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Photoaffinity Labeling of Dopamine D1 Receptors†

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ABSTRACT: A high-affinity radioiodinated D1 receptor photoaffinity probe, (±)-7-[^{125}I]iodo-8-hydroxy-3-methyl-1-(4-azidophenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine ([^{125}I]IMAB), has been synthesized and characterized. In the absence of light, [^{125}I]IMAB bound in a saturable and reversible manner to sites in canine brain striatal membranes with high affinity ($K_D \cong 220$ pM). The binding of [^{125}I]IMAB was stereoselectively and competitively inhibited by dopaminergic agonists and antagonists with an appropriate pharmacological specificity for D1 receptors. The ligand binding subunit of the dopamine D1 receptor was visualized by autoradiography following photoaffinity labeling with [^{125}I]IMAB and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Upon photolysis, [^{125}I]IMAB incorporated into a protein of apparent M_r 74 000 from canine, bovine, and porcine striatal membranes. Labeling was inhibited by dopaminergic agents in a stereoselective manner with a potency order typical of dopamine D1 receptors. In addition, smaller subunits of apparent M_r 62 000 and 51 000 were also specifically labeled by [^{125}I]IMAB in these species. Photoaffinity labeling in the absence or presence of multiple protease inhibitors did not alter the migration pattern of [^{125}I]IMAB-labeled subunits upon denaturing electrophoresis in both the absence or presence of urea or thiol reducing/oxidizing reagents. [^{125}I]IMAB should prove to be a useful tool for the subsequent molecular characterization of the D1 receptor from various sources and under differing pathophysiological states.

Dopamine D1 receptors stimulate the activity of adenylate cyclase, while dopamine D2 receptors inhibit this enzyme [reviewed in Stoof and Kebabian (1984)]. The prototype D1 receptor, located in the bovine parathyroid gland, mediates the release of parathyroid hormone [see Brown and Dawson-Hughes (1983)]. Peripheral dopamine D1 receptors promote renal vasodilatory responses [see Kohli and Goldberg (1987)] and appear to modulate gap junction permeability in electrically coupled cells in the retina (Lasater & Dowling, 1985). In the central nervous system, the exact functional correlate of the D1 receptor has yet to be identified, but evidence suggests that D1 receptor stimulation regulates neuron growth and differentiation (Lankford et al., 1988), elicts some behavioral responses, and exerts modulatory influences on the activity of D2 dopamine receptors [see Waddington (1986) and Clark and White (1987)]. D1 receptors may mediate these effects solely via cAMP-dependent protein kinase activity [see Hemmings et al. (1987)].

In order to examine some of the molecular events associated with various psychomotor, vascular, secretory, and other functions known to be regulated by dopamine, it is helpful to be able to label dopamine receptors in the relevant tissues by means of radioative photoaffinity probes. Although dopamine D2 receptors can now be so identified [see Niznik (1987) for review], the elucidation of the structure of the D1 receptor has been limited, however, by the lack of a specific photoaffinity label for these proteins. Recently, a radioiodinated aminophenyl derivative of the selective D1 antagonist SCH-23390[1] was synthesized and was shown to identify a peptide of M_r 72000 following photoaffinity cross-linking to rat striatal membranes (Amlaiky et al., 1987). The necessity of using heterobifunctional cross-linking reagents and lack of commercial availability of this compound may preclude its use in the subsequent molecular characterization of D1 dopamine receptors.

In this paper, we document the synthesis and characterization of a high-affinity, radioiodinated aryl azide derivative of SCH-23390, (±)-7-[^{125}I]iodo-8-hydroxy-3-methyl-1-(4-azidophenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine ([^{125}I]-IMAB). This ligand binds to D1 dopamine receptors in a specific, reversible, and saturable manner with high affinity and appropriate specificity. Moreover, upon photolysis, [^{125}I]IMAB can be incorporated, with high efficiency, into a protein of apparent M_r ~74 000 representing the neuronal ligand binding subunit of D1 receptors in several species.

Experimental Procedures

Materials. [^{3}H]SCH-23390 (66 Ci/mmol) was obtained from New England Nuclear. Dopamine, serotonin, nor-

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[1] Abbreviations: [^{125}I]IMAB, (±)-7-[^{125}I]iodo-8-hydroxy-3-methyl-1-(4-azidophenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine; MAB, 8-hydroxy-3-methyl-1-(4-azidophenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SCH-23390, (*R*)-(+)-8-hydroxy-7-chloro-2,3,4,5-tetrahydro-3-methyl-1-phenyl-1*H*-3-benzazepine; SCH-23982, (±)-7-iodo-8-hydroxy-2,3,4,5-tetrahydro-3-methyl-1-phenyl-1*H*-3-benzazepine; SKF-38393, 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1*H*-3-benzazepine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; K_D, dissociation constant, HPLC, high-pressure liquid chromatography.

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adrenaline, phenylmethanesulfonyl fluoride, benzamidine, leupeptin, soybean trypsin inhibitor, bovine serum albumin, and prestained molecular weight standards were obtained from Sigma. Electrophoresis reagents were from Bio-Rad. X-ray films (XAR-5) and developing solutions (D-19) were from Kodak. D1 dopaminergic agonists (SKF-38393), antagonists (SCH-23390, butaclamol, eticlopride, spiperone), and other compounds were generously supplied by Research Biochemicals Inc. and various pharmaceutical firms as previously described (Niznik et al., 1988). Canine and porcine brains were from Pel-Freez Biologicals, and bovine brains were obtained fresh from a local abattoir (Bocknek) and stored at −70 °C.

Synthesis of MAB and IMAB. The complete synthesis and chemical characterization of these compounds will be described elsewhere (Baindur et al., 1988). The general synthetic route established by Walter and Chang (1968) for the construction of the 1-aryl-2,3,4,5-tetrahydro-1*H*-3-benzazepine skeleton was employed. Briefly, *p*-nitrostyrene oxide was made by the method of Rafizadeh and Yates (1985) in a one-pot synthesis starting from *p*-nitrobenzaldehyde. Condensation with 4-methoxyphenethylamine gave 1-[{*N*-[(4-methoxyphenyl)-ethyl]amino}methyl]-4-nitrobenzyl alcohol [mp 120–122 °C; 42% yield. Anal. ($C_{17}H_{20}N_2O_4$) C, N, H]. Attempted cyclization employing the standard sulfuric acid–TFA system failed in this case, owing presumably to the destabilization of the incipient carbonium ion intermediate by the 4-nitro group. Cyclization with PPA proved successful, and the required 8-methoxy-1-(4-nitrophenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine [mp 132–134 °C; 78% yield. Anal. ($C_{17}H_{18}N_2O_3$) C, H, N] was obtained. N-Methylation of the above was carried out by the standard Eschweiler–Clarke procedure (Pine & Sanchez, 1971) to obtain 8-methoxy-3-methyl-1-(4-nitrophenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine [mp 116–118 °C; 84% yield. Anal. ($C_{18}H_{20}N_2O_3$) C, H, N]. This was reduced with Raney nickel and hydrazine hydrate in ethanol to yield 8-methoxy-3-methyl-1-(4-aminophenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine (mp 190–192 °C; 92% yield), which was subjected to O-demethylation with boron tribromide to yield 8-hydroxy-3-methyl-1-(4-aminophenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine [mp 230–232 °C; 25% yield; mass spectrum *m/z* 268 (M^+). Anal. ($C_{17}H_{20}N_2O{\cdot}2CH_3SO_3H{\cdot}2H_2O$) C, H, N]. Diazotization of the above with sodium nitrate in 6 N sulfuric acid at 0 °C yielded the diazonium salt intermediate, which was immediately treated with sodium azide at the same temperature to yield MAB [mp 95–100 °C; 64% yield; mass spectrum *m/z* 294 (M^+), 266 ($M - N_2^+$). Anal. ($C_{17}H_{18}N_4O{\cdot}{}^1/_2H_2O$) C, H, N]. Finally, MAB was iodinated with ICl in acetic acid to obtain IMAB [mp 175–180 °C; *m/z* 420 (M^+), 392 ($M - N_2^+$). Anal. ($C_{17}H_{17}N_4O{\cdot}HCl{\cdot}{}^3/_2H_2O$) C, H, N].

Radioiodination of MAB. [^{125}I]IMAB was prepared (by New England Nuclear) by radioiodination of 8-hydroxy-3-methyl-1-(4-azidophenyl)-2,3,4,5-tetrahydro-1*H*-3-benzazepine (MAB). Briefly, MAB (50 μg, 1 μg/μL in MeOH) was added to ~2 mCi of Na^{125}I in 100 μL of 0.2 M sodium phosphate buffer, pH 7.0. Chloramine T (10 μg in 10 μL of 0.2 M sodium phosphate buffer, pH 7.0) was added to this mixture and the iodination reaction allowed to proceed for 2 min at ambient temperature. The reaction was quenched with $Na_2S_2O_5$ (50 μg in 50 μL of H_2O), followed by the immediate addition (200 μL) of 20% acetonitrile and 0.1% trifluoroacetic acid. The entire mixture was purified by reverse-phase HPLC using a Zorbax C-8 column (4.6 mm × 25 cm) and eluted in 0.1% trifluoroacetic acid with a linear gradient of 20–50% acetonitrile at 1 mL/min for 60 min. [^{125}I]MAB (k' = 17.6) was completely separated under these conditions from MAB (k' = 9.4) and all other reactants, yielding a maximal theoretical specific activity of 2200 Ci/mmol. [^{125}I]IMAB was diluted in ethanol to 200 μCi/mL and stored at −20 °C in the dark. The radiochemical yield following HPLC purification was ~50%. [^{125}I]IMAB (R_f = 0.75) was shown to comigrate with nonradioactive IMAB by thin-layer chromatography using silica gel plates (Analtech, 250 μm; 5 × 20 cm) developed in CH_2Cl_2/methanol (9:1 v/v) and in the above reverse-phase HPLC system.

Membrane Preparation. Striata dissected from partially thawed canine, porcine, and bovine brains were Teflon glass homogenized (using 10 up and down strokes) in 20 volumes of ice-cold 25 mM Tris-HCl buffer containing 250 mM sucrose and the following protease inhibitors: 20 mM EDTA, 15 μg/mL benzamidine, 5 μg/mL leupeptin, 5 μg/mL soybean trypsin inhibitor, and 1 mM phenylmethanesulfonyl fluoride (pH 7.4 at 4 °C). Homogenates were centrifuged at 600*g* for 10 min; the supernatant was collected and recentrifuged at 48000*g* for 20 min. The resulting pellet was resuspended in 25 mM Tris-HCl buffer containing 100 mM NaCl and protease inhibitors to yield a final protein concentration of ~4–5 mg/mL. For some experiments, membranes were prepared in 25 mM Tris-HCl buffer alone (pH 7.4 at 4 °C).

Photoaffinity Labeling. Membranes (1 mL) were routinely incubated in the dark with 250 pM [^{125}I]IMAB at a D1 receptor concentration of ~200 pM in a total volume of 5 mL for 90 min at 22 °C in the presence or absence of dopaminergic agents as indicated. Following incubation, samples were centrifuged at 48000*g* for 20 min. Membrane pellets were resuspended in 5 mL of 25 mM Tris-HCl buffer containing 100 mM NaCl, protease inhibitors, and 0.5% bovine serum albumin and recentrifuged. Membrane pellets were resuspended in 1 mL of buffer (without BSA) and irradiated for 35 s as previously described (Niznik et al., 1986a). [^{125}I]-IMAB-labeled membranes were sedimented for 10 min at 12000*g* and prepared for SDS–PAGE as described below.

SDS–PAGE and Autoradiography. Electrophoresis was performed as previously described (Grigoriadis et al., 1988). Briefly, photoaffinity-labeled membranes were washed once in Tris-HCl buffer and incubated with 50 mM Tris, 10% SDS, 10% glycerol, and 5% β-mercaptoethanol, pH 6.8, for 1–2 h at 22 °C. Aliquots (100–300 μg) were loaded on slab gels containing a 12% acrylamide separating gel and a 6% stacking gel and electrophoresed overnight. In an attempt to gain greater resolving power in the molecular weight range of interest, a longer separating gel (~16 cm) was used. Following electrophoresis, gels were dried and exposed to Kodak film with one intensifying screen at −70 °C for various amounts of time. To document quantitative incorporation of [^{125}I]IMAB into D1 receptor subunits, gel regions of interest were cut out and counted in a Packard γ counter at ~80% efficiency. Subsequent autoradiography of the cut gels confirmed that the appropriate section was taken. Molecular weights were determined graphically by plotting the log of the molecular weight of known protein standards versus the R_f (relative migration) of these proteins. The apparent molecular weight of photolabeled receptors was estimated by determining the R_f (from the center of the band) and interpolating this value on the standard curve. The values given are the means of several experiments.

Ligand Binding Assays. For saturation experiments, 50 μL of canine striatal membranes (~10–15 μg of protein) was incubated with 50 μL of [^{125}I]IMAB (10–2000 pM) in the dark for 120 min at 22 °C in a total volume of 0.150 mL of

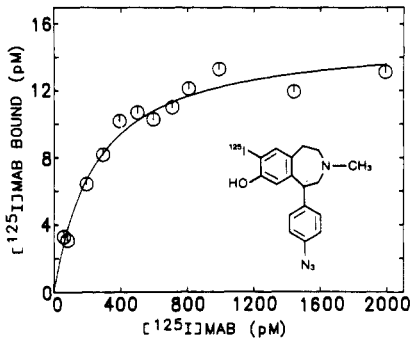

FIGURE 1: Saturation binding of [^{125}I]IMAB to canine striatal membranes. Canine striatal membranes were prepared and incubated in quadruplicate with 10–2000 pM [^{125}I]IMAB for 120 min in the dark and assayed for D1 receptor activity as described under Experimental Procedures. Nonspecific binding was defined by 10 μM (+)-butaclamol. Data were analyzed by the nonlinear curve fitting program LIGAND, with estimated B_{max} and K_D values listed in the text. Inset: Structure of [^{125}I] MAB.

50 mM Tris-HCl buffer containing 120 mM NaCl, 2 mM EDTA, 1.5 mM $CaCl_2$, and 4 mM $MgCl_2$, pH 7.4. For competition binding experiments, 50 μL of membranes (as above) was incubated with ~300 pM [^{125}I]IMAB and varying concentrations of dopaminergic agents (10^{-14}–10^{-3} M) for 120 min at 22 °C in Tris buffer containing sodium chloride. For all experiments, nonspecific binding was defined by 10 μM (+)-butaclamol. Assays were stopped by rapid filtration and monitored for ^{125}I in a Packard γ counter as previously described (Niznik et al., 1988).

Data Analysis. Data from saturation and competition binding experiments were analyzed by the nonlinear least-squares curve fitting program LIGAND run on a Digital micro-PDP 11 as previously described (Niznik et al., 1986b).

Protein Determination. Protein content was assayed with the BCA–protein assay kit (Pierce) as previously described (Niznik et al., 1988).

RESULTS

Synthesis of IMAB. The product described here yields a novel analogue of the potent D1 receptor antagonist SCH-23390. The compound, IMAB, possesses an azidophenyl substituent that renders it as a useful photoaffinity probe for the dopamine D1 receptor. The ability of the precursor MAB to interact with dopamine D1 receptors was assessed in competition binding experiments with [^{3}H]SCH-23390. MAB displayed high affinity for these sites with an estimated K_i value of 3.4 nM. The introduction of an iodo moiety on MAB increased the affinity of the ligand (IMAB) for the receptor by ~10-fold to ~380 pM (data not shown). Thus, compared to the compound SCH-23390 (K_i ~100 pM), both the iodo and azido substituents did not significantly interfere with the ability of IMAB to interact with D1 receptors. Moreover, prephotolyzed IMAB retains the ability to displace [^{3}H]-SCH-23390 binding with a K_D of ~400 pM.

Characteristics of [^{125}I]IMAB Binding to Canine Striatal Membranes. [^{125}I]IMAB bound to canine striatal membranes in a rapid and saturable manner. Equilibrium binding was attained by 60 min at 22 °C (data not shown). The saturable binding of [^{125}I]IMAB to striatal membranes is illustrated in Figure 1. In the presence of 120 mM NaCl, [^{125}I]IMAB bound with high affinity ($K_D \cong 239 \pm 26$ pM) to an apparent homogeneous population of binding sites ($B_{max} = 15 \pm 0.7$ pM). The parent compound [^{3}H]SCH-23390, ($K_D \cong 100 \pm 13$ pM) recognized approximately 40% more binding sites ($B_{max} = 22 \pm 2$ pM) than [^{125}I]IMAB under similar assay

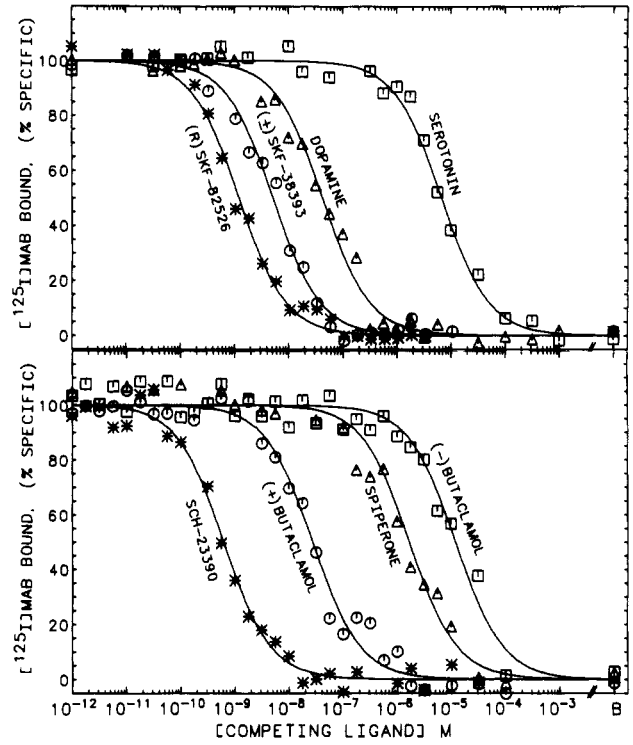

FIGURE 2: Pharmacological selectivity of [^{125}I]IMAB binding to canine striatal membranes. Experiments were performed as described under Experimental Procedures in the presence of 300 pM [^{125}I]IMAB and various concentrations of competing agonists (upper panel) and antagonists (lower panel) as shown. Data are representative of two to three independent experiments.

conditions. This difference was probably due to the racemic nature of the iodinated compound, wherein presumably only the ^{125}I *R* enantiomer bound. The estimated dissociation constant of IMAB for D1 receptors may, therefore, be lower than 400 pM.

The binding of [^{125}I]IMAB to canine striatal membranes exhibited an appropriate D1 dopaminergic pharmacological specificity. As illustrated in Figure 2, dopaminergic agonists and antagonists inhibited the binding of [^{125}I]IMAB in a concentration–dependent and stereoselective manner. For agonists the rank order of potency was (*R*)-SKF-82526 > (±)-SKF-38393 > dopamine ≫ serotonin. Antagonists competed for binding with the following order of potency: SCH-23390 > (+)-butaclamol > spiperone > (−)-butaclamol. Taken together, these data strongly suggest that [^{125}I]IMAB binds to a population of sites in canine striatal membranes that display pharmacological properties similar to those of D1 dopamine receptors identified by the parent probes [^{3}H]-SCH-23390 and [^{125}I]SCH-23892 in both the brain and periphery (Billard et al., 1984; Niznik et al., 1988; Sidhu et al., 1986).

Photoaffinity Labeling of the Neuronal Dopamine D1 Receptor. The major aim of this work was to develop an aryl azide derivative of SCH-23982 that can, upon photolysis, incorporate into the ligand binding subunit of D1 receptors. Figure 3A depicts the results obtained when striatal membranes were incubated with [^{125}I]IMAB and photolyzed and samples subjected to SDS–PAGE and autoradiography. A broad band was labeled at apparent M_r 72000. The specificity of labeling was shown by virtue of the fact that photoincorporation of [^{125}I]IMAB into the M_r 72000 subunit was blocked by 50 nM SCH-23390. Upon lower exposure (Figure 3B) two distinct bands at apparent M_r 74000 and 62000 were seen within the diffuse pattern of labeling. Moreover, a smaller

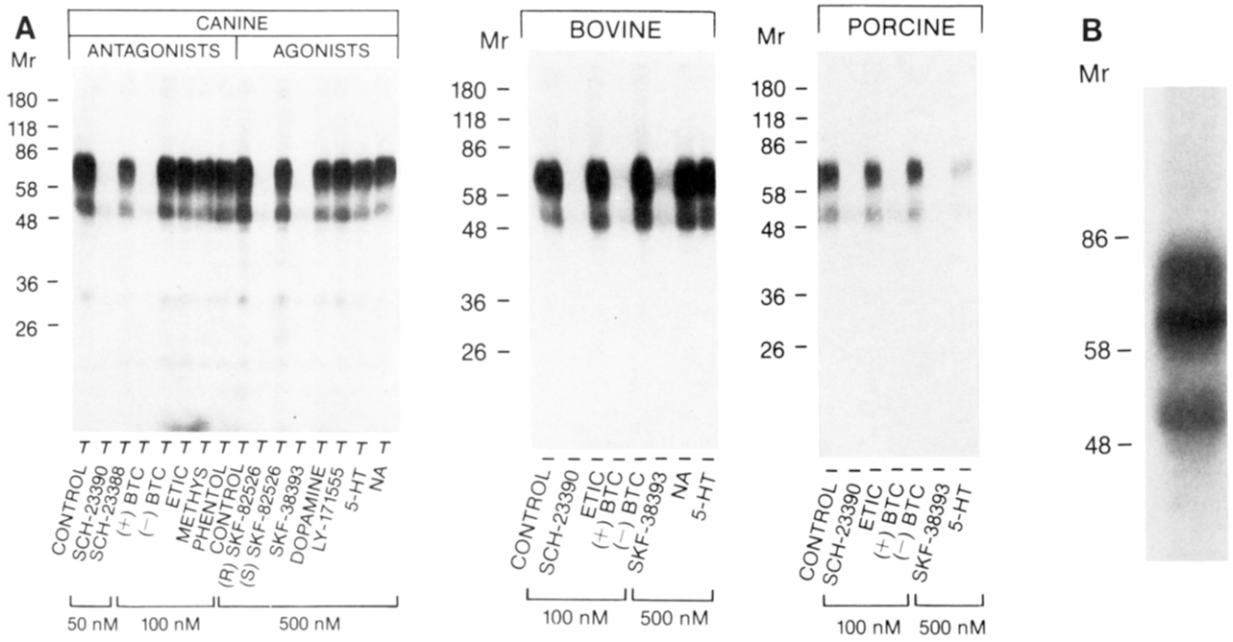

FIGURE 3: Photoaffinity labeling and pharmacological selectivity of [^{125}I]IMAB photoincorporation into striatal membranes of various species. Membranes were prepared and photoaffinity labeled with [^{125}I]IMAB (~200 pM) alone (control) or with [^{125}I]IMAB in the presence of the indicated concentrations of competing ligands as described under Experimental Procedures. Samples were subjected to SDS–PAGE using a 12% acrylamide gel and autoradiography as described. The M_r of known protein standards are shown $\times 10^{-3}$. The results shown are representative of at least five similar experiments. Abbreviations: BTC, butaclamol; ETIC, eticlopride; METHYS, methysergide; PHENTOL, phentolamine; 5-HT, serotonin; NA, noradrenaline; SKF-82526, fenoldopam; LY-171555, quinpirole.

diffuse subunit of apparent M_r 51 000 was also specifically labeled (Figure 3).

The pharmacological specificity of [^{125}I]IMAB photoincorporation was further assessed by examining the ability of various dopaminergic agents to block labeling of these subunits from canine striatal membranes. As seen in Figure 3A, the photolysis-dependent covalent labeling of the subunits at M_r 74 000, 62 000, and 51 000 was stereoselectively blocked by the active isomers of SCH-23390, butaclamol, and the D1 receptor agonist SKF-82526 and by (±)-SKF-38393. In contrast, incubation of [^{125}I]IMAB with a selective D2 receptor antagonist (eticlopride), a serotonergic S_2 receptor antagonist (methysergide), an adrenergic antagonist (phentolamine) or the agonists noradrenaline, serotonin, and the selective D2 agonist LY-171555 did not prevent the incorporation of [^{125}I]IMAB into these subunits. The data strongly suggest that the apparent M_r 74 000, 62 000, and 51 000 proteins are the ligand binding subunits of the canine striatal D1 receptor. Virtually identical results were obtained when bovine or porcine striatal membranes were incubated with [^{125}I]IMAB, photolyzed, and subjected to SDS–PAGE and autoradiography (Figure 3A). A broad band at apparent M_r 74 000 was specifically labeled with an appropriate pharmacological specificity for D1 receptors. As with canine striatal membranes, smaller subunits of M_r 62 000 and 51 000 were also specifically labeled.

Of significant interest was the inability of dopamine (500 nM) to antagonize [^{125}I]IMAB photoincorporation in the ligand binding subunit of D1 receptors (Figure 3A) in all three species studied. This was surprising in light of the rather high affinity displayed by dopamine for [^{125}I]IMAB binding sites (see Figure 2). Figure 4A illustrates the concentration dependency of dopamine to antagonize [^{125}I]IMAB photoincorporation into canine striatal membranes. As can be seen, dopamine inhibited photoaffinity labeling with a pseudo IC_{50} of ~5 μM. Photoaffinity labeling of membranes in the absence of sodium ions, which should increase the affinity of dopamine for the D1 receptor (Niznik et al., 1988), did not

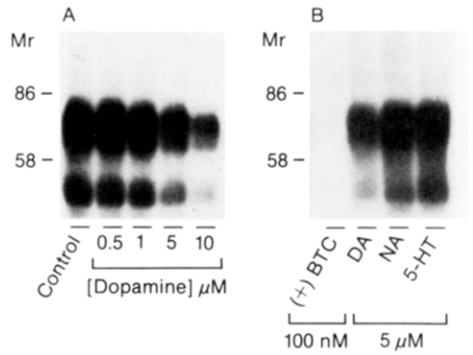

FIGURE 4: Blockade of [^{125}I]IMAB photoincorporation into canine striatal membranes by dopamine. (A) Membranes were incubated with [^{125}I]IMAB (200 pM) in the presence of the indicated concentrations of dopamine and photolyzed and samples subjected to SDS–PAGE and autoradiography as described under Experimental Procedures. (B) Membranes were photolyzed in the presence of the indicated concentrations (5 μM) of dopamine, noradrenaline, and serotonin and subjected to SDS–PAGE as described. Included in this gel is a lane demonstrating nonspecific labeling as defined by 100 nM (+)-butaclamol. The M_r of known standards are shown $\times 10^{-3}$. Abbreviations are as described in Figure 3.

improve the efficiency of dopamine at blocking covalent labeling (data not shown). The molecular mechanism(s) responsible for this effect (if any) is (are) currently unknown. Despite the rather high concentrations of dopamine required to antagonize [^{125}I]IMAB photoincorporation, however, an appropriate rank order of potency for dopamine receptors was obtained when compared to noradrenaline or serotonin. As depicted in Figure 4B, dopamine (5 μM) partially inhibited [^{125}I]IMAB labeling of the D1 receptor binding subunits, while equimolar concentrations of noradrenaline or serotonin did not.

Numerous studies have shown that failure to prevent endogenous protease activity during photoaffinity labeling may result in the generation of lower molecular weight protein fragments displaying the same pharmacological profile as the native receptor (Benovic et al., 1984; Leeb-Lundberg et al., 1984; Nissenson et al., 1987). In order to assess whether both

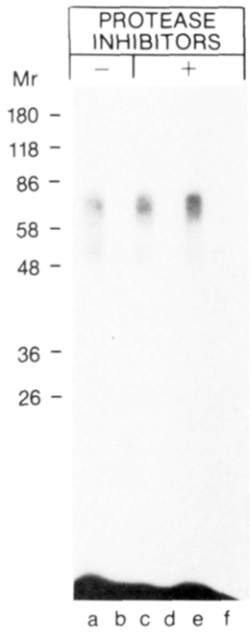

FIGURE 5: Effects of protease inhibitors on the photoaffinity labeling pattern of [^{125}I]IMAB into D1 receptors of canine brain. Membranes were prepared and photolabeled with [^{125}I]IMAB in the complete absence (Tris buffer alone) or presence of protease inhibitors with samples subsequently solubilized in SDS sample buffer in the absence (c, d) or presence (lanes e, f) of multiple protease inhibitors and subjected to SDS–PAGE and autoradiography as described under Experimental Procedures. Included in this gel are lanes b, d, and f, representing nonspecific labeling with 100 nM (+)-butaclamol for each condition. The M_r of known protein standards are shown $\times 10^{-3}$. The experiment was repeated with virtually identical results.

the M_r 62 000 and 51 000 subunits were proteolytically derived receptor fragments from the apparent M_r 74 000 polypeptide, canine striatal membranes were prepared and photoaffinity labeled in the absence or presence of multiple protease inhibitors. As illustrated in Figure 5, preparing and photoaffinity labeling canine striatal membranes in Tris buffer alone did not significantly affect the pattern of [^{125}I]IMAB incorporation into the subunits of apparent M_r 74 000–51 000 (lane a), as compared to samples prepared and photolyzed in the presence of multiple protease inhibitors (lane c) although the efficiency of photoincorporation was slightly increased. Moreover, the inclusion of multiple class-specific protease inhibitors, during membrane solubilization in SDS (lane e), similarly did not prevent the appearance of lower molecular weight subunits. Included in this gel are lanes b, d, and f demonstrating nonspecific labeling as defined by 100 nM (+)-butaclamol. These data would suggest either that an inappropriate protease-inhibitor cocktail was chosen or that the apparent M_r 62 000 and 51 000 labeled subunits are not simply proteolytic degradation products of the M_r 74 000 protein.

Previous work on both the insulin (Helmerhorst et al., 1986) and β-adrenergic (Moxham & Malbon, 1985) receptors has shown that the molecular weight of receptors on SDS–PAGE can vary considerably, depending on the reduction–oxidation state of the protein. In order to investigate the possible subunit structure of the apparent M_r 74 000–51 000 photolabeled proteins, the ability of DTT (or β-mercaptoethanol) and NEM to alter the migration pattern of photolabeled receptors was investigated. NEM (at 10 mM), DTT (10 mM), or β-mercaptoethanol (10%) added during receptor solubilization in SDS sample buffer, in the presence or absence of 8 M urea, did not alter the migration patterns of canine striatal photoaffinity-labeled bands of apparent M_r 74 000–51 000 (data not shown), as compared to samples prepared in SDS buffer alone. These data indicate that the ligand binding subunits of the D1 receptor are probably not linked by intramolecular disulfide bridges.

Discussion

In the present study the synthesis and characterization of an aryl azide derivative of the highly selective D1 dopaminergic antagonist SCH-23390 are described. The analogue, after radioiodination and purification by HPLC, yielded a ligand of high specific radioactivity (~2200 Ci/mmol). Saturation and competitive binding studies using well-defined dopaminergic ligands demonstrate that [^{125}I]IMAB has a specificity and stereoselectivity typical of a D1 dopaminergic ligand. However, upon exposure to UV light, [^{125}I]IMAB photoincorporated into striatal membranes and identified a protein of apparent M_r 74 000 as the major ligand binding subunit of neuronal D1 receptors in a number of species. In addition, two smaller subunits of M_r 62 000 and 51 000 were also labeled with the appropriate pharmacological specificity for D1 dopaminergic receptors. Whether these peptides represent proteolytic receptor fragments is unknown at present, but inclusion of multiple class-specific protease inhibitors previously shown to be useful in preventing receptor degradation in other systems (Benovic et al., 1984; Leeb-Lundberg, 1984; Amlaiky & Caron, 1986) did not prevent the specific labeling of these lower molecular weight species.

The efficiency of photoaffinity labeling with [^{125}I]IMAB was well within the range expected of a photoaffinity probe. About 2% of the ligand that was bound to receptors underwent covalent linkage to the M_r 74 000–62 000 proteins. Interestingly, previous attempts to use an aryl azide derivative of SCH-23390, situated on the 3-phenyl position, to identify the ligand binding subunit of D1 receptors led to much less efficient labeling (Amlaiky et al., 1987).

The identification of an apparent M_r 74 000 polypeptide as the ligand binding subunit of the neuronal dopamine D1 in a number of species is similar to that recently reported (Amlaiky et al., 1987) for rat striatal D1 receptors (M_r 72 000). Previous attempts to label the ligand binding subunit of D1 receptors with various alkylating or photolabile probes such as [^{3}H]dopamine [see Niznik (1987) for review] revealed the existence of apparent M_r 57 000 and 34 500 proteins. The pharmacological specificity of covalent incorporation was not assessed, however, and as such the molecular nature of these peptides and their relation to the M_r 74 000 polypeptide identified here are unknown.

At present it is difficult to ascertain whether the polypeptide at M_r 74 000 represents the ligand binding subunit alone or if it is the functional D1 receptor. The estimated molecular mass of the D1 receptor binding site identified here by photoaffinity labeling with [^{125}I]IMAB corresponds closely, however, to the estimated functional target size of D1 receptors (M_r 79 000) as determined by radiation inactivation (Gredal et al., 1987). Furthermore, it is not clear as to whether the diffuse pattern of [^{125}I]IMAB labeling represents marked proteolytic degradation of the polypeptide at M_r >74 000 or the interference of D1 receptor associated carbohydrates (Niznik et al., 1986b,c) on protein mobility on SDS–PAGE. In any event, it appears that the ligand binding subunit of the D1 receptor is of a smaller molecular weight than that of the dopamine D2 binding subunit (M_r 140 000–94 000) in all tissues examined to date (Amlaiky & Caron, 1986; Jarvie et al., 1988). Final assessment of the molecular weight of the functional D1 receptor will have to await the purification of the protein and its functional reconstitution with appropriate transducer (G-protein) and effector systems.

The introduction of this specific, high-affinity, radioiodinated photoaffinity probe for dopamine D1 receptors should prove to be of value for the subsequent molecular characterization of these receptors from numerous tissues and under various pathophysiological states.

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