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Structural and Ligand Recognition Properties of Imidazoline Binding Proteins in Tissues of Rat and Rabbit

BILJANA LANIER, RITA RADDATZ, VENKATESALU BAKTHAVACHALAM, ISABELLE COUPRY,[1] JOHN L. NEUMEYER, and STEPHEN M. LANIER

Department of Pharmacology, Medical University of South Carolina, Charleston, South Carolina 29425 (B.L., R.R., S.M.L.), Research Biochemicals International, Natick, Massachusetts 01760 (V.B., J.L.N.), and INSERM U388, Pharmacologie Moleculaire et Physiopathologie Renale, Institut Louis Bugnard, Toulouse, France (I.C.)

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SUMMARY

Imidazoline/guanidinium receptive sites (IGRS) belong to a family of membrane proteins that selectively recognize certain pharmacologically active compounds with an imidazoline or a guanidinium moiety. The role of such proteins in the cellular responses elicited by these compounds is unclear, but two members of this protein family are identical to isoforms of monoamine oxidase, an enzyme involved in the metabolism of monoamine neurotransmitters. To characterize the structural and ligand recognition properties of the imidazoline binding proteins, we used the photoaffinity adduct [^{125}I]iodoazidophenoxymethylimidazoline ([^{125}I]AZIPI) to label their ligand binding subunits in selected target tissues (kidney, pancreatic B cells, liver, and salivary gland). Photoaffinity labeling of membrane preparations or subcellular particulate fractions from various rat, rabbit, or hamster tissues indicated two labeled peptides of M_r ~55,000 and ~61,000, the relative tissue distribution of which mirrored the expression of the A or B isoforms of monoamine oxidase. The ligand binding subunit of imidazoline binding proteins was identified on two peptides of M_r ~55,000 and ~61,000 in rat and rabbit kidney, rat liver, rabbit salivary gland, and the pancreatic B cell line RIN-5AH, whereas only an M_r ~61,000 peptide was observed in rat salivary gland and the hamster pancreatic B cell line HIT-T15. Saturation labeling experiments indicated that [^{125}I]AZIPI exhibited similar affinity (K_d ~2–3 nM) for both the M_r ~55,000 and ~61,000 peptides. However, competitive inhibition of photolabeling indicated that the two peptides were distinguished by their affinity for the guanidinium guanabenz or their interaction with potassium. Although some types of imidazoline binding sites are located on the enzyme monoamine oxidase, the nonisoform selective enzyme inhibitor pargyline did not alter photoaffinity labeling of either the M_r ~55,000 or ~61,000 peptide, indicating that imidazolines/guanidiniums and active site inhibitors of monoamine oxidase interact with different domains on the enzyme. In rat kidney and liver, an additional photolabeled peptide of M_r ~25,000 was observed, and its ligand recognition profile was distinct from the M_r ~55,000 and ~61,000 species. In contrast with the mitochondrial location of the larger peptides, subcellular fractionation of liver homogenates indicated that the M_r ~25,000 localized to the plasma membrane.

IGRS belong to a family of membrane proteins that exhibit high affinity for various compounds with an imidazoline and/or guanidinium moiety (1–6).[2] The role of these binding proteins in the functional action of these compounds remains unclear. IGRS or type 2 imidazoline binding proteins in liver and kidney are enriched in mitochondrial membrane fractions, whereas related binding sites are detected at the cell surface (3, 5, 7–9). Functionally, the pharmacology of this system remains poorly defined. The pharmacological characterization of imidazoline binding proteins is also complicated by species variations in ligand recognition, the use of different "selective" radioligands, and failure to eliminate ligand interaction with other hormone/neurotransmitter receptor systems. Relative to the diverse actions (both centrally and in peripheral tissues) of this class of imidazoline/guanidinium compounds in human and the possible existence of an endogenous ligand for imidazoline binding proteins (10–12), the high affinity and specificity of the interaction of these compounds with this family of binding proteins are of particular interest. Such entities may represent an actual receptor in

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[1] Current address: Laboratoire de Biochimie Medicale et Biologie Moleculaire, Universite of Bordeaux II, Bordeaux, France.

[2] The family of imidazoline binding proteins includes proteins termed nonadrenergic imidazoline receptors, imidazole receptors, imidazoline binding sites, and imidazoline/guanidinium receptive sites. Subgroup nomenclature within the family is controversial, but two broad subgroups of imidazoline binding proteins are defined as I1 and I2 binding sites based on differences in their ligand recognition properties. IGRS is most similar to the type 2 imidazoline binding protein subgroup.

ABBREVIATIONS: IGRS, imidazoline/guanidinium receptive sites; [^{125}I]AZIPI, [^{125}I]iodoazidophenoxymethylimidazoline; [^{125}I]AMIPI, [^{125}I]iodoaminophenoxymethylimidazoline; MAO, monoamine oxidase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

the target cell directly linked to the various cell signaling proteins, or these binding sites may be involved in the metabolism or transport of such ligands.

Members of this protein family have been suggested to be heptahelical, G protein-coupled receptors, MAO isoforms, or potassium channels (3, 9, 13, 14). The last suggestion is based on the ability of potassium to allosterically modify ligand affinity and the interaction of selected potassium channel ligands with members of the family of imidazoline binding proteins (3, 8, 9, 15). The apparent identity of two members of the family of imidazoline binding proteins as MAO isoforms is based on the copurification of the two entities, detection of imidazoline binding proteins after heterologous expression of MAO isoforms, and immunoprecipitation of photolabeled imidazoline binding proteins with monoclonal antibodies for MAO isoforms (5, 7, 14). However, differences in apparent molecular weights, tissue distribution, or ligand recognition properties of imidazoline binding proteins indicate the existence of additional members of this family that appear to be unrelated to MAO. There are few clues to the functionality of imidazoline binding proteins, and determination of their primary structure is a major goal of various research groups. To facilitate such efforts, we developed a photoaffinity adduct that exhibits high affinity for members of the family of imidazoline binding proteins (1, 17). In the present report, we used this probe to examine the tissue distribution, ligand recognition properties, and subcellular distribution of imidazoline binding proteins relative to MAO.

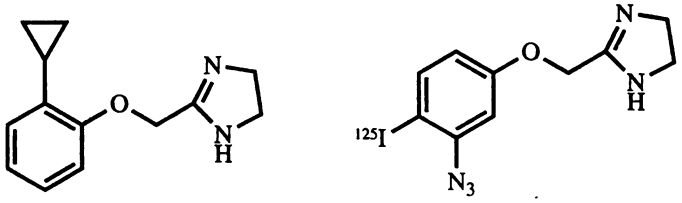
Experimental Procedures

Materials. Carrier-free Na^{125}I and Renaissance Western blot chemiluminescence reagent were purchased from DuPont-NEN (Boston, MA). Idazoxan, guanabenz, amiloride, diazoxide, charybdotoxin, clonidine, glibenclamide, dendrodotoxin, *para*-aminoclonidine, and [5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine] 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, 4-aminopyridine, pargyline, phenylmethylsulfonyl fluoride, Tris, dithiothreitol, ammonium persulfate, and SDS were purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide, bisacrylamide, and electrophoresis-grade Tris were purchased from Bio-Rad (Richmond, CA). Midrange-size standards for SDS-PAGE were obtained from Enprotech or Bio-Rad. Polyclonal antisera to MAOs A and B were generated by immunization of rabbits with a peptide (TNGGQERKFVGGSGQ) shared by both isoforms. Reverse-phase Unibond silica gel plates (HPTLC-RP18F) were purchased from Analtech (Newark, DE), and silica gel 60 plates were purchased from EM Reagents. Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA), and New Zealand White rabbits were obtained from Rabbits LTD (Somerville, SC). Frozen rabbit kidney cortex or salivary gland and rat salivary gland tissue were purchased from Pel-Freez (Rogers, AR). RIN-5AH cells were kindly provided by Dr. Ake Wenmark (University of Washington), and HIT-T15 cells were obtained from American Type Culture Collection.

Membrane preparation, photoaffinity labeling, and immunoblotting. Membranes were prepared from either frozen tissues or freshly isolated cells or tissues as previously described (1, 17). Frozen tissues were minced with scissors in 10 mM Tris·HCl, 5 mM EGTA, 5 mM EDTA, and 10 μM phenylmethylsulfonyl fluroide, pH 7.4, at 4° and then homogenized with a glass Dounce pestle. Membranes were isolated by differential centrifugation and resuspended in membrane buffer containing 50 mM Tris·HCl, 5 mM $MgCl_2$, 0.6 mM EDTA, and 10 μM phenylmethylsulfonyl fluoride, pH 7.4. In some experiments with rat kidney and liver membranes, tissues were processed with buffers containing additional protease inhibitors (1 μg/ml antipain, 1 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin). Protein concentration was determined according to the method of Lowry *et al.* (18). Rabbit kidney membranes were homogenized and centrifuged in a Sorvall RC-5B (SS-34 rotor) at 1000 × *g* for 10 min to generate a low-speed pellet. The supernatant was then centrifuged in the same rotor at 30,000 × *g* to generate a high-speed pellet (17). Unless otherwise noted, photolabeling experiments used the membrane pellet obtained by centrifugation at 30,000 × *g*.

In some experiments, a plasmalemma-enriched fraction of rat liver membranes was prepared according to the method of Prpic *et al.* (19). Particulate fractions enriched in mitochondria were obtained by density gradient centrifugation through Nycodenz as previously described (1). The pancreatic B cell lines RIN-5AH and HIT-T15 were grown as previously described (20), and cell membranes were prepared from confluent cultures. Membrane aliquots were either immediately photoaffinity labeled or frozen at −70° for later use. Immunoblots were generated as previously described (20) using a 1:2000 dilution of antisera. The polyclonal antisera were obtained from rabbits immunized with the peptide TNGGQERKFVGGSGQ corresponding to amino acids 210–227 in MAO A and 202–217 in MAO B. The specificity of the antibody was determined by peptide competition in heterologous expression systems and various tissues. It is unclear whether the two isoforms are recognized equally well by the antisera. Due to the different exposure times of the autoradiographs in the different tissues, it is not possible to compare the relative intensity of photolabeling and the chemiluminescence signal detected by immunoblotting in the different tissues. Although the antisera effectively and specifically recognize the MAO isoforms by immunoblotting, attempts to use the antisera for immunoprecipitation under a variety of experimental conditions were unsuccessful.

The photoaffinity adduct [^{125}I]AZIPI was generated from an analogue (aminophenoxymethylimidazoline) of cirazoline, an imidazoline that exhibits high affinity for the family of imidazoline binding proteins. [^{125}I]AMIPI (17) was converted to the photosensitive azide by diazotization and reaction with sodium azide under reduced light. [^{125}I]AZIPI was purified by thin layer chromatography with silica gel 60 chromatographic plates (dichloromethane/methanol/ammonium hydroxide, 90:9:1) and stored in methanol at −20°. Membrane aliquots (25–250 μg membrane protein) were incubated with 1–2 nM [^{125}I]AZIPI for 45 min at 24° (total volume, 100 μl) and then cooled at 4°. Samples were placed in a Ray-O-Vac photoreactor, and 1 ml of membrane buffer (4°) containing 1 mM dithiothreitol was added to each sample just before photolysis (5 min, 320 nm). Photolabeled membranes were pelleted, resuspended in Laemmli loading buffer, and electrophoresed for 12 hr on 10% or 17% polyacrylamide gels under denaturing conditions. After electrophoresis, the gels were dried under a vacuum and exposed to Kodak XAR-5 film at −70° for 1–8 days. Autoradiographs were analyzed with a 3CX scanner and Image 1.4 software. The area-under-the-peak curve was determined by imaging at least two different exposures of the same gel.



CIRAZOLINE ^{125}I-AZIPI

Fig. 1. Structures of cirazoline and the photoaffinity adduct [^{125}I]AZIPI.

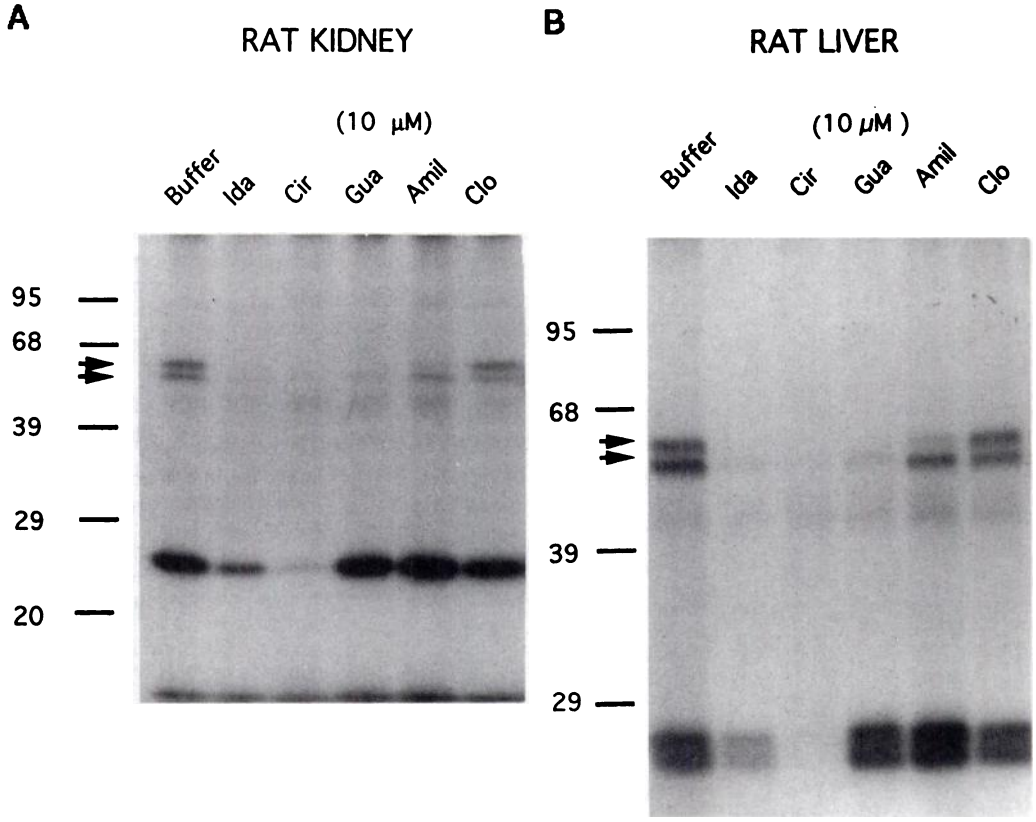


Fig. 2. Photoaffinity labeling of membrane preparations from rat kidney and liver with [^{125}I]AZIPI. Membranes were prepared and aliquots (100–200 μg) photolabeled with [^{125}I]AZIPI (1 nM) in the presence and absence of various competing ligands (10 μM) as described in Experimental Procedures. Photolabeled membranes were solubilized in Laemmli loading buffer (*Buffer*), applied to 10% denaturing polyacrylamide gels, and electrophoresed for 12 hr. A and B, *left*, Migration of nonglycosylated molecular weight standards (Enprotech midrange molecular weight standards) ($M_r \times 10^{-3}$). *Arrows*, major labeled species. Representative of three separate experiments generated with different membrane preparations. *Ida*, idazoxan; *Cir*, cirazoline; *Gua*, guanabenz; *Amil*, amiloride; *Clo*, clonidine.

Results

Identification of the ligand binding subunit of imidazoline binding proteins in rat kidney and liver. The imidazoline cirazoline exhibits high affinity for members of the family of imidazoline binding proteins, and an analogue was derivatized to generate the photoaffinity adduct [^{125}I]AZIPI (Fig. 1). The usefulness of this photoprobe has been previously established (1), and in the present report we used the probe to determine the properties of imidazoline binding proteins in various tissues of rat and rabbit. In rat kidney and liver, the photoprobe covalently incorporated into three major species with M_r of ~25–27,000, ~55,000, and ~61,000 (Fig. 2), whereas only the two larger species were observed in rat brain (21). A panel of ligands that pharmacologically distinguish IGRS was used to compete for photoaffinity labeling in the two tissues. The ligand recognition properties of the corresponding labeled peptides were similar in kidney and liver. Photolabeling of both the M_r ~55,000 and ~61,000 peptides was inhibited by the imidazoline cirazoline, the guanidinium guanabenz, and the α_2 adrenergic receptor antagonist idazoxan, an imidazoline-substituted molecule commonly used to identify IGRS. Photolabeling of the M_r ~61,000 peptide but not the M_r ~55,000 peptide was also blocked by amiloride, a guanidinium molecule that affects various ion transport systems. In rat kidney and liver, [^{125}I]AZIPI also covalently incorporated into M_r ~25,000 peptides (doublet in liver) that exhibited ligand recognition properties distinct from those of the two larger labeled peptides. In contrast to the ligand recognition properties of the two larger labeled peptides, photoaffinity labeling of the M_r ~25–27,000 peptides was inhibited only by cirazoline. Such a site may contribute to the ligand binding detected by radiolabeled cirazoline derivatives in membrane binding assays or by ligand autoradiography in tissue sections. The M_r ~25–27,000 species were not detected in rabbit kidney, rat brain, pancreatic B cells, or rat or rabbit salivary tissue. None of the labeled peptides recognized the α_2 adrenergic receptor antagonist rauwolscine.[3]

Subcellular distribution and ligand recognition properties of photolabeled species in rat liver. To determine the localization of the labeled species in rat liver, fractions enriched for plasmalemma or intracellular organelles were prepared by density gradient centrifugation and photoaffinity labeled with [^{125}I]AZIPI. The M_r ~25–27,000 species were localized to the plasma membrane and were absent in the mitochondrial fraction (Fig. 3). The M_r ~55,000 and ~61,000 labeled peptides were restricted to a particulate fraction enriched in mitochondria, as previously reported (Fig. 3) (1). The relative proportions of the two larger labeled peptides varied among tissues and individual membrane preparations. The appearance of the lower-molecular-weight species was not altered by inclusion of multiple protease inhibitors; it was not observed in the mitochondrial fractions; and it exhibited a ligand recognition profile distinct from that of the M_r ~55,000 and ~61,000 peptides, suggesting that it is not simply a proteolytic product of the larger peptides.

Analysis of imidazoline binding proteins by radioligand binding studies often generate complex data suggesting multiple types and/or affinity states of the labeled entities. However, saturation binding isotherms with the [^{125}I]AMIPI, the nonphotolabile precursor of [^{125}I]AZIPI, were monophasic,

[3] Lanier, B. I., and S. M. Lanier, unpublished observations.

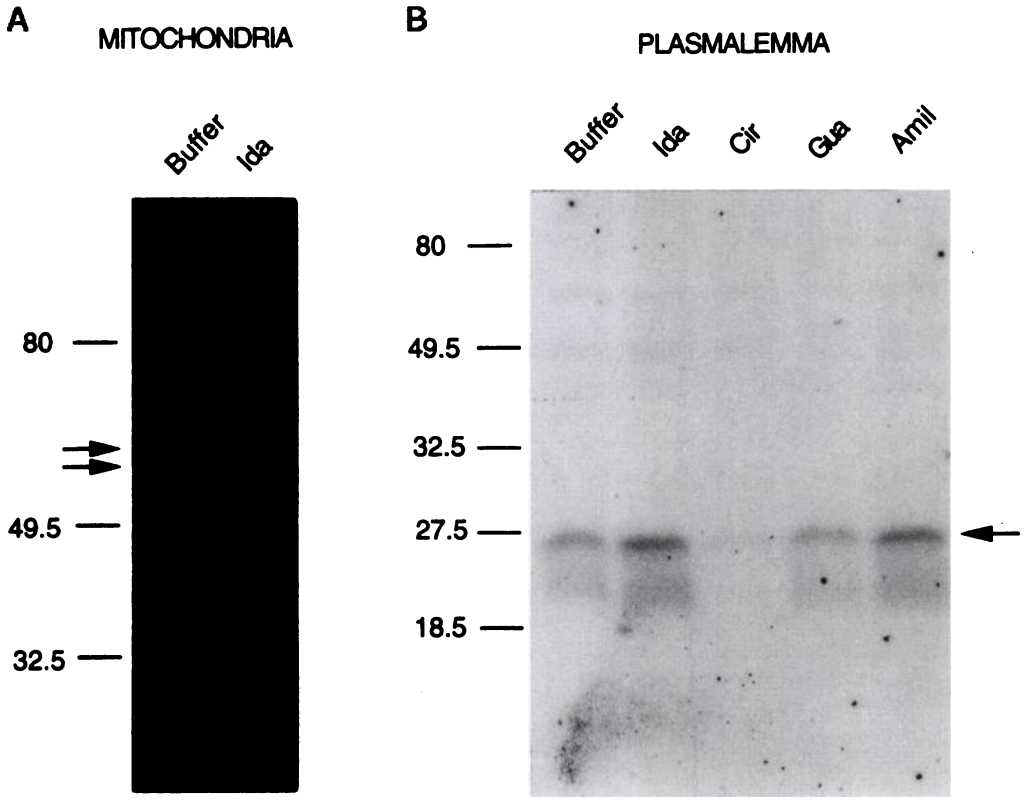


Fig. 3. Subcellular distribution of photolabeled peptides in rat liver. Rat liver homogenate was fractioned by density gradient centrifugation to generate samples enriched in plasmalemma or mitochondria as described in Experimental Procedures. Aliquots (25–100 μg membrane protein) were photoaffinity labeled with [^{125}I]AZIPI (2 nM), and labeled species were visualized after SDS-PAGE and autoradiography. Mitochondrial (*left*) or plasmalemmal (*right*) fractions were applied to 10% and 17% polyacrylamide gels, respectively. Similar results were obtained in two (*right*) or five (*left*) separate experiments. Competing ligand concentration was 10 μM. *Ida*, idazoxan; *Cir*, cirazoline; *Gua*, guanabenz; *Amil*, amiloride.

indicating a population of binding sites that exhibit similar affinity for the radioligand (17). To determine whether the complex binding data reflect differential recognition of the M_r ~55,000 and ~61,000 peptides, we evaluated the effect of increasing ligand concentrations on the photoincorporation of probe into the two labeled peptides in rat mitochondria (Fig. 4). Although cirazoline competed with similar affinity for the labeling of both peptides (IC_{50}, ~50 nM), guanabenz exhibited higher affinity for the M_r ~61,000 peptide (IC_{50}, ~200 nM) versus the M_r ~55,000 peptide (IC_{50}, ~3000 nM) (Fig. 4). These data are consistent with the results of competition binding experiments with [^{125}I]AMIPI in which cirazoline and guanabenz generated monophasic or biphasic competition curves, respectively (17).

Photoaffinity labeling of imidazoline binding proteins in secretory tissue of rat, rabbit, and hamster. Various imidazoline compounds exert poorly understood effects in secretory tissues such as pancreatic B cells, kidney, and salivary gland. To gain insight into the role of these imidazoline binding proteins in these tissues, the photoaffinity adduct was used to label their ligand binding subunit in the pancreatic B cell lines RIN-5AH and HIT-T15 as well as

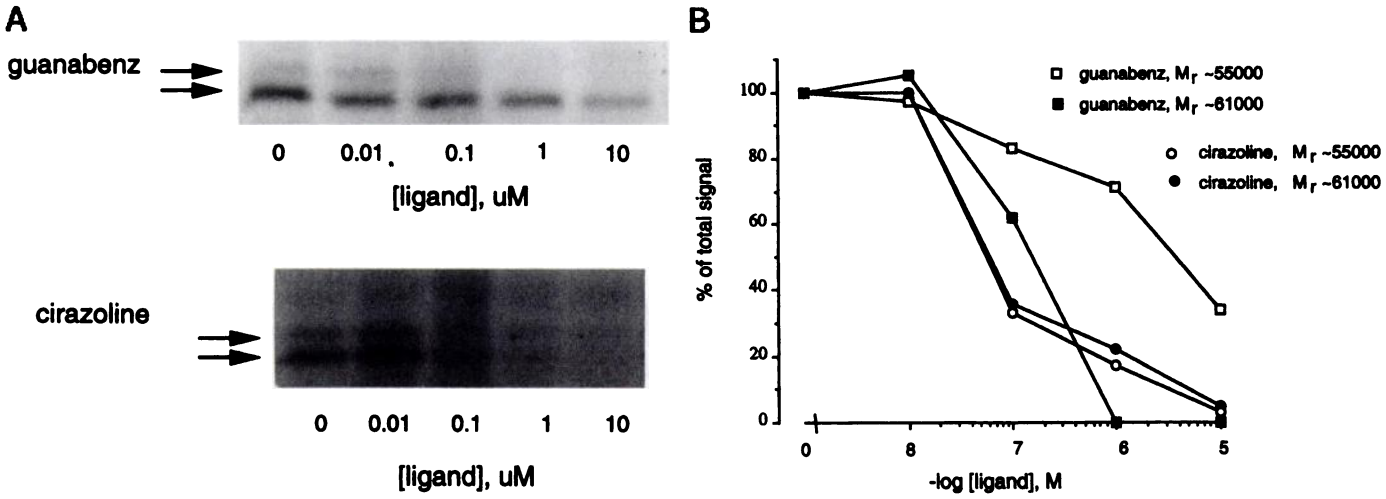


Fig. 4. Inhibition of photoaffinity labeling of rat liver membranes by the guanidinium guanabenz and the imidazoline cirazoline. A, Aliquots (~50 μg) of rat liver membrane fractions enriched in mitochondria were photoaffinity labeled with [^{125}I]AZIPI (0.8 nM) in the absence and presence of increasing concentrations of guanabenz or cirazoline. Autoradiographs were evaluated by densitometric analysis to determine the relative intensity of the labeled species. B, Data were expressed as a percentage of the signal generated in the absence of competing ligand. The experiment was performed twice with essentially identical results. *Arrows*, M_r ~55,000 and ~61,000 peptides.

in rat and rabbit salivary gland. Fig. 5 indicates that the M_r and/or ligand recognition properties differ in the four membrane preparations. In rabbit salivary gland membranes, the labeling of the M_r ~55,000 and ~61,000 (Fig. 5A) peptides was similar to that observed in rat kidney and brain, whereas only the M_r ~61,000 peptide was observed in rat salivary gland (Fig. 5B).

Both the M_r ~55,000 and ~61,000 peptides were expressed in the rat pancreatic B cell line RIN-5AH, whereas only the M_r ~61,000 peptide was expressed in the hamster B cell line HIT-T15 (Fig. 5C). The labeled peptides in the rat pancreatic B cell line did not recognize the imidazoline clonidine, whereas photolabeling of the M_r ~61,000 peptide in the hamster B cell line was blocked by clonidine (Fig. 5C). Clonidine sensitivity is of note due to the high affinity of this ligand for some types of imidazoline binding proteins versus its low affinity for IGRS or type 2 imidazoline binding proteins (22). The differences in the sensitivity of photolabeling to inhibition by clonidine in the rat and hamster pancreatic B cell lines may be due to species differences or the particular properties of the two cell lines. However, ligand autoradiography in rat forebrain sections with [^{125}I]AMIPI indicates selective areas that express binding sites recognized by clonidine (16) as well as cirazoline, which is consistent with the expression of clonidine-sensitive imidazoline binding sites in rat tissue. The expression of a single M_r ~61,000 peptide is not restricted to hamster as it is also observed in the pheochromocytoma cell line PC12 and rat salivary gland

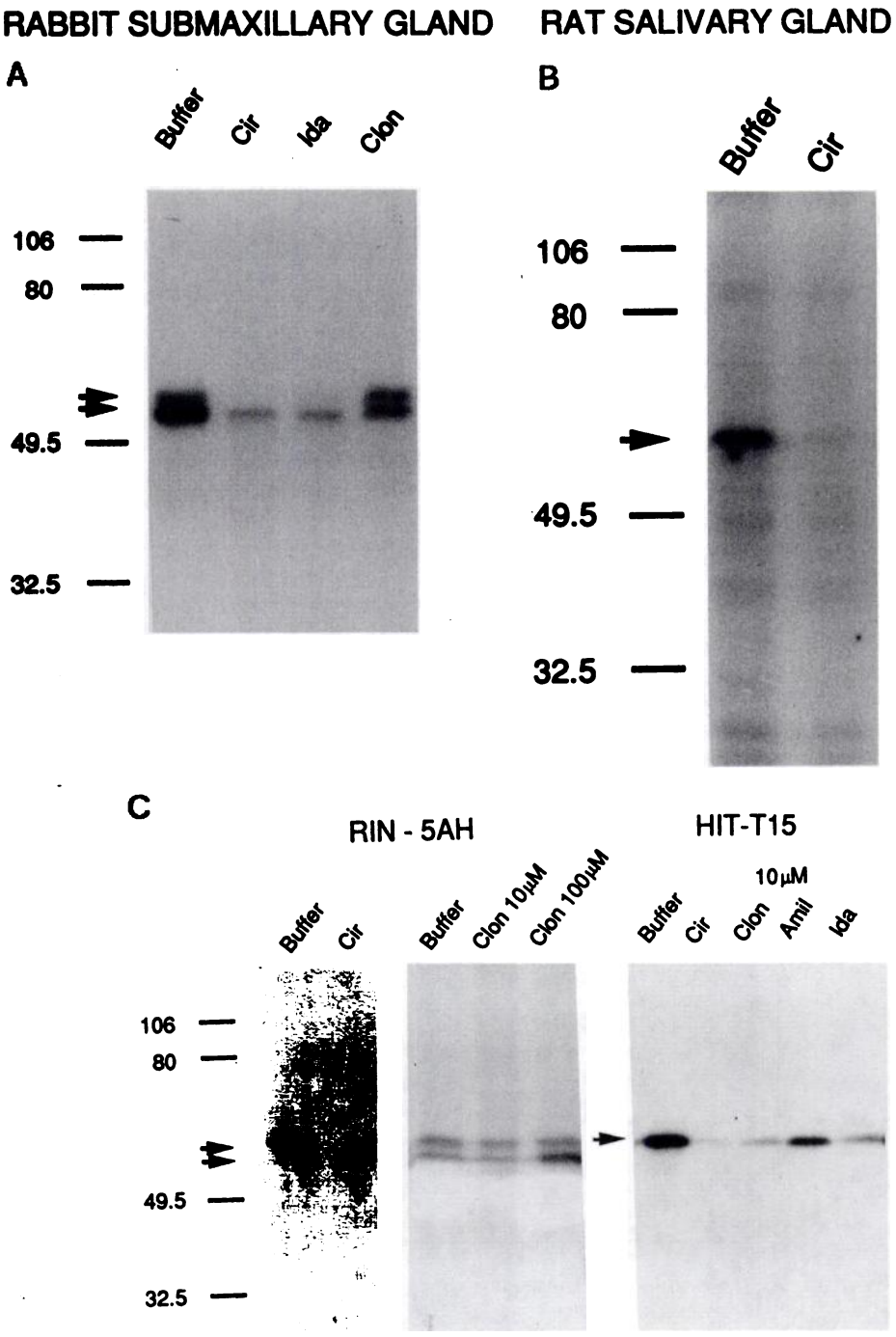


Fig. 5. Photoaffinity labeling of membranes prepared from salivary gland tissue and RIN-5AH or HIT-T15 pancreatic B cell lines. Membranes were prepared, and aliquots (200 μg membrane protein) were photolabeled with [^{125}I]AZIPI (2 nM) in the presence and absence of various competing ligands as described in Experimental Procedures and the legend to Fig. 2. A and B, Rabbit and rat salivary gland membranes, respectively, were prepared from frozen tissue. C, RIN-5AH or HIT-T15 cell membranes were generated from confluent 100-mm tissue culture plates. Similar results were obtained in three experiments with different membrane preparations. Unless indicated otherwise, competing ligand concentration was 10 μM. *Ida*, idazoxan; *Cir*, cirazoline; *Amil*, amiloride; *Clo*, clonidine.

(1) (Fig. 5A). However, the labeled species in the latter two tissues are relatively insensitive to clonidine. The M_r ~61,000 peptides in the rat PC12 cell line and rat salivary gland also differ from each other in their ability to recognize idazoxan (1), further illustrating the ligand recognition complex properties exhibited by imidazoline binding proteins among different tissues of the same species.

The pharmacological profile of ligands competing for photolabeling in membranes of rat salivary gland and RIN-5AH cells was similar to that observed in rat kidney and liver.[3] Saturation labeling experiments were performed with rat tissues to determine the relative affinities of the labeled peptides for the photoaffinity adduct. [^{125}I]AZIPI exhibited similar affinity (K_d, ~2–3 nM) for the M_r ~55,000 and ~61,000 peptides in RIN-5AH and the M_r ~61,000 peptide labeled in rat salivary gland (Fig. 6).

Photoaffinity labeling of imidazoline binding proteins in rabbit kidney. Rabbit kidney membrane preparations were used in initial studies of IGRS; this tissue also served as a source for purification of a single imidazoline binding protein (5, 14). Photoaffinity labeling of rabbit kidney membranes indicated two labeled species of M_r ~55,000 and ~61,000, similar to those expressed in rat tissues (Fig. 7A). In contrast to the results in rat kidney versus salivary gland, rabbit salivary gland membrane preparations expressed photolabeled species similar to rabbit kidney. As was the case for rat mitochondria, the M_r ~55,000 peptide predominates in most membrane preparations, and it also recognized various competing ligands with lower affinity compared with the M_r ~61,000 peptide (Fig. 7A). Photolabeling of both the M_r ~55,000 and ~61,000 peptides was in part inhibited by the imidazoline clonidine. Photoincorporation of [^{125}I]AZIPI was not altered by the α_2 adrenergic receptor antagonist rauwolscine, the α_1 adrenergic receptor antagonist prasozin. or the adrenergic agonist epinephrine (Fig. 7A). Essentially no difference in the labeling pattern was observed in low- versus high-speed membrane fractions obtained by differential centrifugation, a procedure that provides a gross separation of membrane organelles (Fig. 7B).

[^{3}H]Idazoxan is commonly used to identify members of the family of imidazoline binding proteins in various tissues, and its binding properties are influenced by the monovalent cation potassium (8, 15, 17). These effects of potassium appear to involve the M_r ~61,000 peptide as the photoincorporation of [^{125}I]AZIPI into this species was blocked by 100 mM KCl (Fig. 7B). Photolabeling of both the M_r ~55,000 and ~61,000 peptides was blocked by the potassium channel blocker 4-aminopyridine (inhibition of photolabeling of the M_r ~55,000 peptide by 4-aminopyridine was incomplete) but was not altered by the ATP-sensitive potassium channel agonist diazoxide or the potassium channel antagonists dendrotoxin, glibenclamide, or charybdotoxin (Fig. 7B)[3]. The effects of 4-aminopyridine, dendrodotoxin, and diazoxide on [^{125}I]AZIPI photoincorporation paralleled the results obtained in competition binding studies with [^{3}H]idazoxan.[3]

Distribution of IGRS relative to isoforms of MAO. As discussed, several reports indicate a relationship between IGRS and MAO, an enzyme that participates in the metabolism of monoamine neurotransmitters. The M_r of the larger photolabeled peptides in the rat and rabbit tissues is indistinguishable from that of MAO isoforms (MAO A, M_r ~58,000–63,000; MAO B, M_r ~53,000–57,000). To further address this issue, we determined the ability of active site enzyme inhibitors to alter photolabeling of the ligand binding subunit of IGRS. The photolabeling pattern observed in rabbit kidney membranes was not altered by the MAO inhibitor pargyline, which is recognized by both the A and B isoforms of MAO (Fig. 7B). The MAO inhibitors pargyline and clorgyline also did not compete for [^{3}H]idazoxan binding to rabbit kidney IGRS.[3] These data indicate that the two classes of compound interact with distinct sites on the enzyme.

We also addressed the relationship between MAO and IGRS by comparing the tissue distribution of MAO isoforms with that of the ligand binding subunit of IGRS. Immunoblotting of various membrane preparations with polyclonal antisera generated against a peptide shared by both MAO A and B indicated that the distribution of the photolabeled peptides in general corresponded to that of the two MAO isoforms. Thus, in rat liver and kidney, tissues in which both M_r ~55,000 and ~61,000 peptides were photoaffinity labeled with [^{125}I]AZIPI, the MAO antisera detected peptides of a M_r consistent with the presence of MAO A and B (Figs. 2 and 8). MAO A was the predominant enzyme isoform in rat salivary gland and the pancreatic B cell line HIT-T15, tissues in which only the photolabeled M_r ~61,000 peptide was visualized (Figs. 5 and 8). Similarly, the MAO B isoform predominated in tissues where the M_r ~55,000 peptide was the major photolabeled species (Figs. 5, 7, and 8).

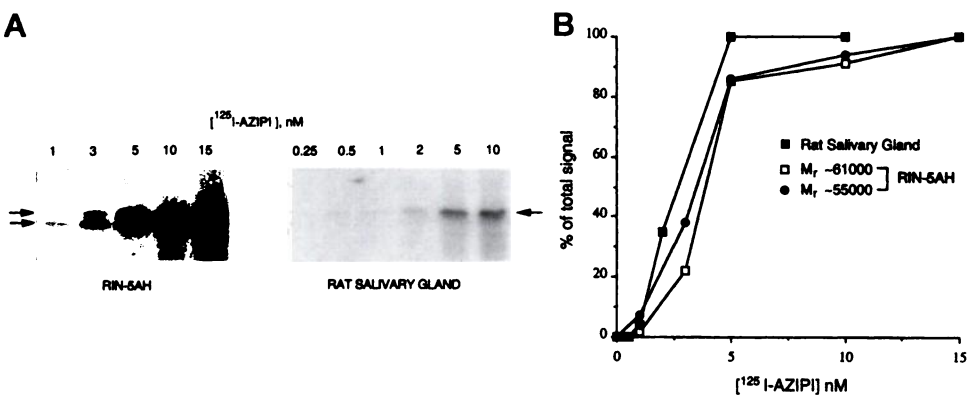


Fig. 6. Ligand recognition properties of the photolabeled species in rat salivary gland and the RIN-5AH pancreatic B cell line. A, Aliquots of membrane protein (100 μg) were photolabeled with increasing concentrations of [^{125}I]AZIPI. The relative amounts of the labeled species were determined by densitometric analysis of the autoradiographs. B, Data were expressed as a percentage of the density observed at the highest concentration of radioligand. The calculated K_d values were ~3 nM for M_r ~55,000 and ~2 nM for M_r ~61,000 for the labeled peptides in RIN-5AH cells and ~3.5 nM for the labeled peptide in rat salivary gland. *Arrows*, M_r ~55,000 or ~61,000 peptides.

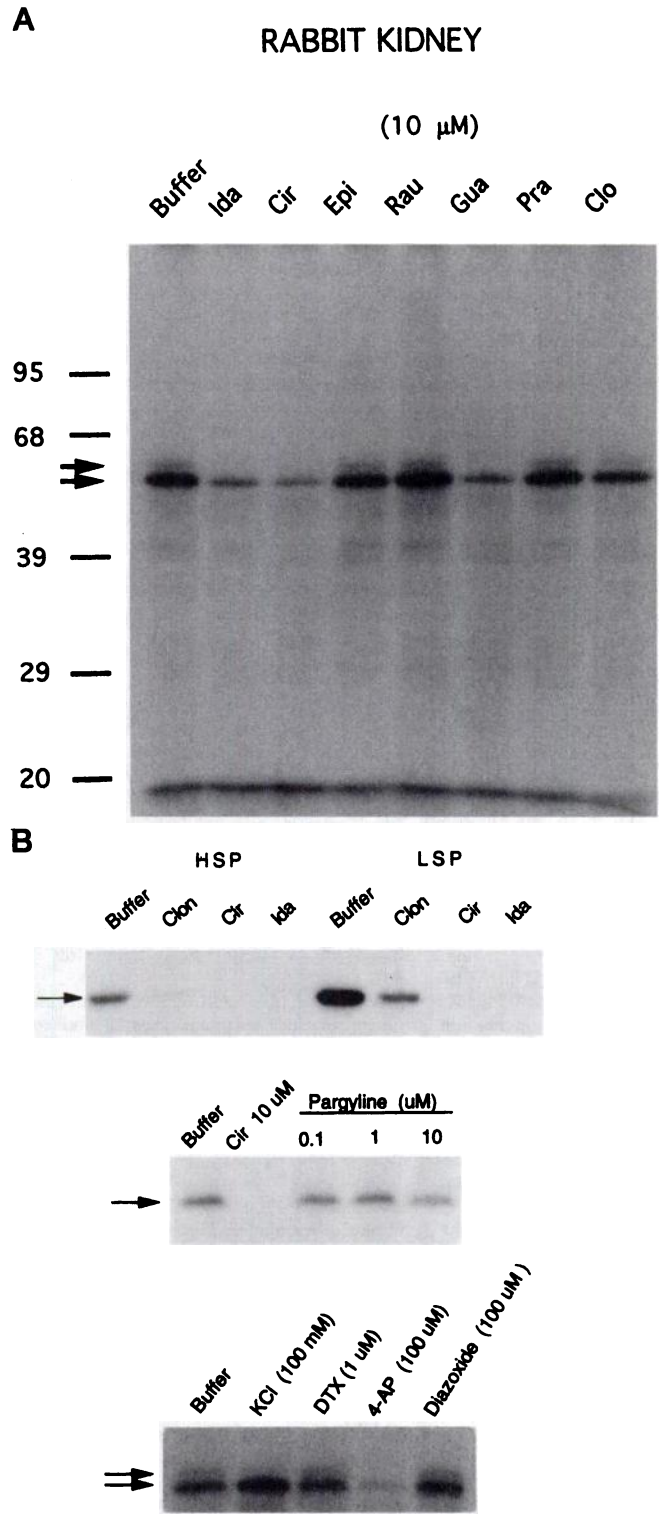


Fig. 7. Photoaffinity labeling of IGRS in rabbit kidney membranes. Rabbit kidney membranes were prepared from frozen or freshly isolated tissues, and 150 μg membrane protein was photolabeled with [^{125}I]AZIPI (1 nM) as described in Experimental Procedures. Samples were electrophoresed as described in the legend to Fig. 1. Representative of the results generated in three to eight experiments with different membrane preparations. The relative amounts of the labeled peptides in rabbit kidney varied in different tissue sources with the M_r ~55,000 peptide predominating over the M_r ~61,000 peptide. Rabbit kidney membranes were homogenized and centrifuged in a Sorvall RC-5B (SS-34 rotor) at 1000 × *g* for 10 min to obtain a low-speed pellet (*LSP*). The supernatant of the 1000 × *g* centrifugation was then centrifuged in the same rotor at 30,000 × *g* to generate a high-speed pellet (*HSP*). Unless otherwise noted, all photolabeling experiments used the membrane pellet generated by centrifugation at 30,000 × *g*. *Arrows*, Labeled M_r ~55,000 and ~61,000 peptides. B, *top*, Competing ligands were used at a concentration of 10 μM. *Ida*, idazoxan; *Cir*, cirazoline; *Gua*, guanabenz; *Amil*, amiloride; *Clo*, clonidine; *Epi*, epinephrine; *Rau*, rauwolscine; *Pra*, prazosin; *4-AP*, 4-aminopyridine; *DTX*, dendrotoxin.

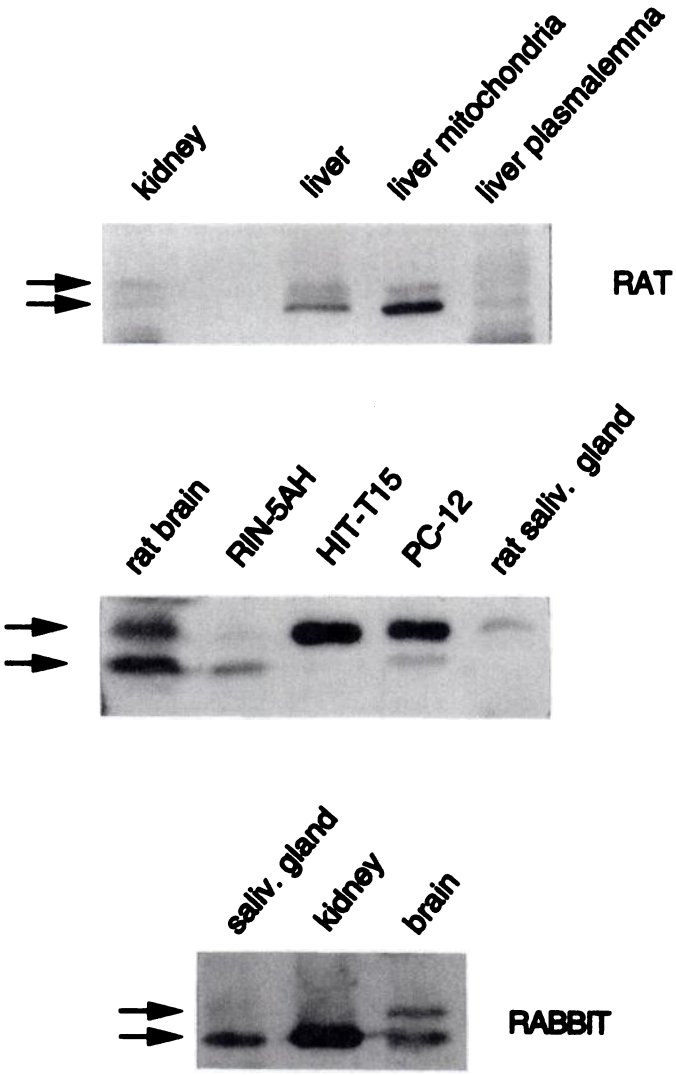


Fig. 8. Tissue distribution of MAO A and B isoforms. Aliquots of membrane preparations used for photoaffinity labeling were electrophoresed on denaturing polyacrylamide gels, transferred to nitrocellulose, and immunoblotted as described in Experimental Procedures. Nitrocellulose transfers were immunoblotted with polyclonal antisera (1:2000) generated in rabbits with a peptide found in the A and B isoforms of MAO. Similar results were obtained with two or three separate immunoblots. Membrane protein loaded (μg) was rat kidney, 300; rat liver, 150; rat mitochondria, 65; rat plasmalemma, 150; rat brain, 200; RIN-5AH, 150; HIT-T15, 40; PC-12, 75; rat salivary gland, 150; rabbit salivary gland, 400; rabbit kidney, 100; and rabbit brain, 250. *Top*, *kidney* and *liver* refer to the high-speed pellet membrane fraction generated as described in Experimental Procedures.

Discussion

Imidazoline binding proteins may represent a functional target for the diverse ligands that these entities recognize. Such a thought was given impetus by the isolation of endogenous substances that also interact with such proteins and the pharmacological actions of these compounds/endogenous substances

that apparently are not mediated by known signaling systems. However, the functionality of such protein binding sites is unclear as no second-messenger system is clearly modified as a result of ligand interaction and some members of the family are localized to the mitochondria in liver and kidney.

Identification of imidazoline binding proteins is a goal of several laboratories. Speculation on the identity of some types of imidazoline binding proteins as a potassium channel/transporter was based on the ability of some potassium channel ligands to compete for [^{3}H]idazoxan binding in rat liver (9) and the allosteric regulation of [^{3}H]idazoxan binding to rabbit kidney IGRS by potassium. However, radioligand binding to the imidazoline binding protein is altered only by certain potassium channel ligands that are relatively nonselective. Inhibition of [^{3}H]idazoxan binding or photoaffinity labeling of rabbit kidney IGRS by 4-aminopyridine may represent an action of the molecule unrelated to its ability to interact with potassium channels.

Stronger evidence indicates that some members of the family of imidazoline binding proteins are MAO. This conclusion is based on the common intracellular location of the two entities, their similar or identical M_r, the copurification of ligand binding with enzyme activity, and heterologous expression systems using cDNA clones encoding MAO isoforms (5, 7, 14). In addition, antibodies to MAO A or B immunoprecipitate photoaffinity labeled IGRS in human placenta and human liver mitochondria (16). However, several points suggest that there are imidazoline binding proteins that are not MAO isoforms. First, the location of monoamine isoforms do not strictly correlate with the expression of IGRS and other members of this protein family. MAO isoforms appear to have a wider distribution than that of imidazoline binding proteins. Second, although relatively high concentrations of selected imidazolines or guanidiniums noncompetitively inhibit the activity of MAO (13, 14), it is difficult to explain most of their functional actions based on a modification of MAO enzymatic activity. Nevertheless, the present report indicates that the two ligand binding subunits of IGRS in rat and rabbit kidney, salivary gland, and pancreatic B cells reside on M_r ~55,000 and ~61,000 peptides, the apparent molecular weight and relative tissue distribution of which are similar to those of the MAO isoforms B and A, respectively. Of note is that both radioligand binding and photolabeling experiments indicate that active site inhibitors (i.e., pargyline) of MAO and imidazoline/guanidinium compounds do not interact with a common binding site (14, 16, 23), suggesting that MAO may subserve additional functions and/or receive multiple regulatory input.

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Send reprint requests to: Stephen M. Lanier, Ph.D., Department of Pharmacology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425.