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Development of a High-Affinity Radioiodinated Ligand for Identification of Imidazoline/Guanidinium Receptive Sites (IGRS): Intratissue Distribution of IGRS in Liver, Forebrain, and Kidney

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SUMMARY

Imidazoline/guanidinium receptive sites (IGRS) are membrane proteins that exhibit high affinity for various compounds with an imidazoline or guanidinium moiety. The structure of these binding sites and their significance in the broad pharmacological action of such ligands are unclear. To address this issue, we developed selective high affinity compounds that could be radioiodinated and used as molecular probes for structural characterization of these proteins. This report describes the synthesis and characterization of such a molecule, 2-(3-amino-4-[^{125}I]iodophenoxy)methylimidazoline ([^{125}I]AMIPI). [^{125}I]AMIPI is structurally related to cirazoline, an imidazoline exhibiting high affinity for IGRS and the family of related imidazoline binding sites. The phenyl-substituted analogue of cirazoline, 2-(3-aminophenoxy)methylimidazoline, was generated by alkylation of acetamidophenol with 2-chloromethylimidazoline. 2-(3-Aminophenoxy)methylimidazoline exhibited high affinity for IGRS in rabbit kidney membranes, as determined in competition binding studies with [^{3}H]idazoxan (K_i = 12.5 ± 7.5 nM), and was radioiodinated by chloramine-T oxidation to yield [^{125}I]AMIPI. The binding properties of [^{125}I]AMIPI were determined in membranes prepared from two representative tissues, rabbit kidney cortex and rat liver. Specific binding of [^{125}I]AMIPI was saturable and of high affinity, as determined by Scatchard analysis of saturation binding isotherms (rabbit kidney, K_d = 2.0 ± 0.9 nM, B_{max} = 554 ± 201 fmol/mg, five experiments; rat liver, K_d = 2.6 ± 1.3 nM, B_{max} = 73 ± 10 fmol/mg, three experiments). [^{125}I]AMIPI binding in rabbit kidney membranes was inhibited by various imidazolines and guanidinium compounds, with the following rank order of potency: cirazoline (K_i = 3.6 ± 0.96 nM) > idazoxan (high-affinity site K_i = 0.2 ± 0.1 nM and low-affinity site K_i = 76 ± 30 nM) > guanabenz (high-affinity site K_i = 1.7 ± 0.84 nM and low-affinity site K_i = 201 ± 72.7 nM) > amiloride (K_i = 625 ± 130 nM) > clonidine (K_i = 2200 ± 200 nM) > *p*-aminoclonidine (K_i = 3422 ± 172 nM). [^{125}I]AMIPI binding was not inhibited by the α_2-adrenergic receptor antagonist rauwolscine or endogenous agonists for neurotransmitter receptors (epinephrine, histamine, serotonin, and dopamine). The rank order of competing ligands is consistent with the definition of the [^{125}I]AMIPI binding site as an IGRS. Receptor autoradiography was used to determine the intratissue distribution of IGRS in rat liver, rat forebrain, and canine kidney. Autoradiograms indicated homogeneous specific binding of [^{125}I]AMIPI in liver. Renal [^{125}I]AMIPI binding was observed as discrete cortical rays. Autoradiograms of rat forebrain tissue sections indicated high densities of specific binding in the hypothalamic arcuate nucleus and the subfornical organ. [^{125}I]AMIPI binding was inhibited by both imidazoline and guanidinium ligands but was not competed for by the selective α_2-adrenergic receptor antagonist rauwolscine. These data indicate that [^{125}I]AMIPI is a high affinity probe that is specifically recognized by the IGRS and thus should facilitate pharmacological and molecular characterization of this entity.

The IGRS is a membrane protein (1–6) recognized by a variety of drugs that affect various aspects of metabolic and cardiovascular function (7–17). These compounds include the α_1-AR agonist/α_2-AR antagonist cirazoline, the α_2-AR antagonist idazoxan, the α_2-AR agonist guanabenz, the ion transport inhibitor amiloride, and other structurally related ligands. The interest in IGRS and other related binding proteins is based on the following: 1) the fact that the diverse effects elicited by these molecules are often difficult to pharmacologically define in the context of known neurotransmitter/hormonal receptor systems, 2) the physical dissociation of IGRS from α-AR (1, 18), and 3) the possible existence of a bioactive endogenous substance that is recognized by these binding sites (19–23).

Recent reports indicate the existence of multiple membrane binding sites for ligands of this class that differ in their ligand recognition properties, their apparent molecular weights, and possibly their subcellular/tissue distribution (3, 24, 25). These sites are often grouped under the general term of imidazoline

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ABBREVIATIONS: IGRS, imidazoline/guanidinium receptive site(s); AR, adrenergic receptor(s); AMPI, 2-(3-aminophenoxy)methylimidazoline; AMIPI, 2-(3-amino-4-[^{127}I]iodophenoxy)methylimidazoline; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SFO, subfornical organ; ARC, arcuate nucleus; [^{125}I]AZIPI, 2-(3-azido-4- [^{125}I]iodophenoxy)methylimidazoline.

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"receptors" (26); however, most of these sites also recognize guanidinium ligands. It is not clear whether the binding sites identified to date actually represent a receptor molecule, in terms of the inherent ability of such an entity to transduce a signal. Imidazoline receptors are further subgrouped as I_1 and I_2 receptors, with the latter being most clearly related to IGRS (26, 27). The radioligands [^{3}H]clonidine, [^{125}I]-clonidine, *p*-[^{3}H] aminoclonidine, [^{3}H]rilmenidine, and [^{3}H]moxonidine are used to identify I_1 receptors (28–30) and [^{3}H]idazoxan is the apparent ligand of choice for I_2 receptors and IGRS (2, 5, 31, 32). The utility of all of these ligands is compromised by their interaction with known receptor systems. The relatively low density of I_1 receptors further complicates their identification with available tools.

The structure of IGRS, its intratissue distribution, and its functional significance remain undefined, due in part to the lack of high affinity, IGRS-selective molecules. An additional unresolved point concerns the subcellular distribution of IGRS, which is found at high density in liver mitochondria (3) and is suggested to be in an intracellular membrane fraction in platelets (18). To facilitate efforts to address these issues, we undertook the development of ligands that could be utilized as molecular probes selective for IGRS. This report describes the synthesis and characterization of one such molecule, [^{125}I]AMIPI.

Experimental Procedures

Materials

[^{3}H]Idazoxan (40 Ci/mmol) and carrier-free Na^{125}I were purchased from Amersham International and NEN/DuPont (Boston, MA), respectively. Idazoxan, guanabenz, amiloride, 5-(*N*,*N*-hexamethylene)amiloride, 5-(*N*-methyl-*N*-isobutyl)amiloride, clonidine, *p*-aminoclonidine, and UK 14,304 were provided by Research Biochemicals Int. (Natick, MA). Cirazoline was a gift from Synthelabo (Paris, France). Rauwolscine was purchased from Atomergic Chemetals (Farmingdale, NY). Prazosin was a gift from Dr. Hess (Pfizer, Groton, CT). (−)-Epinephrine, phenylmethylsulfonyl fluoride, Tris, dithiothreitol, ammonium persulfate, and sodium dodecyl sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide, bisacrylamide, and electrophoresis-grade Tris were purchased from Bio-Rad (Richmond, CA). Glass fiber filters (no. 32) were from Schleicher and Schuell (Keene, NH), and Ecoscint A was from National Diagnostics (Manville, NJ). Reverse phase Unibond silica gel plates (HPTLC-RP18F) were purchased from Analtech (Newark, DE). Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA) and New Zealand White rabbits from Rabbits Ltd. (Somerville, SC). Frozen rabbit kidney cortex was purchased from Pel-Freez (Rogers, AR).

Membrane Preparations

Male New Zealand white rabbits, weighing 2.5–3.5 kg, were anesthetized with pentobarbital. Kidneys were immediately placed in buffer A (5 mM EDTA, 5 mM EGTA, 0.01 mM phenylmethylsulfonyl fluoride, 5 mM Tris, pH 7.4, 4°). The kidney cortex (~2 g) was minced and homogenized by eight passes in a Dounce homogenizer. The homogenate was filtered through two layers of cheesecloth mesh and centrifuged at 1000 × *g* for 10 min at 4° (Sorvall RC-5B, type SS34 rotor) to yield a low-speed pellet (containing nuclei, mitochondria, and undisrupted membrane aggregates). The supernatant was decanted and centrifuged at 47,800 × *g* for 10 min at 4° to yield a high-speed pellet that was generally used for analysis of plasma membrane receptors. Both pellets were washed twice (40 ml) and resuspended by homogenization in ice-cold buffer B (0.6 mM EDTA, 5 mM $MgCl_2$, 0.01 mM phenylmethylsulfonyl fluoride, 50 mM Tris·HCl, pH 7.4), at a protein concentration of 1.5–4 mg/ml. Crude rat liver membranes were prepared as described above and were resuspended in buffer B at a protein concentration of 3–5 mg/ml.

Binding Studies

Radioligand binding studies were performed as described previously (33). In saturation binding studies, increasing concentrations of [^{3}H] idazoxan and [^{125}I]AMIPI were incubated in a total volume of 100 μl (25–150 μg of membrane protein) for 45 min at 24°. Nonspecific binding was determined in the presence of 10 μM cirazoline and represented 15–25% of total binding. Similar degrees of nonspecific binding were defined with 10 μM idazoxan or guanabenz (17–30%). Membranes used in studies with [^{3}H]idazoxan were preincubated with 10 μM rauwolscine to prevent [^{3}H]idazoxan binding to α_2-AR. In competition binding studies, membranes (50–100 μg of protein) were incubated with [^{125}I] AMIPI (0.9–2 nM) and increasing concentrations of competing ligands at 24°. After a 45-min incubation, binding reactions were terminated by addition of 4 ml of wash buffer (100 mM Tris·HCl, pH 7.4) followed by rapid filtration over glass fiber filters. Filters were washed with three 4-ml aliquots of the same buffer (4° for [^{3}H]idazoxan binding or 24° for [^{125}I]AMIPI binding). The [^{125}I]AMIPI radioactivity retained by the filters was determined in a γ counter (model 1261; LKB Wallac) with a counting efficiency of 80%. Filters with ^{3}H-labeled samples were placed in 7 ml of Ecoscint scintillation fluid and counted in a liquid scintillation counter with 50% efficiency. Binding data were analyzed using the nonlinear, least-squares, curve-fitting procedure LIGAND (34). All binding studies were performed in fresh membrane preparations, after determination of the protein concentration by the method of Lowry *et al.* (35).

Synthetic Procedures

Generation of substituted phenoxymethylimidazoline compounds. The imidazoline derivatives RBI-103 [2-(ethyl-5-methoxyphenyl)methylimidazoline], RBI-100 [2-(*p*-acetamidophenoxy)methylimidazoline], and RBI-101 [2-(*m*-acetamidophenoxy)methylimidazoline] were prepared by alkylation of the respective phenol with 2-chloromethylimidazoline hydrochloride in the presence of sodium ethoxide in ethanol. After 16 hr of reflux, the products were isolated as the hydrochloride salts. Alkylation of *m*-acetamidophenol in dimethylformamide yielded the hydrolyzed acetamide product, AMPI (RBI-102) (see Fig. 2).

Synthesis of [^{125}I]AMIPI. Carrier-free Na^{125}I (3 mCi in 30 μl of dilute NaOH) was added to AMPI (~20 μg, dissolved in 30 μl of 0.5 M sodium acetate buffer, pH 5.2). The reaction was initiated by the addition of 6 μl of chloramine-T (1 mg/ml in water, 21.5 nmol). After 1 min the reaction was quenched by addition of 8 μl of sodium metabisulfite (1 mg/ml in water, 42 nmol). The reaction mixture was basified with 20 μl of 1 M NaOH, extracted with ethyl acetate (3 × 300 μl), concentrated, and applied to a reverse phase high performance thin layer chromatography plate (HPTLC-RP18F, octadecyl-modified silica gel, 10 × 20 cm). The plate was developed in methanol/universal (Britton-Robinson) buffer (50:50) at pH 7.0 (36). The universal buffer consisted of 0.2 M NaOH, 0.04 M CH_3COOH, 0.04 M H_3PO_4, and 0.04 M H_3BO_3. The major radioactive spot (R_F = 0.27) was scraped, extracted with methanol (3 × 400 μl), and stored at −20°. The purified radioiodinated species comigrated with the nonradioactive iodinated analogue AMIPI (R_F = 0.27). The major radiolabeled product also comigrated with AMIPI by thin layer chromatography on silica gel 60 plates (EM Reagents), in two different developing systems (ethyl acetate/isopropanol/H_2O/NH_4OH, 50:30:16:4, R_F = 0.59; CH_2Cl_2/methanol/ammonium hydroxide, 90:9:1, R_F = 0.14). The nonradioactive iodinated compound AMIPI was prepared by reaction of AMPI with iodine monochloride in acetic acid at 10°. AMIPI was purified by high performance liquid chromatography on a Waters Bondapak C-18 column, using 30% acetonitrile in phosphate buffer (0.05 M KH_2PO_4) as the eluent.

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Autoradiography of Tissue Sections

Specimens of rat forebrain were obtained from 11 Sprague-Dawley rats (five male and six female, 200–250 g) immediately after decapitation. Canine kidney was obtained from a male animal, and rat liver was from two female Sprague-Dawley rats (200–250 g). Specimens were frozen in dry ice or liquid nitrogen and stored at −70° until processed for autoradiography. The basic autoradiographic technique has been described in detail previously (37–39). Frozen tissues were sectioned at 14 μm on a cryostat. A series of consecutive adjacent sections was mounted on Chrom-Alum-coated glass slides and incubated in membrane buffer (50 mM Tris·HCl, pH 7.4, 0.6 mM EDTA, 5 mM $MgCl_2$) at 25° for 30–40 min. One set of sections was then incubated for 40 min in the same buffer with 0.2–1.2 nM [^{125}I]AMIPI (~2200 Ci/mmol). Additional sets of adjacent sections were incubated with [^{125}I]AMIPI in the presence or absence of cirazoline, clonidine, idazoxan, rauwolscine, or guanabenz (10 μM). Labeled sections were rinsed with fresh buffer for 3 min at 25°, dipped in water at 4°, dried under a cooled and desiccated stream of air, and opposed to SB-5 film (Kodak, Rochester, NY) in cassettes for 1–3 days.

The density of binding sites was estimated by densitometric analysis of autoradiograms of tissue sections and ^{14}C-labeled standards (40). Standards were exposed with each cassette of tissue sections, and different exposure times were used to obtain accurate readings for high, moderate, and low densities of binding according to the linear range of the film. Absorbance values for standards and regions of interest were measured using computerized microdensitometry on an image analysis system (Micro Computer Imaging Device; Imaging Research, Ontario, Canada). Results were corrected for ^{125}I decay. Densitometric measurements were taken from at least two and as many as four different tissue sections in each animal in the absence or presence of each competitor. The percentage inhibition by various competing ligands is presented as mean ± standard error.

Results

Synthesis and binding properties of phenoxymethylimidazoline analogues. The imidazoline and guanidinium analogues in Fig. 1 are representative of the classes of molecules recognized by IGRS. Cirazoline, idazoxan, and guanabenz exhibit high affinity for rabbit kidney IGRS (K_i = 0.1–8 nM), whereas amiloride exhibits 10–100-fold lower affinity. Each of these molecules was considered a candidate for derivatization to generate molecular probes. The effect of structural modification on the binding properties of these compounds was investigated in competition binding studies with [^{3}H]idazoxan in rabbit kidney membranes. Derivatization of idazoxan met with synthetic difficulties, whereas amiloride derivatives exhibited low affinity for IGRS [5-(*N*-methyl-*N*-isobutyl)amiloride, K_i = 1678 nM; 5-(*N*,*N*-hexamethylene)amiloride, K_i = 1888 nM]. In preliminary studies a guanabenz derivative, GB-72 [2-[(2,6-dichlorophenyl-4-amino)methylene]hydrazinecarboximidamide], exhibited a K_i of 50–200 nM. Initial attempts to radioiodinate GB-72 did not yield a radioactive product that specifically bound to rabbit kidney IGRS, and this approach was not pursued.[1]

Removal of the C-2 cyclopropyl moiety from the parent molecule cirazoline did not alter affinity for IGRS, and our efforts focused on the derivatization of the phenyl ring of this ligand. The amino-substituted compound (RBI-102) retained high affinity for IGRS (K_d = 12.5 ± 7.5 nM). Introduction of bulkier substituents on the phenyl ring[2] or on the bridging atoms between the phenyl and imidazoline rings (Fig. 2) tended to decrease the affinity for IGRS. The amino-substituted derivative AMPI was radioiodinated and investigated as a potential ligand for IGRS (Fig. 3).

Binding properties of [^{125}I]AMIPI in membrane preparations. [^{125}I]AMIPI binding in rabbit kidney and rat liver membranes was saturable and of high affinity. Scatchard analysis of saturation binding isotherms indicated that in rabbit kidney membranes [^{125}I]AMIPI bound to an apparently homogeneous population of sites (high-speed pellet, n_H = 0.98, K_d = 2.0 ± 0.9 nM, B_{max} = 554 ± 201 fmol/mg, five experiments; low-speed pellet, K_d = 3.7 ± 1.38 nM, B_{max} = 326 ± 87, five experiments) (Fig. 3; Table 1). Data analysis by LIGAND indicated that the data were best fit to a one-site model. The density of [^{125}I]AMIPI binding sites was similar to that identified with [^{3}H]idazoxan, a radioligand commonly utilized to identify IGRS.

Kinetic studies indicated that specific [^{125}I]AMIPI (0.9 nM) binding required ~30 min to reach equilibrium at 24° (Fig. 4) and remained stable for at least 90 min. The apparent association rate constant (k_{app}) was 0.24 ± 0.05 min^{-1}. Analysis of [^{125}I]AMIPI dissociation indicated that ~60–70% of specifically bound probe dissociated within 60 min after a 40-fold dilution of the incubation mixture. However, ~20–40% of the probe specifically bound at time 0 was still observed 120 min after dilution. Similar results were obtained when 10 μM cirazoline was included in the dilution buffer to prevent probe rebinding. In parallel studies with [^{3}H]idazoxan, ~20% of specifically bound radioligand was also observed 120 min after dilution (data not shown). A dissociation rate constant for [^{125}I]AMIPI (1 nM) was caculated after subtraction of nondissociable radioligand from total radioligand bound. An [^{125}I]AMIPI dissociation rate constant of 0.03 ± 0.003 min^{-1} was determined and was used to calculate k_1 ($k_1 = k_{app} - k_2/[[^{125}I]AMIPI]$), resulting in a K_d (k_2/k_1) of 0.17 ± 0.02 nM. In rat liver membranes, [^{125}I]AMIPI also bound to an apparently homogeneous population of sites (high-speed pellet, K_d = 2.6 ± 1.3 nM, B_{max} = 73 ± 10 fmol/mg; low-speed pellet, K_d = 2.98 ± 2.5 nM, B_{max} = 204 ± 25 fmol/mg) (Fig. 5).

Specific [^{125}I]AMIPI binding represented 80–85% of total ligand bound at ligand concentrations of 1 nM and increased linearly with increasing protein from 13 to 190 μg (r = 0.99) (Fig. 6A). Specific [^{125}I]AMIPI binding could be detected with <13 μg of membrane protein. Similar levels of nonspecific binding were observed with 10 μM cirazoline, idazoxan, or guanabenz (data not shown). Monovalent cations decreased specific [^{125}I]AMIPI binding in a concentration-dependent manner (Fig. 6B). Potassium salts exhibited a greater potency and maximal effect, relative to NaCl. The percentage inhibition was 32 ± 4% for KCl, 35 ± 3% for potassium acetate, and 13 ± 1% for NaCl at salt concentrations of 300 mM.

Specificity of [^{125}I]AMIPI binding. To characterize the ligand recognition properties of the [^{125}I]AMIPI binding site, a series of competition binding studies were performed. Compounds with imidazoline and guanidinium moieties inhibited specific [^{125}I]AMIPI binding in rabbit kidney membranes with a rank order of potency consistent with the definition of the binding site as an IGRS (cirazoline > idazoxan > guanabenz > amiloride > clonidine > *p*-aminoclonidine). Cirazoline competed with highest potency (K_i = 3.6 ± 0.96 nM) (Fig. 7).

[1] I. Coupry, A. Grodski, A. Parini, H. J. Hess, and S. M. Lanier, unpublished observations.

[2] B. Ivkovic, M. Pigini, S. M. Lanier, and L. Brasili, unpublished observations.

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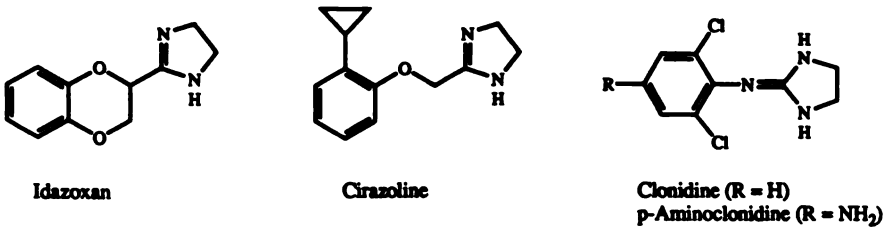
IMIDAZOLINE COMPOUNDS



Idazoxan | Cirazoline | Clonidine (R = H); p-Aminoclonidine (R = NH_2)

GUANIDINIUM COMPOUNDS

Guanabenz | Amiloride (R_1,R_2 = H); MI-amiloride (R_1 = CH_3, R_2 = isobutyl); HM-amiloride (R_1, R_2 = hexamethylene)

Fig. 1. Chemical structure of imidazoline and guanidinium compounds.

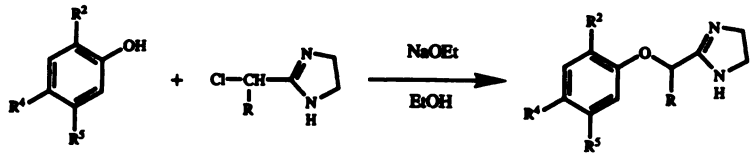


Fig. 2. Generation of substituted phenoxymethylimidazoline derivatives. The reaction sequence leading to the synthesis of the phenoxymethylimidazoline derivatives and recognition of the ligands by IGRS in rabbit kidney membranes are shown. K_i values were obtained in competition binding studies with [^{3}H]idazoxan (8–10 nM) and are expressed as the mean ± standard error. Data represent the average of three to five experiments with different membrane preparations. *NaOEt*, sodium ethoxide; *EtOH*, ethanol.

	R	R2	R4	R5	Ki(nM)
CIRAZOLINE	H	cyclopropyl	H	H	5 ± 1.4
RBI- 100	H	H	$NHCOCH_3$	H	28 ± 4.9
RBI- 101	H	H	H	NHCOCH3	612 ± 255
RBI- 102	H	H	H	NH_2	12 ± 7.5
RBI- 103	H	CH_2CH_3	H	OCH_3	27 ± 3.5

Amiloride, an inhibitor of numerous ion transport systems, exhibited lower affinity (K_i = 625 ± 130 nM) (Table 2).

The selective α_2-AR agonists clonidine, *p*-aminoclonidine, and UK 14,304, all of which have an imidazoline moiety, competed with >400-fold lower affinity, relative to the imidazoline cirazoline (K_i = 2200 ± 200 nM, 3422 ± 172 nM, and 1500 ± 170, respectively). [^{125}I]AMIPI binding was not inhibited by the endogenous AR agonist (−)-epinephrine (Fig. 6A) or agonists for other neurotransmitter receptors, including dopamine, serotonin, histamine, and the histamine metabolite imidazole-4-acetic acid. The α_2-AR-selective antagonist rauwolscine, the α_1-AR-selective antagonist prazosin, the α-AR agonist oxymetazoline, and the β-AR antagonist propranolol did not inhibit [^{125}I]AMIPI binding at concentrations of 10 μM (Table 2). A similar rank order of potency for competing ligands was observed in rat liver membranes (data not shown). Competition binding curves with idazoxan and guanabenz were shallow (n_H = 0.6–0.8) and could be resolved into two sites of high and low affinity (idazoxan, K_i = 0.2 ± 0.1 nM for 24%, K_i = 76 ± 30 nM for 76%; guanabenz, K_i = 1.7 ± 0.84 nM for 47%, K_i = 201 ± 72 for 53%) (Fig. 7).

Intratissue distribution of IGRS in rat forebrain, rat liver, and canine kidney. Although IGRS or related imidazoline-binding proteins are found in crude homogenates of central and peripheral tissues, the intratissue distribution of these sites is unknown. The high affinity and high specific activity of [^{125}I]AMIPI offer several advantages for tissue section autoradiography, relative to other probes that interact with other receptor systems and are of low specific activity. To address this issue we examined [^{125}I]AMIPI binding in sections from three tissues, i.e., forebrain, kidney, and liver.

Autoradiograms of rat forebrain tissue sections incubated with 0.2–1.2 nM [^{125}I]AMIPI indicated high densities of specific binding (75–80%) in the SFO and the hypothalamic ARC (Fig.

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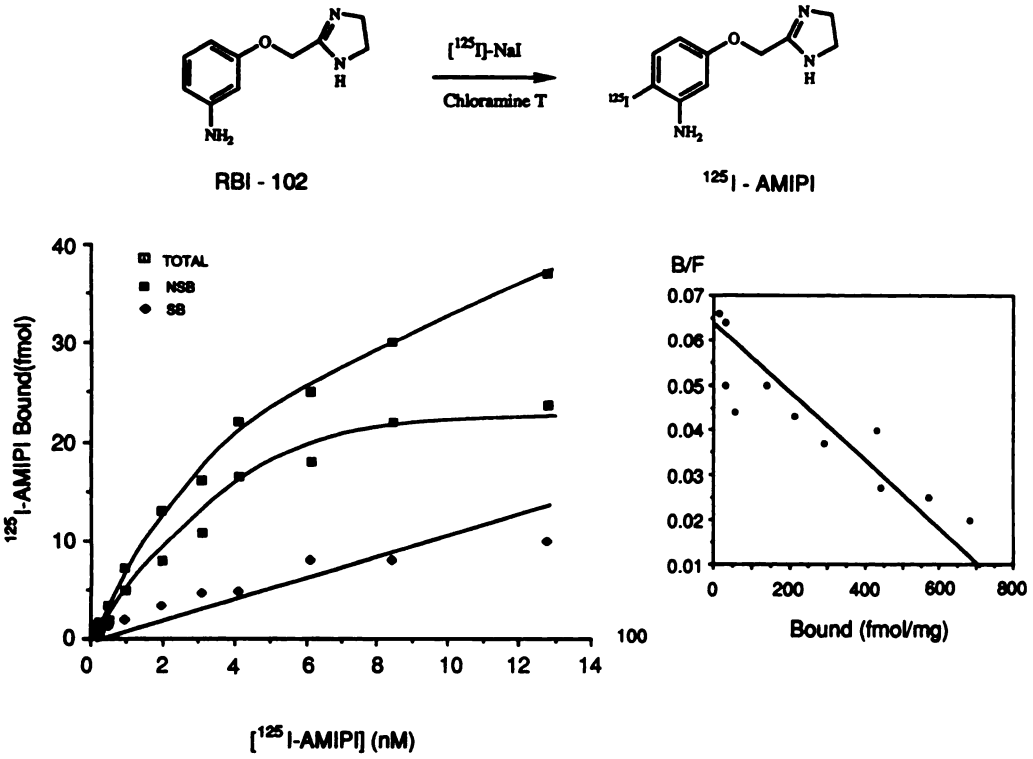

Fig. 3. Equilibrium binding studies with [^{125}I]AMIPI in rabbit kidney membranes. RBI-102 was radioiodinated as described in Experimental Procedures. Radioligand binding in rabbit kidney membranes (high-speed pellet, 38 μg of membrane protein) was performed as described in Experimental Procedures, using increasing concentrations of the radiolabeled probe (0.10–13 nM). The percentage specific binding ranged from 75–80% at a concentration of 1 nM (total ligand bound, 28,406 dpm) to 28% at 10 nM (total ligand bound, 125,100 dpm). *Inset*, Scatchard analysis of specific [^{125}I]AMIPI binding. In this particular experiment, the K_d value was 4.5 nM. *NSB*, nonspecific binding; *SB*, specific binding.

TABLE 1

Comparison of [^{3}H]idazoxan and [^{125}I]AMIPI saturation binding parameters in rabbit kidney membranes

Rabbit kidney membranes (high- and low-speed pellets) were incubated with increasing concentrations of [^{3}H]idazoxan (0.4–60 nM) or [^{125}I]AMIPI (0.1–22 nM). The dissociation constant (K_d) and the maximum number of sites (B_{max}) were calculated from equilibrium data using a computer-assisted transformation of the saturation isotherm (34). Results are expressed as means ± standard errors (five experiments).

Radioligand	K_d	B_{max}
	nM	*fmol/mg of protein*
High-speed pellet		
[^{3}H]idazoxan	8.6 ± 1.8	779 ± 168
[^{125}I]AMIPI	2.0 ± 0.9	554 ± 201
Low-speed pellet		
[^{3}H]idazoxan	9.5 ± 0.29	490 ± 139
[^{125}I]AMIPI	3.7 ± 1.38	326 ± 87

8). Autoradiograms from male and female rats were qualitatively similar. Quantitative analysis revealed a specific binding density of 1235 ± 205 fmol/mg of protein in the SFO (seven experiments) and of 1264 fmol/mg of protein in the ARC (one experiment) at 0.9 nM. [^{125}I]AMIPI binding was inhibited by both imidazoline and guanidinium ligands but was not competed for by the selective α_2-AR antagonist rauwolscine. Idazoxan competed for 95 ± 5% (six experiments) of the specific binding in the SFO, and guanabenz competed for 99 ± 1% (four experiments) in the SFO and 99 ± 1% (three experiments) in the ARC. In contrast, there was minimal competition with rauwolscine in either the SFO (11 ± 4%, four experiments) or the ARC (16 ± 12%, three experiments). Clonidine also effectively competed with radioligand binding in both the ARC and SFO (91 ± 6%, three experiments).

In sections of liver, approximately 70% of [^{125}I]AMIPI binding was inhibited by cirazoline and other IGRS-selective ligands. Specific binding (~636 fmol/mg of protein) was uniformly distributed across the tissue. In renal sections, specific heterogeneous [^{125}I]AMIPI binding was observed in the renal cortex. [^{125}I]AMIPI binding in renal cortex was characterized by cortical rays as well as punctate localizations. At this level of resolution it was difficult to define anatomical structures; however, the pattern was consistent with previous reports indicating a high density of IGRS in purified membrane preparations of renal proximal tubules (2). [^{125}I]AMIPI binding was inhibited by guanabenz, idazoxan, and clonidine but not the selective α_2-AR antagonist rauwolscine. However, clonidine competed with less effectiveness (68–80% of specific binding was inhibited) in kidney and liver, consistent with the lower apparent affinity of clonidine in competition binding studies (Fig. 7).

Discussion

In 1984, Bousquet *et al.* (14) demonstrated that the centrally mediated effects of certain imidazoline ligands on blood pressure could be dissociated from those of catecholamines. The action of the latter is elicited through α_2-AR activation, resulting in a decrease in sympathetic drive to the periphery. Because several imidazoline ligands are useful as α-AR agonists/antagonists, the dissociation of some of their actions from those of catecholamines suggested the existence of novel sites of action for these compounds, i.e., the "imidazoline-preferring receptor." Similar observations by other groups and the suggestion of a possible endogenous substance for such sites provided additional evidence for the existence of such an entity. Subsequently, radiolabeled imidazolines were used to identify specific binding sites in various tissues, including brain and kidney. These sites shared the common property of not recognizing agonists for known monoamine receptors but differed in their ligand recognition properties. Initially, many of the radioligand binding studies were complicated by the interaction of the

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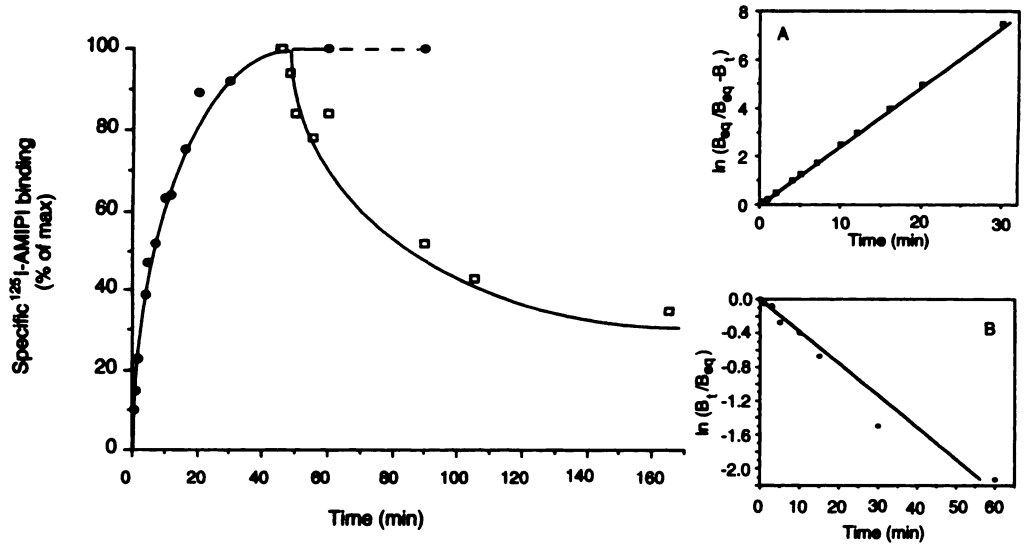

Fig. 4. Kinetics of [^{125}I]AMIPI binding to rabbit kidney membranes. In the association reaction, 150 μg of membranes (high-speed pellet) were added to test tubes containing [^{125}I]AMIPI (0.9 nM final concentration) and either 10 μM cirazoline or buffer B, at 24°. At the times indicated, the incubation was terminated by vacuum filtration as described in Experimental Procedures. In the dissociation reaction, the reaction mixture at equilibrium was diluted with 4 ml of buffer B and the incubation was continued at 24° for the times indicated. The apparent rate constant (k_{app}) for the pseudo-first-order association reaction was calculated from the slope of the line (*inset A*) relating $\ln[B_{eq}/(B_{eq} - B_t)]$ and time (determined by linear regression analysis, $r = 0.99$), where B_{eq} is the amount of [^{125}I]AMIPI bound at equilibrium and B_t is the amount bound at each time t. The second-order rate constant (k_1) was calculated according to the equation $k_1 = (k_{app} - k_2)/[[^{125}I]AMIPI]$, where k_2 is the dissociation rate constant. This latter constant (k_2) was determined from the dissociation reaction, where k_2 is the slope of the line (*inset B*) relating $\ln(B_t/B_{eq})$ and time (determined by linear regression analysis, $r = 0.82$), B_t is the amount of specific binding at each time t, and B_o is the amount bound at equilibrium before dilution. Dissociation kinetics were analyzed (*inset B*) after subtraction of the nondissociable portion of [^{125}I]AMIPI binding, as indicated in the text. The results shown are representative of three separate experiments using different membrane preparations. □, dissociation; ●, association.

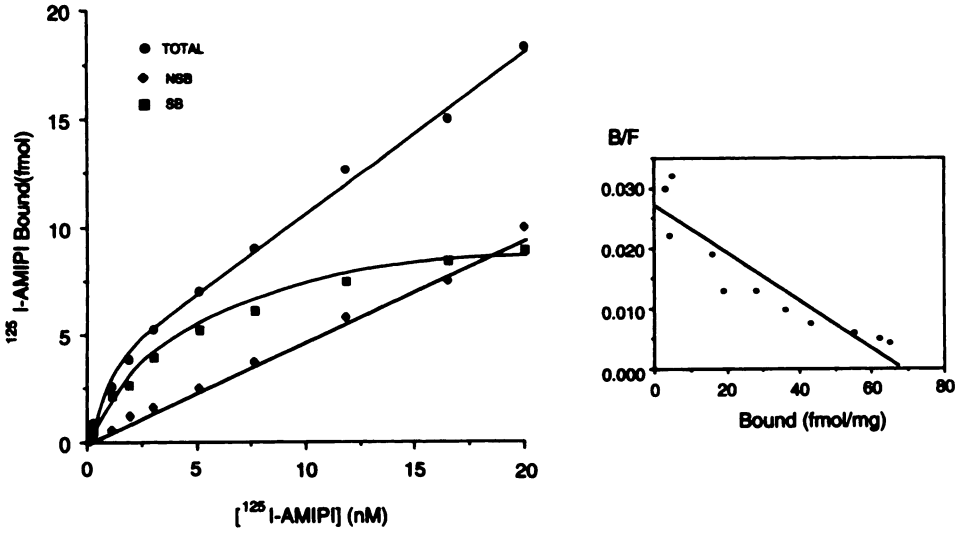

Fig. 5. Equilibrium binding studies with [^{125}I]AMIPI in rat liver membranes. Saturation binding isotherms were performed as described in Experimental Procedures, using increasing concentrations of [^{125}I]AMIPI (0.13–20 nM) with the high-speed membrane pellet (125 μg of membrane protein). The percentage specific binding ranged from 80% at a concentration of 1 nM (total ligand bound, 10,150 dpm) to 43% at a ligand concentration of 10 nM (total ligand bound, 71,075 dpm). *Inset*, Scatchard analysis of specific [^{125}I]AMIPI binding. The K_d value in this experiment was 2.5 nM. *NSB*, nonspecific binding; *SB*, specific binding.

labeled ligands with various neurotransmitter receptors, particularly α_2-AR. However, Parini *et al.* (1) successfully dissociated such a postulated receptive site from α_2-AR after detergent solubilization of membranes. Based on the ability of the receptive site to recognize ligands with both imidazoline and guanidinium substituents, the site was termed the IGRS. The IGRS is apparently distinct from the imidazoline receptor identified with [^{3}H]clonidine or its analogues, based on ligand recognition properties, tissue distribution, and possibly subcellular localization.

To facilitate the identification and structural characterization of IGRS and other members of this family of binding sites, we attemped to develop high affinity radiolabeled molecules of high specific activity that selectively recognize these sites. Such molecules could be used in radioligand binding assays and would allow intratissue or subcellular localization of these sites in fixed sections or in intact cells. An additional goal was to design such molecules so that they could be functionalized for use as photoaffinity adducts or in ligand affinity chromatography.

A series of aryloxymethylimidazolines were generated, yielding molecules with various substitutions at positions R_2, R_4, and R_5 on the phenyl ring. None of the phenyl substitutions dramatically affected the interaction of the compounds with IGRS. Of particular interest was the high affinity exhibited by the amino-substituted derivative AMPI. The presence of the

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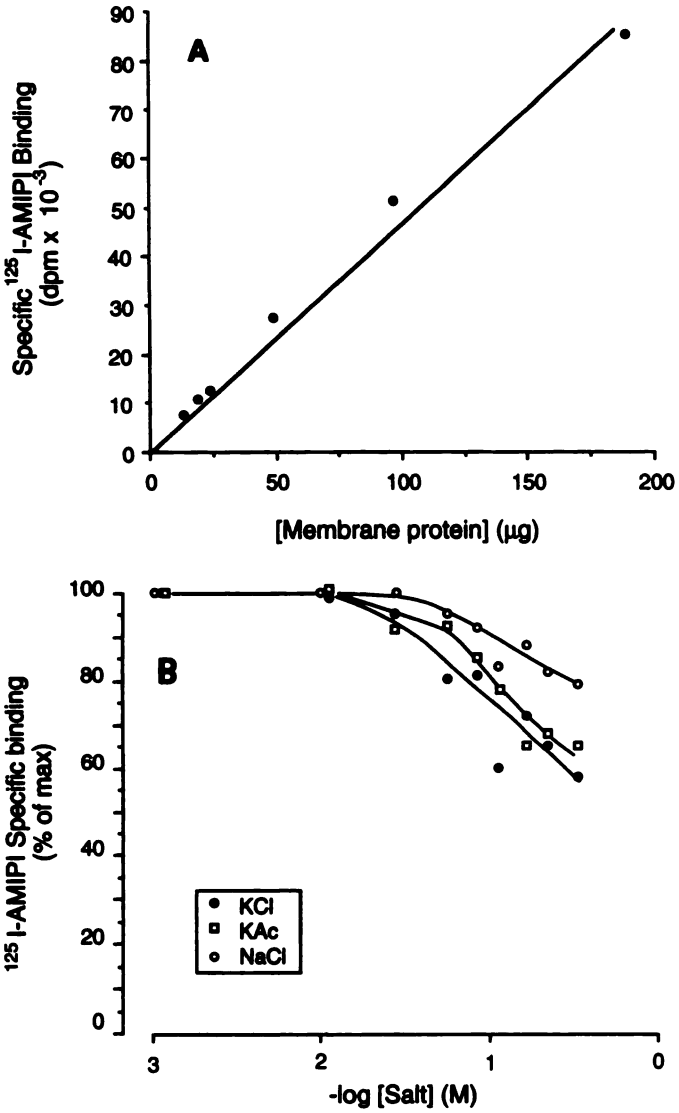

Fig. 6. Effect of membrane protein concentration and monovalent cations on [^{125}I]AMIPI binding in rabbit kidney membranes. A, [^{125}I]AMIPI (0.8 nM) was incubated with increasing amounts of membrane protein as described in Experimental Procedures. The data are representative of three experiments. B, Rabbit kidney membranes were incubated with 1 nM [^{125}I]AMIPI and increasing concentrations of KCl, potassium acetate (*KAc*), and NaCl. The data are representative of three experiments using different membrane preparations and are expressed as a percentage of specific [^{125}I]AMIPI binding in the absence of salts.

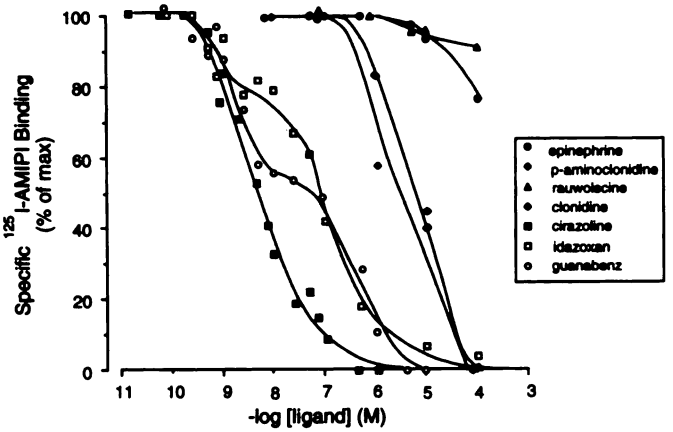

Fig. 7. Inhibition of [^{125}I]AMIPI binding by ligands in rabbit kidney membranes. The effect of increasing concentrations of competing ligands on [^{125}I]AMIPI binding (0.8–1 nM) was determined in rabbit kidney membranes (50–100 μg of membrane protein) as described in Experimental Procedures. In these studies nonspecific binding (inhibited by 10 μM cirazoline) represented 17–25% of total counts bound (~30,000 dpm). Competition curves with idazoxan and guanabenz were shallow and were best fit to a two-site model (idazoxan, one versus two sites, $F = 4.63$, $p = 0.029$; guanabenz, one versus two sites, $F = 12.77$, $p = 0.001$). The data are presented as the average of duplicate determinations and are representative of three to five separate experiments using different membrane preparations.

TABLE 2

Inhibitory binding constants (K_i) for ligands in competition binding studies with [^{125}I]AMIPI

Competition binding studies were performed in rabbit kidney membranes with ~1 nM [^{125}I]AMIPI, in the presence or absence of increasing concentrations (10^{-11} to 10^{-4} M) of competing ligands. K_i values were calculated according to the Cheng and Prusoff equation (41) and are presented as means ± standard errors obtained from two to four experiments.

Competing ligand	K_i	n_H
	nM	
Cirazoline	3.6 ± 0.96	0.87 ± 0.08
Idazoxan	0.2 ± 0.1[a]	0.66 ± 0.02
	76 ± 30	
Guanabenz	1.7 ± 0.84[a]	0.44 ± 0.07
	201 ± 72.7	
Amiloride	625 ± 130	0.85 ± 0.05
Clonidine	2200 ± 200	0.79 ± 0.02
p-Aminoclonidine	3422 ± 172	0.89 ± 0.01
UK 14,304	1500 ± 170	0.80 ± 0.02
Rauwolscine	—[b]	
Oxymetazoline	—[b]	
Prazosin	—[b]	
Propranolol	—[b]	
Epinephrine	—[b]	
Dopamine	—[b]	
Serotonin	—[b]	
Histamine	—[b]	
Imidazole-4-acetic acid	—[b]	

[a] Idazoxan and guanabenz competition binding curves were best fit by a two-site model.

[b] —, No inhibition of [^{125}I]AMIPI binding was observed at competing ligand concentrations of 10 μM.

phenylamine allowed further derivatization of the molecule to generate the radioiodinated ligand [^{125}I]AMIPI. The binding of the radioiodinated probe was saturable, reversible, and inhibited by imidazoline or guanidinium ligands with a pharmacological specificity consistent with the identification of the binding site as IGRS.

Scatchard analysis of saturation binding isotherms indicated that [^{125}I]AMIPI apparently recognizes a single class of homogeneous binding sites. Similarly, competition curves with the imidazoline cirazoline were monophasic. However, the benzodioxan idazoxan and the guanidinium guanabenz apparently interact differently with subpopulations of the sites identified with [^{125}I]AMIPI, based on the biphasic curves observed in competitive binding assays. The populations of high affinity binding sites for idazoxan and guanabenz quantitatively differ, suggesting a heterogeneous group of binding proteins. It is not clear whether the multiple sites resolved in idazoxan and guanabenz competition curves represent distinct proteins or different conformational states of the same receptive site that do not interconvert under these experimental conditions. The biphasic competition curves were also observed in the presence of the nonhydrolyzable GTP analogue guanyl-5′-ylimidodi-

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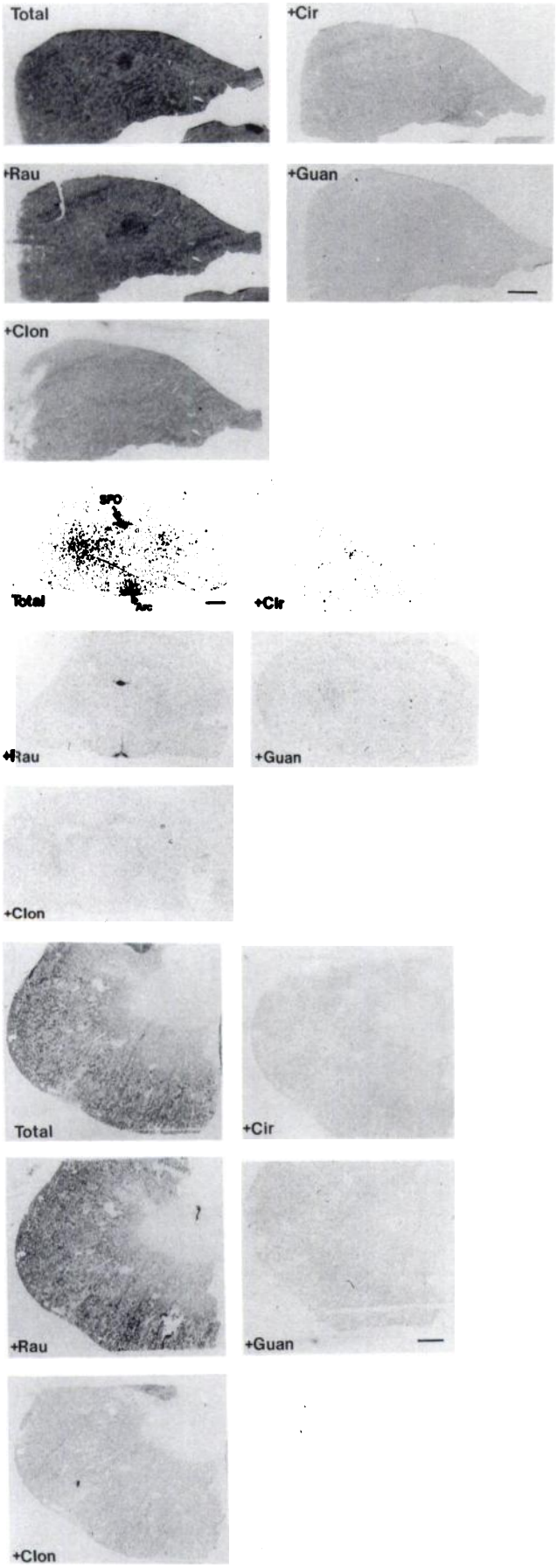

phosphate.[3] The biphasic nature of the competition curves for some ligands is consistent with the presence of multiple binding sites identified by the photoaffinity adduct [^{125}I]AZIPI, a derivative of [^{125}I]AMIPI. It is not clear whether the different populations of sites are found at distinct subcellular locations. The availability of the radioiodinated probe [^{125}I]AMIPI described herein and its photolabile adduct [^{125}I]AZIPI (25) should facilitate efforts to define these issues.

The utility of [^{125}I]AMIPI as a molecular probe is also indicated by results of tissue section autoradiography. The apparently homogeneous distribution of binding sites in liver contrasts with the discrete localization of IGRS in forebrain and kidney. The apparent high density of IGRS in the SFO and hypothalamic ARC is of particular interest relative to the action of these various imidazoline and guanidinium compounds on food intake (42) and cardiovascular function. The SFO is one of several circumventricular organs that are deficient in the "blood-brain barrier" and may actually monitor levels of various circulating hormones (43, 44). The SFO is an important area for regulation of thirst and expresses receptors for several circulating hormones, including angiotensin II, endothelin, and atriopeptin. The ARC of the hypothalamus is an important regulator of various endocrine functions. A localization of IGRS in the ARC was suggested by previous binding studies with [^{3}H]idazoxan in the presence of α_2-AR-selective ligands (45). However, other reports on the intratissue location of [^{3}H]idazoxan binding in the brain (46–47) utilized sections that did not include the SFO or the ARC. The availability of [^{125}I]AMIPI should facilitate further detailed mapping of the family of imidazoline-binding proteins in the central nervous system.

Differences in ligand recognition properties define two distinct groups in this family of binding sites, 1) one with high affinity for the imidazolines clonidine and cirazoline and low affinity for guanidiniums such as guanabenz and amiloride (radioligand, *p*-[^{3}H]aminoclonidine or other radiolabeled analogues of clonidine) and 2) one with high affinity for the imidazolines cirazoline and idazoxan and the guanidinium guanabenz and low affinity for clonidine analogues (radioligand, [^{3}H]idazoxan). Additional entities are defined by sites that share the properties of the second group but either exhibit a higher affinity for clonidine or do not recognize amiloride or idazoxan. The latter sites and members of group 2 are all defined as IGRS, based on their ability to recognize representative members of the chemical class. The relationship among all of these sites is unclear, and observed differences in binding properties may represent species differences, interlaboratory variability, or the existence of distinct receptor subtypes. A

[3] B. Ivkovic, V. Bakthavachalam, W. Zhang, J. L. Neumeyer, and S. M. Lanier, unpublished observations.

Fig. 8. Photomicrographs of [^{125}I]AMIPI binding (0.22 nM) in serial 14-μm coronal sections of rat liver (A), rat forebrain (B), and dog kidney (C). Tissue sections were prepared and incubated with [^{125}I]AMIPI (0.2 nM) as described in Experimental Procedures. Competition studies using adjacent sections from the same animal indicate that binding was displaced by cirazoline (*+Cir*), guanabenz (*+Guan*), and clonidine (*+Clon*) but not rauwolscine (*+Rau*). Competing ligands were used at 10 μM. *Total*, no competing ligand. In A, sections were cut at a slight angle to include both the SFO and the hypothalamic ARC. *Scale bar* = 1 mm. The autoradiographs in A and B are representative of three to seven experiments using different animals and/or different batches of radioligand.

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common feature of all of these sites is that they recognize the imidazoline cirazoline, leading to the potential utility of the radioiodinated ligand [^{125}I]AMIPI described in the present study.

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