

Analysis of the Pharmacological and Molecular Heterogeneity of I₂-Imidazoline-Binding Proteins using Monoamine Oxidase-Deficient Mouse Models

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ABSTRACT

The I₂ subgroup of imidazoline-binding sites was identified as monoamine oxidases (MAOs), but it is unclear whether there are I₂-binding sites located on proteins distinct from MAOs. To address this issue, we characterized I₂-binding proteins in liver and brain of wild-type and MAO A- and MAO B-deficient mice. I₂-binding sites were identified using [³H]idazoxan and the photoaffinity adduct 2-[3-azido-4-[¹²⁵I]iodophenoxy]methylimidazoline ([¹²⁵I]AZIPI). [³H]idazoxan labeled binding sites with ligand recognition properties typical of I₂ sites in both brain and liver of wild-type mice. High-affinity, specific [³H]idazoxan binding were not altered in MAO A knockout (KO) mice. In contrast, [³H]idazoxan binding was completely abolished in both liver and brain of MAO B KO mice. In wild-type mice, [¹²⁵I]AZIPI

photolabeled three proteins with apparent molecular masses of ~28 (liver), ~61 (brain), and ~55 kDa (liver and brain). The photolabeling of each protein was blocked by the imidazoline cirazoline (10 μM). Photolabeling of the ~61- and ~55-kDa proteins was not observed in MAO A and B KO mice, respectively. In contrast, photolabeling of the liver ~28-kDa protein was still observed in MAO-deficient mice, indicating that this protein is unrelated to MAOs. These data indicate that I₂ imidazoline-binding sites identified by [³H]idazoxan reside solely on MAO B. The binding sites on MAO A and the liver ~28-kDa protein may represent additional subtypes of the family of the imidazoline-binding sites.

I₂-binding sites (I₂BS) are members of a family of imidazoline-binding proteins that recognize imidazolines, guanidiniums, and structurally/related derivatives with nanomolar affinity and do not recognize agonists for known neurotransmitter systems (Michel and Ernsberger, 1992; Parini et al., 1996). These binding sites were originally detected using [³H]idazoxan (Coupry et al., 1987; Limon et al., 1992), and at present, this radioligand is considered the “reference” ligand to identify I₂BS. More recently, it has been shown that I₂BS can be labeled with high affinity by other radioligands including 2-[3-amido-4-[¹²⁵I]iodophenoxy]methylimidazoline (Ivkovic et al., 1994) and its photoaffinity derivative

2-[3-azido-4-[¹²⁵I]iodophenoxy]methylimidazoline ([¹²⁵I]AZIPI) (Lanier et al., 1993). Whereas [³H]idazoxan appears selective for I₂BS, [¹²⁵I]AZIPI may recognize multiple imidazoline-binding proteins (Lanier et al., 1993, 1995).

I₂BS were identified as monoamine oxidases (MAOs), using different experimental approaches (Tesson et al., 1995; Raddatz et al., 1997), two mitochondrial enzymes involved in the degradation of endogenous (e.g., epinephrine, norepinephrine, dopamine, and serotonin) and dietary (e.g., tyramine) (Shih et al., 1999) amines. However, recent studies suggested the existence of I₂ imidazoline-binding sites located on proteins distinct from MAOs. First, a polyclonal antibody directed against a 70-kDa imidazoline-binding protein purified from bovine adrenal medulla (Wang et al., 1992, 1993) revealed two peptides in human and rat brain with apparent molecular masses different from those reported for MAO A (~61 kDa) and MAO B (~55 kDa) (Escriba et al., 1994); second, a ~25-kDa protein was detected in rat liver using the photoaffinity probe [¹²⁵I]AZIPI (Lanier et al., 1995)

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ABBREVIATIONS: I₂BS, I₂-imidazoline-binding sites; MAO, monoamine oxidase; [¹²⁵I]AZIPI, 2-[3-azido-4-[¹²⁵I]iodophenoxy]methyl imidazoline; KO, knockout.

Third, [³H]idazoxan-binding sites in 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid-solubilized rat brain membranes were eluted from a size exclusion chromatography column at four fractions corresponding to apparent molecular masses of 130 to 140, 70, 50, and 35 kDa (Escriva et al., 1995).

Although these observations suggest the existence of I₂BS distinct from MAOs, the wide tissue distribution and relatively high levels of MAO A and B complicate the identification and characterization of additional members of the I₂ subgroup of imidazoline-binding proteins. To address this issue, we characterized imidazoline-binding proteins in tissues from mice in which the gene encoding MAO A (Cases et al., 1995) and MAO B (Grimsby et al., 1997) was disrupted and thus did not express the respective proteins.

Materials and Methods

Drugs and Chemicals. Polyvinylidene difluoride membranes were purchased from New England Nuclear Life Science Products (Paris, France). [³H]Idazoxan (42 Ci/mol) was obtained from Amersham International (Buckinghamshire, England), and rauwolscine was from Roth (Karlsruhe, Germany). Acrylamide, bisacrylamide, and Tween 20 were purchased from Bio-Rad (Ivry/Seine, France). Cirazoline was a gift from Synthelabo (Paris, France). The precursor for the synthesis of the photoaffinity adduct 2-[3-azido-4-[¹²⁵I]iodophenoxy]methylimidazoline was kindly provided by Dr. Bakthavachalam at Research Biochemicals International (Natick, MA). Atipamezole, dexmetomidine, and demetomidine were a gift from Mika Scheinin (University of Turku, Finland). All remaining drugs and chemicals were purchased from Sigma (Paris, France).

Membrane Preparation. MAO A- and B-deficient mice and their corresponding C3H and 129/Sv wild-type mice were generated as previously described (Cases et al., 1995; Grimsby et al., 1997). Experiments in rats were performed in the Sprague-Dawley strain. Ten- to 12-week-old mice and rats were sacrificed, and tissues were removed and rinsed in ice-cold buffer containing 1 mM MgCl₂, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 50 mM Tris-HCl, pH 7.4 (buffer A). After homogenization in a Dounce homogenizer (pestle A), tissues were filtered through two layers of cheesecloth mesh and centrifuged at 600g for 10 min at 4°C. The supernatant was decanted and centrifuged at 28,000g for 30 min. The resulting pellet was washed twice in buffer A and stored at -80°C. Membrane protein concentrations were determined by the Bio-Rad protein assay based on the technique described by Bradford (1976) using bovine γ -globulin as a standard.

Immunoblots. Membrane proteins were solubilized in loading buffer (60 mM Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 1% β -mercaptoethanol, and 0.05% bromphenol blue) at 100°C for 5 min and subjected to 9% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes with a semidry electroblotter (Trans-blot, Bio-Rad) for 1 h at 450 mA. The blots were blocked with 5% nonfat dried milk in wash buffer (phosphate buffered saline, pH 7.5, and 0.1% Tween 20) overnight at 4°C, washed twice, and incubated for 1 h at room temperature with a rabbit polyclonal antisera obtained from rabbits immunized with the peptide TNGGQERKQFVGGSGQ corresponding to amino acids 211 to 225 in MAO A and 202 to 216 in MAO B. After the blots were washed, they were incubated with peroxidase-labeled anti-rabbit IgG in wash buffer for 30 min. After the blots were washed extensively, the antibody was detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to Amersham Hyperfilm-MP film.

Radioligand Binding. For saturation-binding isotherms, membranes (200–300 μ g) were incubated with various concentrations (1–85 nM) of [³H]idazoxan at 24°C for 60 min in a final volume of 125

μ l of buffer A in the presence of 10⁻⁵ M rauwolscine to mask α 2-adrenoceptors. Incubation was stopped by vacuum filtration (Whatman GF/C) with 3 \times 5 ml washes with buffer containing 10 mM Tris-HCl, pH 7.4, at 4°C. Filters were placed in 4 ml of Packard Emulsifier-Safe scintillation fluid, and bound radioactivity was counted in a liquid scintillation spectrometer (Packard, model Tri-Carb 4000) at 56% efficiency. Nonspecific binding was defined in the presence of 10⁻⁵ M cirazoline and represented 30 to 50% of the total binding depending on the radioligand concentrations used.

[³H]Idazoxan-binding parameters were evaluated using a nonlinear least square curve-fitting procedure (Prism GraphPad, San Diego, CA), and statistical comparisons of the results were obtained by using the Student's unpaired *t* test. Results are expressed as the means \pm S.E.M.

Photoaffinity Labeling. The cirazoline derivative, 2-(3-amino-4-iodophenoxy)methylimidazoline was iodinated and converted to the photolabile azide [¹²⁵I]AZIPI for use as a photoaffinity adduct as described previously (Lanier et al., 1993). Membranes were incubated in reduced light with 1 to 2 nM [¹²⁵I]AZIPI for 30 min at 24°C, chilled on ice, and diluted 10-fold with buffer A containing 2 mM dithiothreitol. Samples were immediately photolyzed at 4°C for 5 min in a photolysis chamber (320 nm). Photolabeled membranes were centrifuged, solubilized in sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10% SDS, 2- β -mercaptoethanol, and 0.05% bromphenol blue) at 100°C for 5 min, and finally subjected to SDS-(9%)-polyacrylamide gel electrophoresis. After the sample was electrophoresed, the gels were dried under vacuum and analyzed using a 445 SI phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Results

Imidazoline-binding sites were characterized in membrane preparations from liver and brain because these tissues may express a heterogeneous population of I₂BS (Escriva et al., 1994, 1995; Lanier et al., 1995) and they differ for the relative expression of each MAO isoform (Saura et al., 1992). The actual protocol used for "crude" membrane preparation was selected because it was similar to that used by other authors to show the existence of I₂-binding sites located on proteins distinct from MAO (Escriva et al., 1994, 1995, 1996).

Binding studies were carried out at [³H]idazoxan concentrations ranging from 1 to 85 nM in the presence of the α 2-adrenoceptor antagonist rauwolscine (10⁻⁵ M) to prevent radioligand binding to α 2-adrenoceptors. In MAO A and MAO B parent strains, [³H]idazoxan interacted with a saturable, high-affinity population of binding sites (Figs. 1 and 2). Competition studies indicated that [³H]idazoxan binding was inhibited by a series of imidazoline and guanidinium derivatives with the following order of potency: cirazoline (pK_i 8.83 \pm 0.12) > guanabenz (pK_i 8.35 \pm 0.11) > amiloride (pK_i 6.7 \pm 0.06) \gg clonidine (pK_i 5.2 \pm 0.11). This pharmacological profile is in agreement with that previously reported for I₂BS (Regunathan and Reis, 1996). According to the relative expression of the MAO isoforms in liver and brain, [¹²⁵I]AZIPI photolabeled an ~55-kDa protein in liver and two ~55- to 61-kDa proteins in brain of wild-type mice (Figs. 1 and 2). The ~55- and ~61-kDa species were previously identified as MAO B and MAO A, respectively, by purification/amino acid sequencing and immunoprecipitation of the photolabeled peptides by monoclonal antibodies to MAO B and MAO A (Raddatz et al., 1995; Tesson et al., 1995).

[³H]Idazoxan binding in liver and brain membrane preparations of MAO A KO mice was similar to that found in wild-type mice (Table 1 and Fig. 1). In contrast, photoaffinity

labeling experiments and immunoblot analysis in brain of MAO A-deficient mice indicated the absence of the ~61-kDa peptide (Fig. 1). These results indicate that [³H]idazoxan, which is considered a reference ligand to identify I₂BS, may exhibit a low affinity for the imidazoline-binding site located on MAO A. To address this issue, we performed binding experiments in wild-type and MAO A KO mice using an [³H]idazoxan (135 nM) concentration higher than that required for saturation of the high-affinity sites. At this concentration, specific [³H]idazoxan binding in membranes from wild-type mice increased by 51% above that observed in saturation experiments (Fig. 3). The increase in binding capacity was much less evident in liver, which contains lower amounts of MAO A. The increase in binding capacity was completely abolished in MAO A KO mice, indicating that the low affinity [³H]idazoxan-binding site is associated with MAO A. These results indicate that, at the concentrations used in typical radioligand-binding studies to identify I₂BS, [³H]idazoxan does not label the MAO A imidazoline-binding site. In contrast, this binding site is labeled by the photoaffinity probe [¹²⁵I]AZIPI.

Experiments performed in MAO B KO mice showed that, compared with wild-type mice, [³H]idazoxan-specific binding was completely abolished in both liver and brain (Table 1 and Fig. 2). Two observations indicated that only nonspecific binding accounted for [³H]idazoxan binding to liver and brain

membranes from MAO B KO mice: first, ligand binding was not saturable and was a linear function ($r = 0.95$; Fig. 2), and second, it was not inhibited by 10 μ M cirazoline, guanabenz, amiloride, or clonidine. To verify that [³H]idazoxan binding to I₂BS distinct from MAO B could be detected in partially purified plasma membranes, we generated membrane fractions 5- to 6-fold enriched in 5'-nucleotidase, a plasma membrane marker. Although enriched in 5'-nucleotidase, such membrane preparations also contained mitochondrial membranes as reflected by the presence of MAO, determined by enzyme assays. Despite the enrichment of plasma membranes, which are supposed to contain additional I₂-binding proteins, [³H]idazoxan-specific binding was abolished in both liver and brain of MAO B KO mice (data not shown). These results indicate that, as previously reported in human and rabbit liver (Remaury et al., 1998), the expression of MAOs is necessary for the detection of [³H]idazoxan binding to I₂BS. The loss of [³H]idazoxan binding to liver and brain membranes was associated with the disappearance of the immunodetectable and photolabeled ~55-kDa peptide. These data indicate that, in both brain and liver, 1) [³H]idazoxan binds with high affinity exclusively to MAO B and 2) this binding site is also labeled by [¹²⁵I]AZIPI.

In addition to the I₂BS on MAO B, [¹²⁵I]AZIPI also labeled an ~28-kDa peptide in liver (Fig. 4). [¹²⁵I]AZIPI photoincorporation into the ~28-kDa peptide was still observed in liver

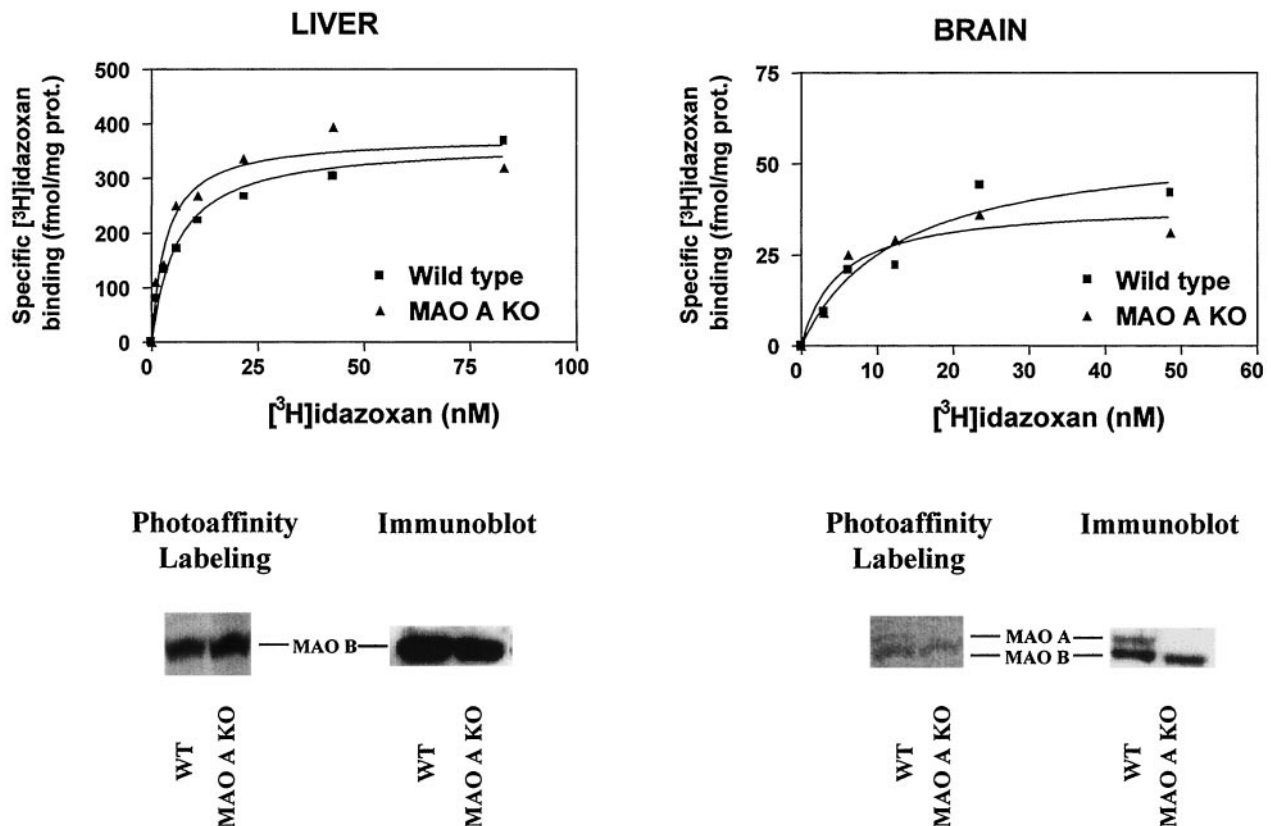


Fig. 1. [³H]idazoxan binding, [¹²⁵I]AZIPI photoaffinity labeling, and MAO immunodetection in wild-type (WT) and MAO A KO mice. Top, liver or brain membranes were incubated with increasing concentrations of [³H]idazoxan (1–85 nM) for 60 min at 25°C. Cirazoline (10 μ M) was used to define nonspecific binding, and rauwolfscine (10 μ M) was added to the incubation medium to mask α 2-adrenoceptors. Bottom, for photolabeling experiments, membranes (300 μ g) were incubated with [¹²⁵I]AZIPI (1–2 nM). After an SDS-polyacrylamide gel electrophoresis, the gels were dried under vacuum and exposed for 24 h in a Storage Phosphor Screen for detection with a Molecular Dynamics 445 SI PhosphorImager. For immunoblots, liver and brain membranes (50 μ g) were electrophoresed on a 9% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The blot was incubated with a rabbit anti-MAO A/B polyclonal antiserum. Immunoreactive proteins were identified as described under *Materials and Methods*. These figures are representative of three independent experiments.

from MAO A and MAO B KO mice, indicating that this peptide is unrelated to MAOs. Based on the loss of high affinity [³H]idazoxan binding in the MAO B KO mice, it is

also apparent that the ~28-kDa peptide does not recognize the I₂-imidazoline ligand [³H]idazoxan. The ligand recognition properties of the ~28-kDa peptide were compared with

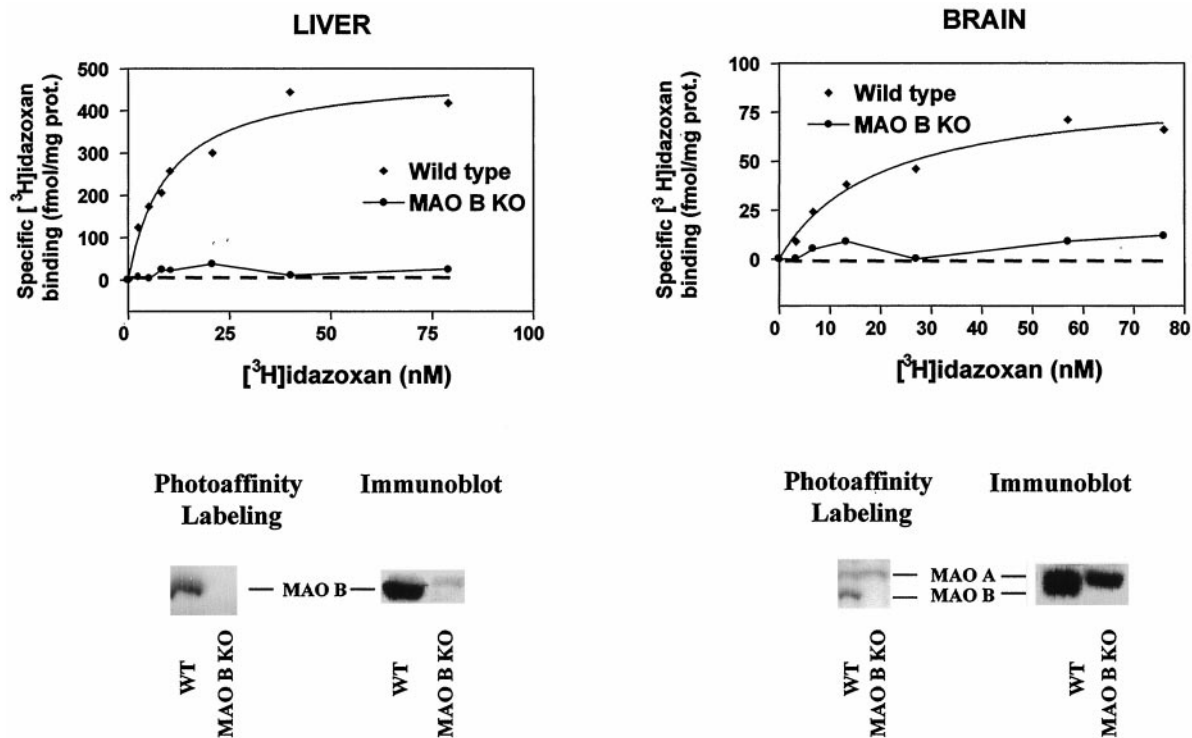


Fig. 2. [³H]idazoxan binding, [¹²⁵I]AZIPI photoaffinity labeling, and MAO immunodetection in wild-type (WT) and MAO B KO mice. [³H]idazoxan-binding (top) photolabeling experiments and immunoblots (bottom) were performed as described in the legend to Fig. 1. The results are representative of three independent experiments.

TABLE 1
 [³H]idazoxan-specific binding in wild-type and MAO-deficient mice
 Values represent the means ± S.E.M. of three independent experiments.

	Wild-Type MAO A		MAO A KO		Wild-Type MAO B		MAO B KO
	<i>B</i> _{max}	<i>K</i> _d	<i>B</i> _{max}	<i>K</i> _d	<i>B</i> _{max}	<i>K</i> _d	
	fmol / mg protein		fmol / mg protein		fmol / mg protein		
	nM		nM		nM		
Liver	364 ± 12.5	6.1 ± 0.9	375 ± 16	3.5 ± 1	455 ± 16.5	8.7 ± 0.9	N.D.
Brain	62 ± 6.5	11.5 ± 2.5	41 ± 6.5	6.4 ± 2.2	68 ± 5.5	9.4 ± 2.1	N.D.

N.D., not detectable.

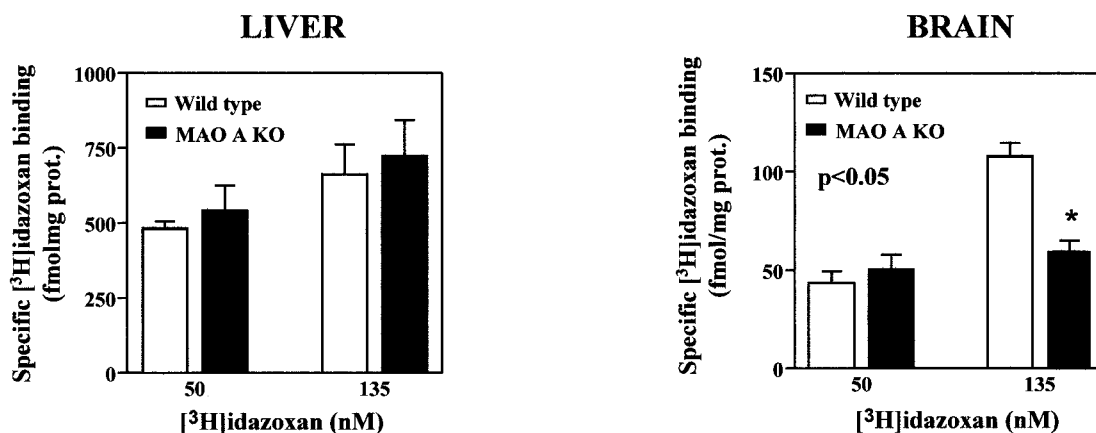


Fig. 3. Low-affinity [³H]idazoxan binding in liver and brain membranes from wild-type and MAO A KO mice. Membranes (100 μg) were incubated with 50 or 135 nM [³H]idazoxan for 60 min at 25°C. Cirazoline (10 μM) was used to define nonspecific binding, and rauwolscine (10 μM) was added to the incubation medium to mask α₂-adrenoceptors. Results are the means ± S.E.M. of three independent experiments. **P* < .05 compared with the control.

those of the I₂BS located on MAO B. Experiments were performed in rat liver, the tissue where the ~28-kDa peptide was first identified (Lanier et al., 1995). As shown in Fig. 5, [¹²⁵I]AZIPI photoincorporation into the ~28-kDa peptide was inhibited by cirazoline, dexmedetomidine, detomidine, atipamezole, and to a lesser extent, the histamine receptor antagonist cimetidine and the α₂-adrenoceptor agonist oxymetazoline (Fig. 5). In contrast, photolabeling of the imidazole binding domain on MAO B was not blocked by atipamezole, oxymetazoline, and cimetidine. These data indicate that the imidazole-binding sites located on the ~28-kDa peptide and MAO B differ in their pharmacological profiles. The fact that the ~28-kDa peptide in rat liver does not exhibit high affinity for the typical I₁ (e.g., clonidine) and I₂ (e.g., idazoxan and guanabenz) ligands (Lanier et al., 1995)

indicates that it may identify an additional subtype of imidazole-binding protein.

Discussion

During the last years, one of the major debates in the field of the imidazole-binding sites concerned the existence of a pharmacological and molecular heterogeneity of this protein family and, more particularly, the exclusive localization of the I₂ subtype on MAOs (Eglen et al., 1998). Using MAO-deficient mice, we show that I₂BS, as identified by high-affinity [³H]idazoxan binding, is in fact located exclusively on MAO B. Indeed, in both brain and liver, two organs supposed to contain a heterogeneous population of I₂BS, we found that the loss of MAO B led to the concomitant disappearance of specific, high-affinity [³H]idazoxan binding. Surprisingly, we did not observe any modification of [³H]idazoxan binding in MAO A-deficient mice compared with their wild-type parent strain. This was an unexpected result because MAO A was previously considered a member of the I₂ subgroup of the imidazole-binding sites based on two major observations: first, expression of human placenta MAO A in yeast led to the coexpression of [³H]idazoxan-binding activity (Tesson et al., 1995), and second, [¹²⁵I]AZIPI, which was considered a selective I₂ photoaffinity probe, labeled MAO A in various tissues and cells (Lanier et al., 1995). Our results indicate that the imidazole-binding site on MAO A displays, in fact, a low affinity for [³H]idazoxan. Indeed, as shown by the experiments performed in brain and liver of wild-type and MAO A KO mice, [³H]idazoxan concentrations higher than those required for saturation of the high-affinity I₂BS were

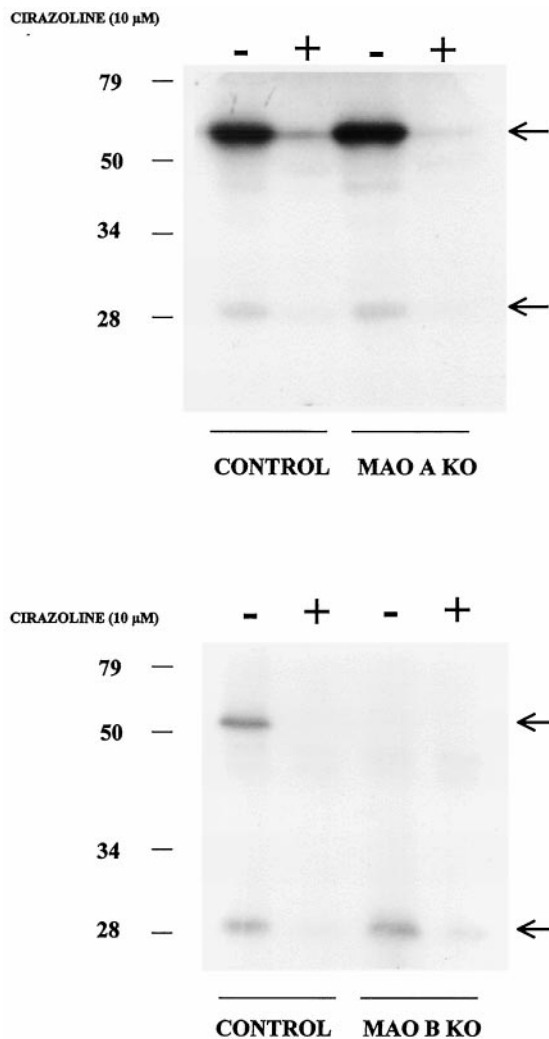


Fig. 4. Photoaffinity labeling of imidazole-binding proteins in wild-type and MAO KO mice liver. Membranes were prepared from the liver of wild-type or MAO KO mice, and aliquots (100 μg of membrane protein) were covalently labeled with the imidazole photoaffinity adduct [¹²⁵I]AZIPI in the presence (+) or absence (-) of cirazoline (10 μM) as described under *Materials and Methods*. Solubilized samples were electrophoresed under denaturing conditions on 8% SDS-polyacrylamide gels. Autoradiographs were obtained by exposing the dried gels at -70°C for 1 to 3 days. Arrows indicate the migration of the ~55 and ~28-kDa proteins. Similar results were obtained in two experiments. The migration of midrange molecular mass standards is indicated by the numbers to the left of the autoradiograph ($M_r \times 10^{-3}$). Each experiment was repeated twice with similar results.

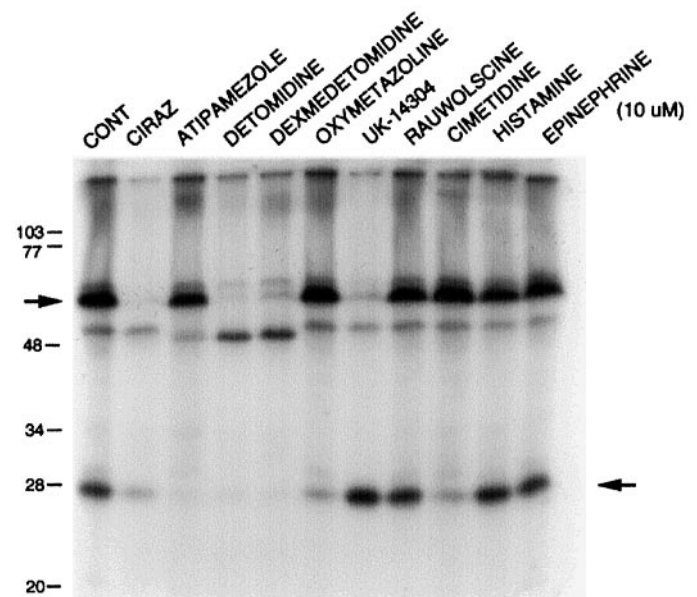


Fig. 5. Ligand recognition properties of the ~55- and ~28-kDa imidazole-binding proteins. Membranes were prepared from rat liver and covalently labeled with [¹²⁵I]AZIPI in the absence or presence of competing ligands (10 μM) as described under *Materials and Methods*. Membranes were solubilized and electrophoresed under denaturing conditions on 8% SDS-polyacrylamide gels. Autoradiographs were obtained by exposing the dried gels at -70°C for 1 to 3 days. Arrows indicate the migration of the ~55- and ~28-kDa proteins. Although not indicated by an arrow, small amounts of MAO A (~61 kDa) were also photoaffinity labeled. The migration of midrange molecular mass standards is indicated by the numbers to the left of the autoradiograph ($M_r \times 10^{-3}$). Each experiment was repeated twice with similar results.

required to label MAO A. The low affinity of MAO A for [³H]idazoxan may not be limited to mice but could a common property of the imidazoline-binding site located on this MAO isoform. Indeed, a relatively low affinity of MAO A for idazoxan has been also reported in human placenta (Diamant et al., 1992), liver (Raddatz et al., 1995), and yeast transformed with recombinant cDNA encoding human placenta MAO A (Tesson et al., 1995). These data suggest that, if the high affinity for idazoxan is a characteristic required to define an I₂BS, the imidazoline-binding site of MAO A may not belong to the I₂BS subtype.

The ~28-kDa protein labeled by [¹²⁵I]AZIPI is clearly unrelated to MAO A, MAO B, and I₂BS. Indeed, the loss of [³H]idazoxan binding observed in MAO B KO mice was not associated with the disappearance of [¹²⁵I]AZIPI photoincorporation into the ~28-kDa peptide. In addition, the imidazoline-binding site on the ~28-kDa peptide also differs from the I₂BS on MAO B in ligand recognition properties. In addition to their different affinities for idazoxan, these two imidazoline-binding sites show marked differences concerning the affinity for at least three compounds, atipamezole, oxymetazoline, and cimetidine. It was previously reported that [³H]atipamezole identifies an imidazoline-binding site distinct from the I₁ and I₂ subtypes in neonatal and adult rat lung (Sjoholm et al., 1995). This site exhibits some of the pharmacological properties of the liver ~28-kDa imidazoline-binding protein, namely, the inhibition by detomidine and dexmedetomidine and the low affinity for clonidine, UK-14304, and histamine. In contrast, the lung [³H]atipamezole and the liver ~28-kDa imidazoline-binding sites differ for their affinity for cirazoline, cimetidine, and oxymetazoline. At present, further studies are necessary to define whether the lung [³H]atipamezole and the liver ~28-kDa imidazoline-binding sites are the same entity.

Our results allow us to better define the selectivity of [³H]idazoxan and [¹²⁵I]AZIPI for different imidazoline-binding proteins. This could be particularly interesting because these two ligands are currently used to identify and characterize imidazoline-binding proteins. Based on our results, [³H]idazoxan appears to be extremely selective for the I₂BS on MAO B and, therefore, should be considered the reference ligand to define this subgroup of imidazoline-binding sites. In contrast, [¹²⁵I]AZIPI displays less selectivity and should identify various members of the imidazoline-binding protein family.

In conclusion, the use of MAO-deficient mice allowed us to demonstrate the exclusive localization of I₂BS on MAO B and supplied new insight into the pharmacological and structural heterogeneity of imidazoline-binding sites. Based on our results, MAO B-deficient mice may represent an ideal model to investigate the functional properties of I₂BS.

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