was inhibited by a number of ligands (Fig. 1B) with a rank order of potency similar to that expected for the IGRS (Ref. 6, Table I). Neither rauwolscine (10 μM) nor (−)-epinephrine (100 μM) competed for [3H]idazoxan binding in the fall-through fraction from the heparin-agarose affinity matrix. [3H]Rauwolscine binding could be eluted from the heparin-agarose column with a high salt buffer as described for the α₂-adrenergic receptor in other tissues (13, 20). α₂-adrenergic receptor agonists and antagonists with a rank order of potency consistent with these results are listed in Table I. The inability of rauwolscine to interact with the heparin-agarose affinity matrix contrasts with our previous experience with not only the IGRS but also other adrenergic receptor subtypes. The inability of [3H]idazoxan binding to IGRS in the fall-through fraction from the heparin-agarose affinity matrix. The data represent the average of triplicate determinations obtained in three to six experiments with three different membrane preparations. ND, not determined.

### Table I

<table>
<thead>
<tr>
<th>Competing ligand</th>
<th>α₂-Adrenergic receptor</th>
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<tbody>
<tr>
<td>Cirazoline</td>
<td>0.8 ± 0.12</td>
</tr>
<tr>
<td>(±)-Idazoxan</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Guanabenz</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>(−)-Idazoxan</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>30 ± 4.1</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>2,800 ± 265</td>
</tr>
<tr>
<td>Histamine</td>
<td>10,000 ± 950</td>
</tr>
<tr>
<td>BHT-920</td>
<td>=</td>
</tr>
<tr>
<td>(−)-Epinephrine</td>
<td>102 ± 64</td>
</tr>
</tbody>
</table>

* No inhibition of [3H]idazoxan binding was observed at competing ligand concentrations of 10 μM.

![Fig. 1. A, separation of IGRS from α₂-adrenergic receptors (α₂-AR) on a heparin-agarose affinity matrix. In this experiment, 21 ml of solubilized preparation was applied on a 7-ml column of a heparin-agarose affinity matrix at a flow rate of 1.5 column volumes h⁻¹. The 21 ml of solubilized preparation contained ~6,300 fmoles of IGRS and ~900 fmoles of α₂-adrenergic receptor. The fall-through from the resin contained ~180 fmoles of α₂-adrenergic receptor and ~5,980 fmoles of IGRS. Seventy-seven percent of the α₂-adrenergic receptor retained by the resin could be recovered by elution with a high salt buffer as described for the α₂-adrenergic receptor in other tissues (13, 20). Binding activity was determined as described under "Experimental Procedures" utilizing 10 nM [3H]idazoxan for IGRS and 20 nM [3H]rauwolscine for α₂-adrenergic receptors. The results shown are representative of six experiments utilizing six different solubilized preparations. B, pharmacological specificity of solubilized IGRS binding in the fall-through fraction from the heparin-agarose affinity matrix. This fraction is devoid of the α₂-adrenergic receptor, which was retained by the heparin-agarose resin. Aliquots (400 μl) of the fall-through material were incubated with 4 nM [3H]idazoxan and increasing concentrations of competing ligand as described under "Experimental Procedures." O, cirazoline; □, guanabenz; Δ, tolozolmine; ●, UK14304. Nonspecific binding was determined in the presence of 1 μM clonidine and represented ~20% of total radioligand bound. The results shown are the average of triplicate determinations and are representative of six separate experiments performed with six different solubilized preparations. C, effect of trypsin on the binding of [3H]idazoxan to IGRS in the fall-through fraction from the heparin-agarose affinity matrix. Approximately 3,600 fmoles of IGRS from the fall-through fraction was incubated with or without 0.1% (w/v) trypsin at 20 or 37°C in a total volume of 10 ml for 10 min. The reaction was terminated by the addition of a 10-fold excess of soybean trypsin inhibitor (SBTT) and by cooling in ice-cold water at 4°C prior to radioligand binding assays. In these experiments equilibrium binding was determined as described under "Experimental Procedures." 0, α₂-AR; □, control; △, trypsin; ●, trypsin + SBTT.

S. M. Lanier, unpublished observations.
with an \( \alpha_2 \)-adrenergic receptor binding site.

To determine if ligand recognition by IGRS is sensitive to limited proteolysis, the fall-through fraction from the heparin-agarose resin was exposed to the serine proteinase trypsin. As shown in Fig. 1C, \([3H]\)idazoxan binding is reduced by trypsin treatment in a temperature-dependent manner (control at 20°C: \( B_{max} = 183 \) fmol/mg, \( K_D = 4.7 \) nM; trypsin-treated at 20°C: \( B_{max} = 112 \) fmol/mg, \( K_D = 13.8 \) nM; control at 37°C: \( B_{max} = 237 \) fmol/mg, \( K_D = 4.8 \) nM; trypsin-treated at 37°C: \( 80 \) fmol/mg, \( K_D = 9.9 \) nM). This finding indicates that IGRS is a protein and not a lipid component of the membrane. Ligand recognition by adrenergic receptors is, in contrast, resistant to trypsin digestion likely due to the existence of the ligand binding domain as a hydrophobic pocket in the membrane bilayer (21-27). These data again suggest that the IGRS differs structurally from adrenergic and related G-protein coupled receptors.

In porcine brain, human platelet, and rabbit kidney the \( \alpha_2 \)-adrenergic receptor is apparently glycosylated via oligosaccharides \( N \)-linked to asparagine residues (13, 20, 28-30). To determine whether the IGRS is similarly glycosylated, the crude solubilized rabbit kidney preparation was applied to a wheat germ lectin resin. Whereas the \( \alpha_2 \)-adrenergic receptor readily adsorbed to the lectin resin, the IGRS was found in the fall-through fractions (Fig. 2). Similarly, the IGRS in the fall-through fraction from the heparin-agarose resin did not adsorb to the lectin resin. These data indicate that the IGRS is apparently devoid of the complex- or hybrid-type \( N \)-linked oligosaccharides that are required for adsorption by the wheat germ lectin resin. Passage of either the crude solubilized material or the post-heparin material over the lectin resin resulted in a 10-fold purification of the IGRS. Again, \([3H]\) idazoxan binding was inhibited by the various ligands with a rank order of potency expected for the IGRS.

These data indicate that the IGRS and the \( \alpha_2 \)-adrenergic receptor are distinct membrane-receptor proteins that differ in their ligand recognition properties, in their associated glycan moieties, and in their biophysical properties. It is not clear what effector cell response is mediated by the IGRS, although it may play a role in central nervous system control of blood pressure, acid secretion by parietal cells and/or the regulation of electrolyte and water absorption in the gut and kidney (7, 8, 11, 12, 31). Preliminary studies indicate that in isolated rabbit proximal tubule cells cirazoline and idazoxan modulate \(^{22}\)Na influx by a mechanism that is independent of the \( \alpha_2 \)-adrenergic receptor. The precise mechanism involved is under investigation. Neither of these compounds affect the epithelial sodium channel in toad bladder, and the effect of these compounds on \(^{22}\)Na influx occurs in the presence of an inhibited Na\(^+\)/K\(^+\)-ATPase.

As \([3H]\)idazoxan binding to IGRS is insensitive to epinephrine and norepinephrine, these catecholamines cannot be physiological agonists for the IGRS. One potential endogenous agonist at the IGRS is clonidine-displacing substance, a small non-protein, non-catechol compound partially purified from calf brain (18). This material contracts gastric fundus smooth muscle (32), inhibits the twitch response of rat vas deferens (33) and produces centrally mediated effects on

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**Footnotes:**


Preincubation of trypsin with soybean trypsin inhibitor (1%, w/v) prevented the effect of trypsin on IGRS binding (TRYPsin + STII).

These results are the average values of triplicate determinations and are representative of three separate experiments performed with two different solubilized receptor preparations.


systemic blood pressure (8, 34), all of which appear unrelated to α2-adrenergic receptor activation. As shown in Fig. 3, the clonidine-displacing substance effectively inhibited [3H]imidazoxan binding after physical separation of IGRS from the α2-adrenergic receptor. These observations suggest that the IGRS may actually be the membrane receptor mediating the actions of the clonidine-displacing substance. The identification of IGRS as a membrane protein that is distinct from the α2-adrenergic receptor and that recognizes an endogenous clonidine-displacing substance should facilitate the localization and functional characterization of this potential hormonal/neurotransmitter-receptor system.

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REFERENCES


Imidazoline/Guanidinium Receptive Site