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ACCELERATED COMMUNICATION

Identification of the Binding Subunit of the D_1-Dopamine Receptor by Photoaffinity Crosslinking

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SUMMARY

The D_1-dopamine receptor from rat striatum has been successfully identified by photoaffinity crosslinking using a newly synthesized radioiodinated derivative of the selective D_1-antagonist SCH-23390. This compound, (*R,S*)-5-(3′-aminophenyl)-8-chloro-2,3,4,5-tetrahydro-3-methyl-[1H]-3-benzazepin-7-ol(SCH-38548), has been radioiodinated by a chloramine T procedure yielding three radioiodinated products. One of these separated congeners (with $R_f = 0.35$ on thin layer chromatography; CH_2Cl_2/MeOH/triethylamine; 82.5:17.5:0.01) binds reversibly to rat striatal membranes with high affinity ($K_D \cong 200$ pM), appropriate stereoselectivity, and D_1-dopaminergic specificity. [^{125}I]SCH-38548 can be covalently incorporated into a peptide of $M_r \cong$ 72,000 using the heterobifunctional crosslinking reagent *N*-succinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate. Covalent incorporation of [^{125}I]SCH-38548 into the $M_r \cong$ 72,000 peptide can be blocked by dopaminergic agents with D_1-dopaminergic specificity (for agonists: SKF-38393 > apomorphine > dopamine; for antagonists: SCH-23390 >>>, SCH-23388 and *cis*-flupentixol >>> *trans*-flupentixol). The D_1-dopaminergic selectivity and specificity of the labeling were further demonstrated by the fact that other antagonists such as domperidone, ketanserin, phentolamine, and alprenolol did not compete for the covalent labeling of the $M_r \cong$ 72,000 peptide. These results indicate that the ligand-binding subunit of the D_1-dopamine receptor resides on peptide distinct from that of the D_2-dopamine receptor (M_r = 94,000). This new radioligand should be useful in the molecular characterization of the D_1-dopaminergic receptor from various sources.

Dopamine receptors have been classified on the basis of pharmacological and biochemical properties into two subtypes, D_1 and D_2. D_1-receptors are linked to a stimulation of adenylate cyclase, whereas D_2-receptors are coupled to an inhibition of this enzyme (1). In the central nervous system the interaction of dopamine with these receptors affects various motor and behavior functions (1, 2). In the parathyroid gland, D_1-receptors mediate dopaminergic stimulation of parathyroid hormone secretion (3), whereas in the anterior and intermediate lobe of the pituitary gland D_2-receptors mediate the dopaminergic inhibition of prolactin and α-melanocyte-stimulating hormone release, respectively (4, 5). A similar classification has been proposed for the dopamine receptors of the renal and cardiovascular systems; however, these have been designated DA_1 and DA_2 (6).

To determine the mechanism by which hormones mediate their physiological effect requires the identification and characterization of the different components involved. Recently, many of the catecholamine receptors have been identified and purified, in particular, β_1, β_2 (7, 8), α_1, α_2 (9, 10), and D_2 (11). The characterization of these receptors has been possible because of the availability of the biospecific methods of photoaffinity labeling and affinity chromatography. Recently, several tritiated and iodinated radioligands [[^{3}H]SCH-23390 (12), [^{3}H]SKF-(*R*)-83566 (13), and [^{125}I]SCH-23982 (14)] have been developed for measurement of ligand binding to D_1-dopamine receptors. These compounds are more selective and specific than tritiated thioxanthines, [^{3}H]flupentixol or [^{3}H]piflutixol, used earlier which bind to both D_1- and D_2-receptors and have high levels of nonspecific binding (15). However, selective biospecific tools for the identification of the D_1-receptor protein have not yet been available. In this report we describe the development of a derivative of the D_1-antagonist SCH-23390 substituted in the phenyl ring with an amino group in the 3′-position. After iodination this compound yields a high specific

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ABBREVIATIONS: SANPAH, *N*-succinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate; TLC, thin layer chromatography; HF, hydrogen fluoride; EDTA, ethylenediaminetetraacetate, SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EtOAc, ethyl acetate; EtOH, ethanol; MeOH, methanol; TEA, triethylamine; [^{125}I]N_3-NAPS, *N*-(*p*-azido-*m*-[^{125}I]iodophenethyl)spiperone.

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radioactivity probe that binds to the D_1-dopaminergic receptor in rat striatal membranes with high affinity and appropriate specificity. The binding is reversible, saturable, and stereospecific. Using the heterobifunctional crosslinking reagent SANPAH, [^{125}I]SCH-38548, by virtue of its arylamine, can be covalently incorporated into a peptide of M_r = 72,000, representing the binding subunit of the D_1-dopamine receptor.

Experimental Procedures

Materials

Rats (Sprague-Dawley) were from Charles River Breeding Laboratories (Wilmington, MA). SANPAH was from Pierce Chemical Company (Rockford, IL). All materials were from sources described previously (16) or as indicated in the text.

Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. NMR spectra were recorded at 90 MHz on a Varian EM-390 spectrometer, at 79.5 MHz on a CFT-20 spectrometer, and at 300 MHz. Mass spectra were determined on a Varian CH5 spectrometer at 70 eV and a port temperature of 180°. TLC was performed using Analtech silica gel GF plates (250 μm).

Methods

Synthesis of SCH-38548. *α-[[[2-(3-Chloro-4-methoxyphenyl)-ethyl]methylamino]methyl]-3-nitrobenzene-methanol.* A mixture of *N*-methyl-3-chloro-4-methoxybenzeneethanamine (17) (8.96 g, 0.045 mol) and 3-nitrostyrene oxide (18) (8.41 g, 0.05 mol) was heated in an oil bath at 100° for 2 hr, after which the reaction mixture was dissolved in ethanol and treated with ethereal HCl. Dilution with ether and cooling gave the crystalline hydrochloride (13.2 g), m.p. 169–172°. NMR (free base, $CDCl_3$): δ2.42 (3, s, NCH_3), 3.89 (3, s, OCH_3), 4.75 (1, m, ArC<u>H</u>OH), 6.88–8.33 (7, m, aromatic).

N-[3-[1-O-Acetyl-2-[[2-(3-chloro-4-methoxyphenyl)ethyl]methylamino]ethyl]-phenyl]acetamide. A solution of the preceding product (2.0 g) in a mixture of 40 ml of glacial acetic acid and 10 ml of acetic anhydride was hydrogenated over 200 mg of 10% palladium-carbon at 40–50 psi. Uptake of hydrogen ceased after 30 min. Catalyst was filtered, and the filtrate was concentrated *in vacuo*. The residue was treated with cold 5% NaOH, and the gummy solid product was extracted into $CHCl_3$. The extracts were washed with water, dried over anhydrous sodium sulfate, and evaporated to give 1.3 g of pale yellow oil. TLC ($CHCl_3$/EtOH/NH_4OH, 50:3:1) showed one major product, R_f = 0.44. NMR ($CDCl_3$):δ2.03 (3, s, $NHCOC\underline{H}_3$), 2.10 (3, s, $OCOC\underline{H}_3$), 2.33 (3, s, NCH_3), 3.83 (3, s, OCH_3), 5.80 (1, m, ArC<u>H</u>OAc).

N-[3-[2-][2-(3-Chloro-4-methoxyphenyl)ethyl]methylamino]-1-hydroxyethyl]phenyl]acetamide. The preceding material (6.1 g) was dissolved in 80 ml of methanol and a solution of 2.0 g of K_2CO_3 in 20 ml of water was added. The resulting solution was stirred at room temperature (pH 10) for 4 hr. Solvents were evaporated at 30° *in vacuo;* then, the residue was diluted with water and extracted with CH_2Cl_2-ether. The extracts were dried over anhydrous $MgSO_4$ and filtered, and the filtrate evaporated to a colorless oil. NMR ($CDCl_3$): δ2.04 (3, s, $NHCOC\underline{H}_3$), 2.32 (3, s, NCH_3), 3.80 (3, s, OCH_3), 4.54(1, t, *J*=6 Hz, ArC<u>H</u>OH).

N-[3-(7-Chloro-2,3,4,5-tetrahydro-8-methoxy-3-methyl-1H-3-benzazepin-1-yl)-phenyl]acetamide. The entire material obtained above was dissolved in 15 ml of nitrobenzene contained in a polyethylene bottle protected from atmospheric moisture, and ~25 ml of anhydrous HF were added. The resulting solution was stirred at room temperature for 20 hr, and excess HF was then removed by passing a slow stream of nitrogen through the mixture. The resulting residue was poured into a well-stirred ice-cold K_2CO_3 solution. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2. The combined extracts were then extracted with 5% HCl. The acid extracts were washed well with ether, then basified with dilute NaOH solution. The precipitated gum was extracted into CH_2Cl_2, and the solution was washed with water and dried over anhydrous $MgSO_4$. Filtration and evaporation of the filtrate *in vacuo* gave 4.3 g of glassy solid. TLC (EtOAc/EtOH/NH_4OH, 100:3:1) showed three major products, R_f = 0.65, 0.47, and 0.31. A portion (2.9 g) of this material was dissolved in ethyl acetate and column chromatographed over 200 g of Merck Kieselgel 60 G, eluting initially with EtOAc/EtOH/NH_4OH, 100:3:1. Fractions containing the fastest running component were collected, and the eluant was changed to EtOAc/EtOH/NH_4OH, 50:3:1. Fractions containing the next two components were thus obtained. Appropriate fractions were combined and evaporated to give the individual components.

Product R_f = 0.65 was assigned the structure *N*-[3-[2-[[2-(3-chloro-4-methoxyphenyl)ethyl]methylamino]-1-fluoroethyl]phenyl]acetamide on the basis of its NMR spectrum ($CDCl_3$): δ2.13 (3, s, $NHCOC\underline{H}_3$), 2.40 (3, s, NCH_3), 3.80 (3, s, OCH_3), 5.46 (ddd, J_{HF} = 50 Hz, J_{HH} = 8 Hz, 3 Hz, ArC<u>H</u>FCH_2—).

The major product R_f = 0.31 (1.1 g, oil) was designated the benzazepine. NMR ($CDCl_3$): δ2.06 (3, s, $NHCOC\underline{H}_3$), 2.33 (3, s, NCH_3), 3.63 (3, s, OCH_3), 4.20 (1, m, ArC<u>H</u>Ar), 6.33 (1, s, H-9), 7.10 (1, s, H-6).

The structure of product R_f = 0.47 is unknown.

5-(3′-Aminophenyl)-8-chloro-2,3,4,5-tetrahydro-3-methyl-1H-3-benzazepin-7-ol (SCH-38548). A solution of 500 mg of the preceding product, R_f = 0.31, in 5 ml of 48% HBr was heated for 1½ hr at 130° in an oil bath. The hot mixture was diluted with 60 ml of boiling water, and the pH of the resulting solution was adjusted to ~8 by addition of small portions of $NaHCO_3$. The precipitated gummy solid was filtered and partitioned between water and $CHCl_3$. The organic layer was dried over anhydrous $MgSO_4$ and filtered. The filtrates were evaporated to dryness, and the residue recrystallized from acetonitrile to give 141 mg of product, m.p. 221–223°.

Analysis ($C_{17}H_{19}ClN_2O$): C, 67.43; H, 6.33: N, 9.25. Found: C, 67.65; H, 6.37; N, 9.46. MS: 302(M), 301(M−1), 287, 271, 258, 245. NMR (79.5 MHz, d^6-dimethyl sulfoxide): δ2.63 (3, s, NCH_3), 4.02 (1, dd, *J*=8 Hz, 2 Hz, ArC<u>H</u>RAr), 4.93 (2, s, NH_2), 6.34 (1, s, 9-H), 6.97(1, d, *J*=8 Hz, 5′-H), 7.06 (1, s, 6-H), 9.63 (1, s, OH). The protons at 4.93 and 9.63 were exchangeable with D_2O.

Authentic iodinated materials were obtained by iodination as described below: extraction of reaction products into methylene chloride, and column chromatography on Merck Kieselgel 60 G eluting with $CHCl_3$/EtOH/NH_4OH, 100:3:1.

Structures were established by 300 MHz NMR spectroscopy (d^6-dimethyl sulfoxide) of the aromatic region. (5-(3-Amino-2-iodophenyl)-8-chloro-2,3,4,5-tetrahydro-3-methyl-1H-3-benzazepin-7-ol): δ6.15 (s, 1, H-6), 6.26 (dd, 1, *J*=8 Hz, 2 Hz, H-4′), 6.34 (dd, 1, *J*=8 Hz, 2 Hz, H-6′), 6.89 (t, 1, *J*=8 Hz, H-5′), 7.20 (s, 1, H-9). (5-(3-Amino-4 (or 6)-iodophenyl)-8-chloro-2,3,4,5-tetrahydro-3-methyl-1H-3-benzazepin-7-ol): δ6.09 (s, 1, H-6), 6.35 [dd, 1, *J*=8 Hz, 2 Hz, H-6′(H-4′)], 6.52 (d, 1, *J*=2 Hz, H-2′), 7.11 (s, 1, H-9), 7.43 (d, 1, *J*=8, H-5′). The coupling pattern of the aromatic protons does not permit us to distinguish between the two regioisomers 4- and 6-iodo.

Radioiodination of SCH-38548. Radioiodination was performed by procedures previously described for other arylamine-containing compounds (16). Briefly, SCH-38548 (3 μg, 9 nmol, 1 μg/μl in MeOH) was added to 1 M sodium acetate buffer (8 μl, pH 5.6) and Na[^{125}I] (8 μl, 3 mCi, 1.3 nmol, > 350 mCi/ml in 0.1 N NaOH) in a polypropylene Microfuge tube. To the mixture was added chloramine T (3 μl, 12 nmol, 1 μg/μl in H_2O) at room temperature. The reaction was stopped after 1 min with $Na_2S_2O_5$ (3 μl, 15.7 nmol, 1 μg/μl in H_2O). The complete mixture was spotted across a 5 × 20 thin layer silica gel plate (E. M. Merck, F-254 with fluorescent indicator) and chromatographed in the solvent system (CH_2Cl_2/MeOH/TEA, 82.5:17.5:0.01). Radioactive bands were detected by autoradiography, and material was extracted in ethyl acetate with 0.1% TEA and stored at ~0.5–1.0 mCi/ml in EtOH-0.1% phenol at −20°. The three major products (bands *I, II,* and *III,* Fig. 1) were assessed for their ability to bind to rat striatal membranes. Both band I and II products gave specific binding. The iodinated product band II was chosen because of its highest level of specific binding (>70–80%). Band I was found to co-migrate in several

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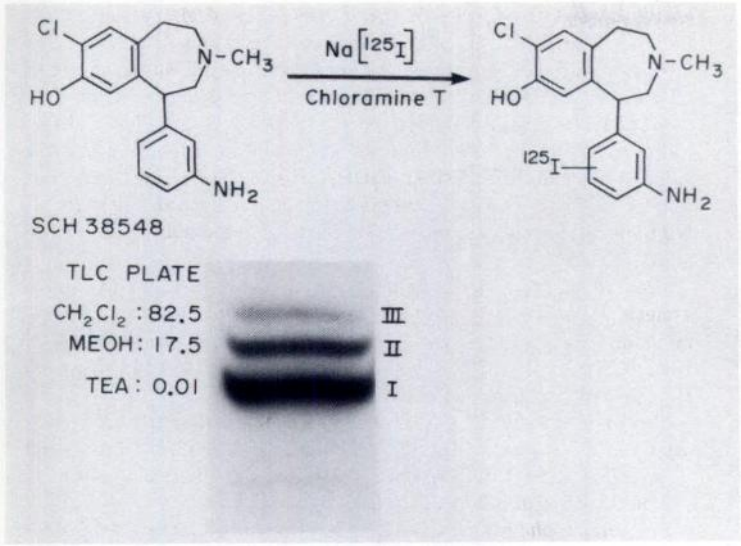

Fig. 1. Structures of SCH-38548 and the radioiodination reaction. SCH-38548 was radioiodinated by a chloramine T procedure as described in Experimental Procedures. The various radioiodinated isomers were separated by chromatography on TLC plates. An autoradiogram of a typical TLC pattern is presented. The material in band *II* was used as the ligand for these studies.

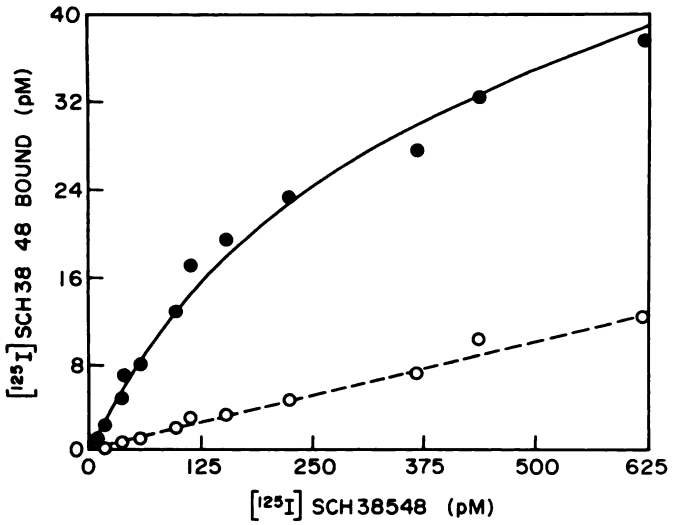

Fig. 2. Binding isotherm of [^{125}I]SCH-38548 to rat striatal membranes. Membranes were incubated for 1 hr with concentrations of [^{125}I]SCH-38548 ranging from 10 to 625 pM in the presence and absence of 100 nM SCH-23390. Results were analyzed by computer-assisted nonlinear regression analysis (25) K_D = 165 pM, B_{max} = 500 fmol/mg.

solvent systems with the authentic 4 (or 6)-iodo derivative of SCH-38548 [5-(3-amino-4 (or 6)-iodophenyl)-8-chloro-2,3,4,5-tetrahydro-3-methyl-1H-3-benzazepin-7-ol]. Because this iodo derivative co-migrates with the major iodinated product (band I), this compound is most likely the 4-iodo derivative based on the reactivity of the *ortho* position to the 3-amino derivative. The iodinated product band II, which has been used in these studies, has not been yet conclusively identified. The compound is not the 2-iodo derivative of SCH-38548 based on differential TLC migration. Based on the fact that the radioiodinated products were separated from the starting compound (SCH-38548), a specific activity for the labeled material of 2200 Ci/mmol should be assumed. However, this value would appear to be an overestimate since [^{125}I]SCH-38548 identifies fewer sites (500 fmol/mg) than are identified by [^{3}H]SCH-23390 in rat striatum (700–900 fmol/mg) (12, 19).

Conversion of [^{125}I]SCH-38548 to the 3-azidophenyl derivative was performed essentially as described previously for the D_2-dopaminergic probe [^{125}I]N_3-NAPS (16).

Membrane preparation. Rat striatum membranes were prepared essentially as described previously (16).

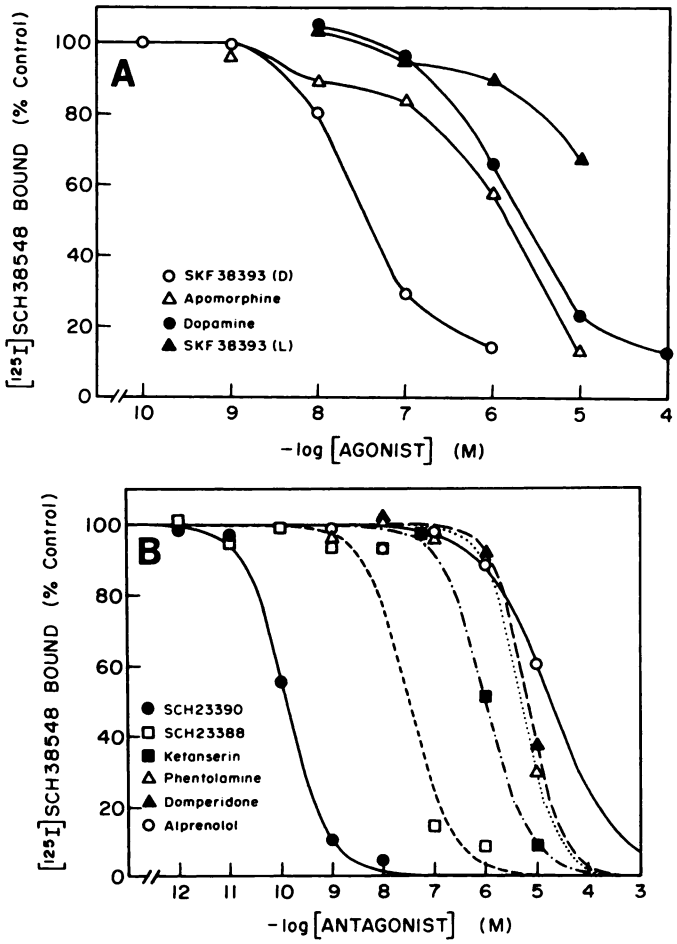

Fig. 3. Competition curves of dopaminergic agonists (A) and antagonists (B) for the [^{125}I]SCH-38548 binding to rat striatal membranes. Experiments were performed as described in Experimental Procedures in the presence of the various concentrations of competitors shown. Data are representative of two to three experiments.

Ligand binding. Glass tubes in duplicate received 375 μl of 50 mM Tris-HCl, pH 7.4, 25°, 100 mM NaCl, 1 mM EDTA, 6 mM $MgCl_2$, 50 μl of various concentrations of drugs, 25 μl of a solution of [^{125}I]SCH-38548 (final concentration 150–200 pM) in water, and 50 μl of mem-

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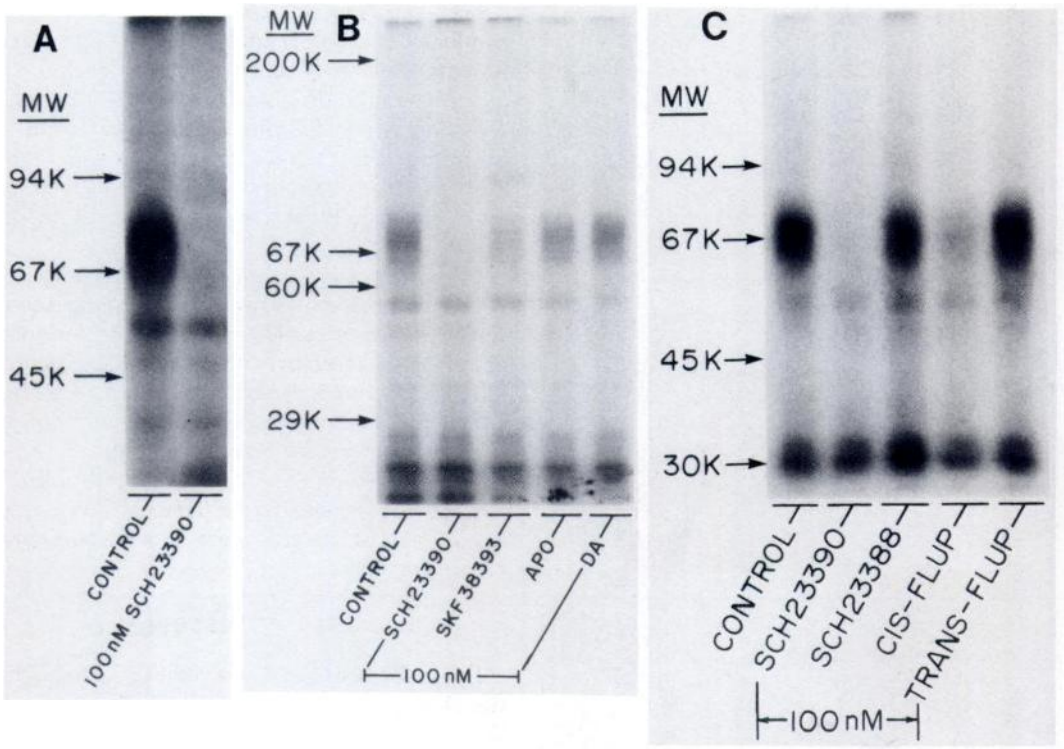

Fig. 4. Photoaffinity crosslinking and pharmacological specificity of [^{125}I]SCH-38548 in rat striatal membranes. A. Membranes were incubated with 500 pM [^{125}I]SCH-38548 for 60 min in the presence and absence of SCH-23390 as described in Experimental Procedures. Samples were processed as described in Experimental Procedures for photoaffinity labeling and SDS-PAGE. An equivalent amount of protein was loaded on each SDS-gel lane. The results shown are typical of 10–15 experiments. B. Membranes were incubated as described above with [^{125}I]SCH-38548 in the presence of 100 nM SCH-23390 and a 100 nM concentration of each of the agonists shown. Results are representative of 2 experiments. C. Membranes were incubated as described above with 100 nM concentrations each of *cis*- and *trans*-flupentixol, SCH-23390, and SCH-23388. Results are representative of 4 experiments.

branes (~50 μg/assay). Tubes were incubated at 25° for 1 hr and rapidly filtered under vacuum through Whatman GF/C filters with four 4-ml rinses of 25 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$. The nonspecific binding was defined using 100 nM SCH-23390.

Photoaffinity crosslinking. Membranes were suspended in 25 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, pH 7.4, at 25° to a receptor concentrations of ~200 pM in a 1-ml volume with 500 pM [^{125}I]SCH-38548 for 60 min at 25° in the presence or absence of various agents. After this incubation, the membranes were washed by repeated centrifugation (three times) with ice-cold 10 mM Na_2HPO_4/100 mM NaCl, pH 7.4, and resuspended in 1 ml of the same buffer. Under dark conditions, 20 μl of a solution of 1 mM SANPAH (freshly dissolved in dimethyl sulfoxide) were added, reacted for 15 min at 25°, and stopped with 30 μl of 1 M glycine. The mixture was photolyzed as described previously (16). Membranes were pelleted by centrifugation and solubilized in 150 μl of 10% SDS, 10% glycerol, 25 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol for 30 min at 25°. Polyacrylamide slab gels (8%) were run (16). Identical amounts of protein were applied to the lanes (200–350 μg). A nitrene scavanger such as *p*-aminobenzoic acid (10 μM) did not affect the pattern of photoaffinity crosslinking.

Results

Synthesis of SCH-38548. The synthesis described here yields a novel analog of the D_1-selective antagonist SCH-23390. This compound, SCH-38548, possesses a 3-aminophenyl substituent which renders it a useful probe for the biochemical characterization of the receptor. This substituent does not markedly diminish the ability of the compound to interact with the D_1-receptor, as a K_D of ~1.5 nM was obtained for SCH-38548 in competition for [^{3}H]SCH-23390 binding (not shown). This amino substituent affords the compound two desirable properties: 1) the compound can be radioiodinated, as shown in Fig. 1, to yield several radioiodinated isomers which can be separated chromatographically; and 2) the amino group represents a functional group which renders the compound usable as a probe for direct photoaffinity or crosslinking labeling of the receptor. As shown below, the radioiodinated SCH-38548 retains high affinity for binding to the D_1-receptor.

Characteristics of [^{125}I]SCH-38548 binding to rat striatal membranes. [^{125}I]SCH-38548 binds to rat striatal membranes in a rapid and reversible fashion. Equilibrium is attained after 30–40 min at 25° and the addition of the antagonist SCH-23390 (100 nM) leads to a rapid dissociation of the receptor-bound ligand (data not shown). The binding of [^{125}I]SCH-38548 is of high affinity and to one homogeneous population of sites (Fig. 2). The nonspecific binding defined using 100 nM SCH-23390 represents 10–15% of the total binding. The equilibrium dissociation constant (K_D) calculated is ≅200 pM with a number of sites of ~500 fmol/mg in the membrane preparations used (Fig. 2). Binding of [^{125}I]SCH-38548 to rat striatal membranes displays the appropriate D_1-dopaminergic specificity (12). Agonists compete for binding with the following order of potency: SKF-(*R*)-38393 > apomorphine > dopamine > SKF-(*S*)-38393 (Fig. 3A). For antagonists, binding showed stereoselective properties, with SCH-23390 >>> SCH-23388 the less potent isomer of SCH-23390. In addition, ketanserin (serotonergic), phentolamine (α-adrenergic), domperidone (D_2-dopaminergic), and alprenolol (β-adrenergic) compete for [^{125}I]

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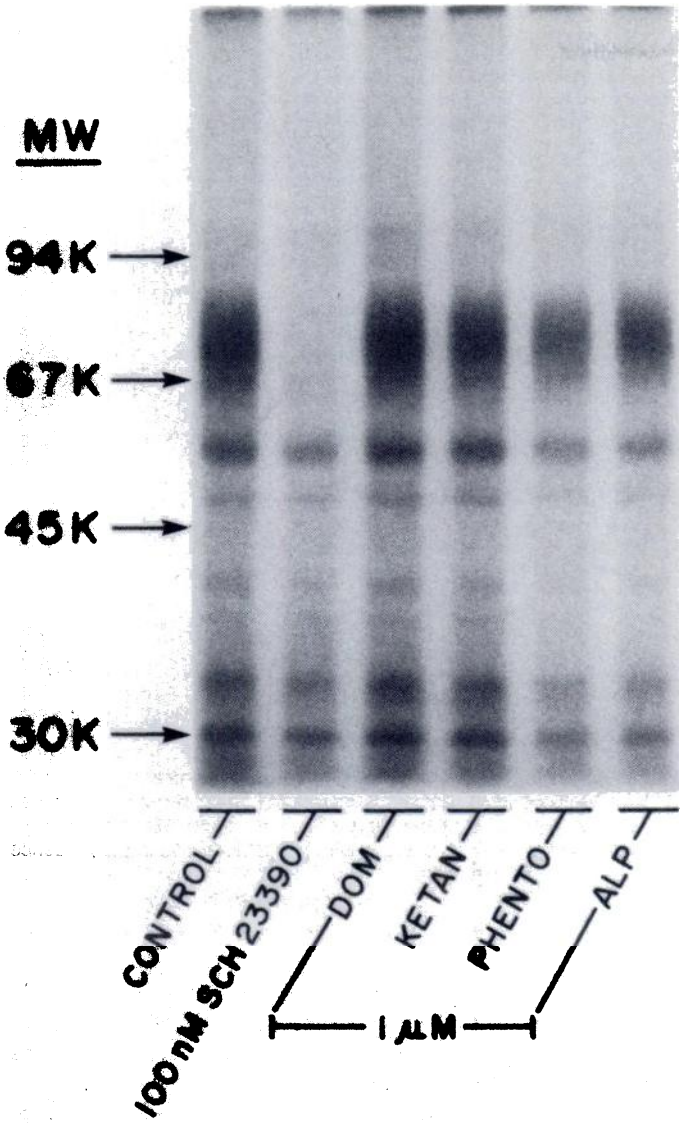

Fig. 5. Photoaffinity crosslinking and pharmacological selectivity of [^{125}I] SCH-38548 incorporation into rat striatal membranes. Membranes were incubated as described in Experimental Procedures in the presence of 100 nM SCH-23390 and a 1 μM concentration of the various antagonists shown. Results are representative of two experiments.

SCH-38548 binding only with extremely low potency (Fig. 3B). These results indicate that [^{125}I]SCH-38548 binds to a population of sites in rat striatal membranes that possess the properties of the D_1-dopaminergic receptor subtype.

Photoaffinity crosslinking of [^{125}I]SCH-38548 into the D_1-receptor-binding subunit of rat striatal membranes. The goal of this study was to use this probe to identify the ligand-binding subunit of the D_1-receptor by covalent labeling. Fig. 4A shows the results obtained when rat striatal membranes are incubated with [^{125}I]SCH-38548, reacted with SANPAH, and photolyzed and the samples subjected to SDS-PAGE. A broad peptide band is labeled at M_r = 72,000. The specificity of the labeling is shown by the fact that covalent incorporation of [^{125}I]SCH-38548 into the M_r = 72,000 peptide is blocked by 100 nM SCH-23390. The pharmacological specificity of the covalent labeling can be further assessed (Fig. 4B). When [^{125}I]SCH-38548 is incubated with various agonists at a concentration of 100 nM, the ability of these various agonists to block the covalent incorporation of [^{125}I]SCH-38548 shows an order of potency equivalent to their relative order of efficacy in D_1-ligand binding studies: SKF-(*R*)-38393 > apomorphine > dopamine. In addition, the labeling of the M_r = 72,000 peptide is stereoselective, since SCH-23388 and *trans*-flupentixol do not block the incorporation, whereas the active isomer SCH-23390 and *cis*-flupentixol completely block the labeling (Fig. 4C).

Fig. 5 shows the selectivity of the labeling. Whereas SCH-23390, the selective D_1-antagonist, blocks the incorporation of the probe into the M_r = 72,000 peptide, domperidone (D_2-antagonist), ketanserin (S_2-antagonist), phentolamine (α-adrenergic antagonist), and alprenolol (β-adrenergic antagonist) do not significantly alter the amount of probe photocrosslinked into the D_1-receptor-binding subunit. A few other lower M_r peptides (~30–50) are labeled by the probe; however, these bands do not reveal specific labeling. These results suggest that the M_r = 72,000 peptide represents the D_1-dopaminergic ligand-binding subunit.

Discussion

[^{125}I]SCH-38548 is a new radioiodinated ligand that binds to the D_1-dopamine receptor with high affinity and appropriate D_1 pharmacology. The binding is saturable and stereoselective. The binding of [^{125}I]SCH-38548 is inhibited by various D_1-agonists and -antagonists with affinities comparable to those determined for competition with [^{3}H]SCH-23390 binding. Because of the presence of a free arylamine on SCH-38548, not only can this compound be radioiodinated, but the label probe can be incorporated into the receptor-binding subunit using a bifunctional crosslinker. This study shows that a major broad peptide centered at M_r = 72,000 can be labeled specifically by [^{125}I]SCH-38548 and SANPAH in rat striatal membranes. This peptide represents the D_1-receptor ligand-binding site since this peptide is labeled with appropriate D_1 pharmacological specificity, selectivity, and stereoselectivity. The D_1-agonist ligands SKF-(*R*)-38393, (−)-apomorphine, and dopamine show the same typical order of potency in inhibiting covalent labeling of the M_r = 72,000 band as they do in competing for the binding of [^{125}I]SCH-38548 or [^{3}H]SCH-23390. Moreover, whereas the antagonist SCH-23390 and *cis*- and *trans*-flupentixol inhibited incorporation of the labeled probe with appropriate selectivity and stereoselectivity, antagonists selective for other receptor systems, D_2, S_2, and α- and β-adrenergic, did not affect labeling of these receptor-binding sites, attesting to the selectivity and specificity of the [^{125}I]SCH-38548 probe.

Other investigations have previously attempted to use the photoaffinity labeling approach to identify the D_1-dopamine receptor. Light-dependent incorporation of [^{3}H]dopamine into a peptide of M_r = 57,000 was demonstrated by Kuno and Tanaka (21). However, in that study, the specificity of [^{3}H] dopamine labeling was not assessed. Moreover, dopamine binds with rather low affinity (K_D = 1.4 μM) to the D_1-receptor (12) and is not selective for D_1-receptor only; thus, the identity of this M_r = 57,000 peptide remains an open issue.

The size of the D_1-dopamine receptor-binding site identified here by photoaffinity crosslinking (M_r = 72,000) corresponds closely to the size determined by radiation inactivation. Nielsen *et al.* (22) reported an estimate of 79,500 for the target size of the rat striatum receptor. The peptide revealed by crosslinking

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is centered at M_r = 70,000–72,000; however, it migrates on SDS-PAGE as a broad band from $M_r \cong$ 65,000 to 80,000. Moreover, shorter exposure of autoradiograms (cf. Fig. 4B, *control* lane) occasionally reveals the presence of discrete and diffused bands within the 65,000–80,000 broad band pattern. Such heterogeneous patterns of labeling have previously been observed with visualization of other membrane receptors by photoaffinity labeling (8, 23). These broad labeling patterns are consistent with heterogeneity of the various carbohydrate substitutents of membrane proteins (23). However, we cannot totally exlude the possibility that partial proteolysis of a single $M_r \cong$ 80,000 peptide may be responsible for the generation of this apparent heterogeneity.

Covalent labeling of receptors with affinity or photoaffinity probes has been a useful method in the characterization of these proteins. Direct photoaffinity probes are usually preferable to affinity ligands such as those with bromacetamide alkylating groups because a greater specificity can be achieved. Crosslinking of ligands with heterobifunctional reagents such as that reported here can also be used but may give lower levels of covalent incorporation than direct photosensitive probes. The extent of incorporation achieved in this study was 1–2% of the original ligand bound. Interestingly, attempts to label the rat striatum D_1-receptor with the azide derivative of [^{125}I]SCH-38548 led to much less efficient labeling. This may reflect the absence of a reactive group in the binding site within the proximity of the azide group. Although activated azides should incorporate into any covalent bond, the presence of a nucleophil within the proximity of the azide renders the process more efficient (24).

The results presented here suggest that the D_1-dopamine receptor ligand-binding subunit resides on a peptide of M_r ~72,000. This peptide is different from that which has been identified by photoaffinity labeling of the D_2-dopamine receptor (M_r = 94,000) of various tissues (16, 25). [^{125}I]SCH-38548 should represent a useful probe for further biochemical characterization of the receptor protein from various sources. The ability to visualize the receptor in crude membrane preparations with this probe provides a means of examining the influence of various factors or pathophysiological situations on the biochemical properties of the receptor. Moreover, this high affinity ligand, which possesses a functional arylamine, may be useful in the development of a biospecific affinity matrix for purification of the receptor.

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