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Molecular cloning and expression of a D_1 dopamine receptor linked to adenylyl cyclase activation

(rat striatum/cDNA/polymerase chain reaction/NS20Y neuroblastoma cell/cAMP)

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ABSTRACT **In order to clone the D_1 dopamine receptor linked to adenylyl cyclase activation, the polymerase chain reaction was used with highly degenerate primers to selectively amplify a cDNA sequence from NS20Y neuroblastoma cell mRNA. This amplification produced a cDNA fragment exhibiting considerable sequence homology to guanine nucleotide-binding (G)-protein-coupled receptors that have been cloned previously. To characterize this cDNA further, a full-length clone was isolated from a rat striatal library by using the cDNA fragment as a probe. Sequence analysis of this cDNA clone indicated that it is indeed a member of the G-protein-coupled receptor family and exhibits greatest homology with the previously cloned catecholamine receptors. Northern blot analysis of various neural tissues revealed a transcript of ≈4 kb that was predominantly located in the striatum with lesser amounts in the cortex and retina. In contrast, no mRNA was detected in the cerebellum, hippocampus, olfactory bulb, mesencephalon, or pituitary. *In situ* hybridization analysis also revealed a high abundance of mRNA in the striatum as well as in the olfactory tubercle. To establish the identity of this cDNA, we performed transient expression experiments in COS-7 cells. [^{3}H]SCH-23390, a D_1-selective radioligand, exhibited specific, saturable binding only in cells that were transfected with this cDNA. Competition binding analysis with a variety of dopaminergic ligands demonstrated a D_1 dopaminergic pharmacology. In addition, dopamine as well as other D_1-selective agonists stimulated cAMP accumulation in transfected COS-7 cells. We conclude that we have cloned a cDNA encoding the D_1 dopamine receptor linked to the activation of adenylyl cyclase activity.**

Dopamine receptors belong to a large superfamily of receptors that are linked to their signal-transduction pathways via guanine nucleotide-binding regulatory (G) proteins. Pharmacological, biochemical, and physiological criteria have been used to describe two subcategories of dopamine receptors referred to as D_1 and D_2 (1). D_1 receptors have been classically defined as being linked to the stimulation of adenylyl cyclase activity (2, 3). In contrast, activation of D_2 receptors results in various responses (4), including inhibition of adenylyl cyclase activity, inhibition of phosphatidylinositol turnover, increase in K^+ channel activity, and inhibition of Ca^{2+} mobilization. The molecular characterization of D_2 dopamine receptors has been facilitated by the cloning of a cDNA encoding a rat D_2 receptor (5). More recently, this receptor has been shown to exist as two protein isoforms that are derived from a single gene yet produced by alternative RNA splicing (6–11). This splice variation occurs in a region of the receptor that may be involved in G-protein coupling, suggesting that these receptor isoforms may activate different signal-transduction pathways.

Evidence has also accumulated suggesting heterogeneity in the D_1 category of dopamine receptors. D_1 receptors have recently been described in renal tissue that stimulate phospholipase C activity independently from that of adenylyl cyclase (12–14). We have shown, using *Xenopus* oocyte expression experiments, that rat striatal mRNA encodes D_1 receptors that are coupled to phospholipase C and Ca^{2+} mobilization in a cAMP-independent fashion (15). These data suggest that there may be multiple D_1 receptors linked to different signal-transduction pathways or that a single, multifunctional D_1 receptor exists. To investigate these possibilities and to characterize the D_1 receptor(s) on a molecular level, we have attempted to clone the D_1 receptor subtype expressed in mouse NS20Y neuroblastoma cells, which is coupled to the stimulation of cAMP generation (16).§

METHODS

Polymerase Chain Reaction (PCR). Poly(A)$^+$ RNA was prepared from NS20Y cells exactly as described (15). First-strand cDNA synthesis was performed using this RNA template in the presence of avian myeloblastosis virus reverse transcriptase (Promega) and 1 μg of oligo(dT) primer. This cDNA was then submitted to 30 cycles of PCR amplification in a total volume of 100 μl with 1 μM each of the oligodeoxynucleotide primers 5′-GTCGACCCTGTGC(or T)GC(or T)C(or G)ATCAG(or C)CATG(or T)GAT(or C)C(or A)GC(or G)TA-3′ and 3′-AAGT(or C)G(or A)G(or C)G(or T)AGACGACCG(or A)AC(or G)GGGAAGAAGT(or A)ATTCGAA-5′ in the presence of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin–Elmer/Cetus). The timing used was 1.5 min at 93°C, 2 min at 55°C, and 4 min at 72°C followed by a 7-min extension at 72°C. Individual bands were isolated by gel electrophoresis and subcloned into pGEM-9Zf(−) (Promega). Minipreparations of plasmid DNA were prepared for insert sequencing as described below.

cDNA Library Screening and DNA Sequencing. A rat striatal cDNA library constructed in the λ ZAP II vector (Stratagene) was screened (6) with a PCR fragment that was ^{32}P-labeled by nick-translation. High-stringency washing of the filters was performed with 0.15 M NaCl/0.015 sodium citrate, pH 7/0.1% SDS at 65°C prior to autoradiography. λ phage found to hybridize to the probe were subsequently plaque-purified. *In vivo* excision and rescue of the nested pBluescript plasmids from the λ ZAP II clones were performed according to the Stratagene protocol. Nucleotide

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Abbreviations: G protein, guanine nucleotide-binding protein; PCR, polymerase chain reaction.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M35077).

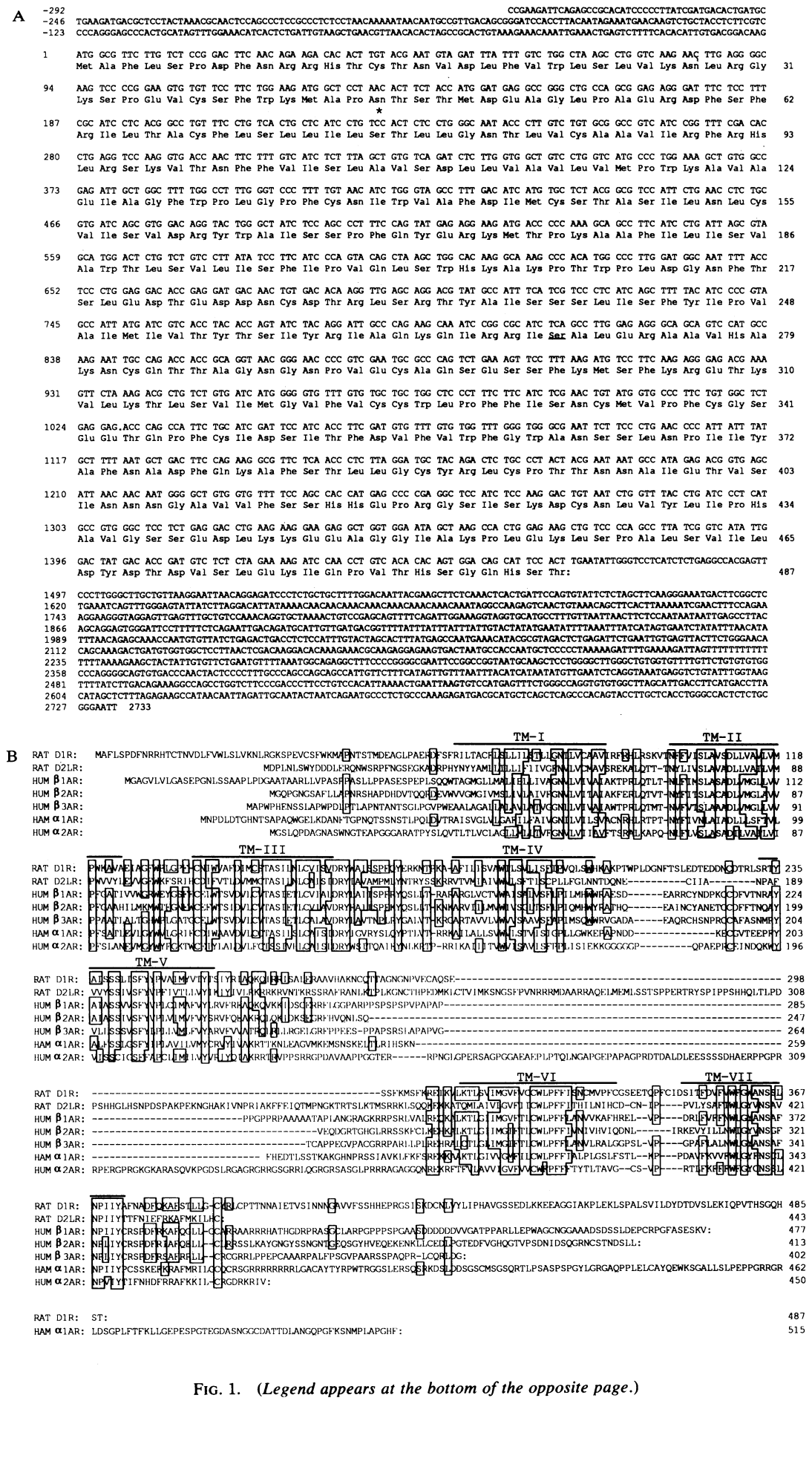

FIG. 1. (*Legend appears at the bottom of the opposite page.*)

sequence was analyzed by the Sanger dideoxynucleotide chain-termination method with Sequenase (United States Biochemical) on denatured doubled-stranded plasmid templates. Primers were synthetic oligonucleotides that were either vector-specific or derived from prior sequence information.

mRNA Analysis. Northern blot and *in situ* hybridization histochemical analyses were performed exactly as described (6), using oligonucleotide probes derived from nucleotides 489–537, 594–642, and 829–877 in pB73D1. These were radiolabeled using terminal deoxynucleotidyltransferase with either [α-^{32}P]dATP (Northern blots) or [α-[^{35}S]thio]dATP (*in situ* hybridization).

Expression Studies. A full-length cDNA insert, including the multiple cloning site of the λ ZAP II vector, was amplified from the purified λ phage clone by using T3 and T7 promoter primers and PCR as described above. The reaction product was subcloned into the *Not* I/*Kpn* I restriction sites in a modified expression vector, pCD-SRα (17). This construct was used to transiently transfect COS-7 cells by the calcium phosphate precipitation technique (18). After 72 hr, membranes were prepared and assayed for D_1 receptor binding activity with [^{3}H]SCH-23390 (DuPont/NEN) exactly as described (16). Intact cells were used for cAMP assays (19). Protein concentrations were determined using the bicinchoninic acid (BCA) reagent (Pierce) as described (20).

RESULTS AND DISCUSSION

To clone the D_1 dopamine receptor linked to adenylyl cyclase activation, we used the PCR method to selectively amplify cDNA sequences from mRNA purified from mouse NS20Y neuroblastoma cells, which express this receptor subtype (16). Poly(A)$^+$ RNA was used to synthesize cDNA by reverse transcription and this cDNA was subjected to PCR amplification with a pair of highly degenerate primers derived from the third and sixth transmembrane regions of the previously cloned adrenergic, D_2 dopamine, and serotonin receptors. This process resulted in the amplification of several cDNA fragments, which were preliminarily characterized by DNA sequence analysis (data not shown). One of these fragments exhibited considerable sequence homology to previously cloned G-protein-coupled receptors and was used to screen a rat striatal cDNA library in order to isolate a full-length clone. One clone (pB73D1) with an insert of ≈3.6 kilobases (kb) was isolated and found to hybridize strongly with the ^{32}P-labeled PCR probe on dot blot analysis (data not shown). The nucleotide sequence of this cDNA exhibited >90% homology in the region of the PCR fragment, the divergence of which is probably attributable to species differences (mouse vs. rat).

The complete nucleotide and deduced amino acid sequence for clone pB73D1 is shown in Fig. 1*A*. The longest open reading frame in this cDNA codes for a 487-residue protein of 54,264 Da. Although the neighboring sequence of the first ATG in this reading frame is similar to Kozak's consensus initiation sequence (23), the third methionine codon, at position 46, actually provides a better match (Fig. 1*A*). Additional work will thus be necessary to definitively assign the translational start site for this mRNA. The 292-nucleotide sequence preceding the long open reading frame contains a

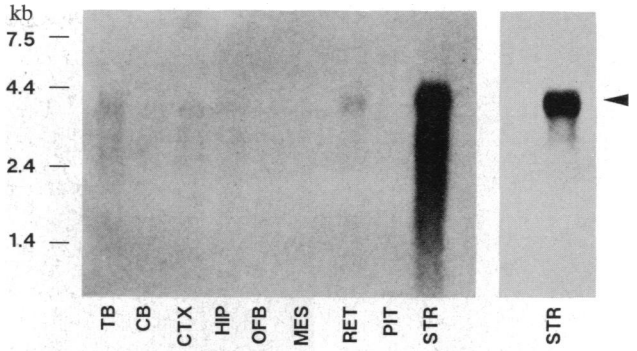

FIG. 2. Northern blot analysis of D_1 receptor transcripts in rat brain and other tissues. Each lane contained 3 μg of poly(A)$^+$ RNA. Hybridizations were performed using ^{32}P-labeled oligonucleotides as described (*Methods* and ref. 6). Locations of RNA size markers are indicated. (*Left*) Ten-day autoradiographic exposure. Lanes: TB, total brain; CB, cerebellum; CTX, cortex; HIP, hippocampus; OFB, olfactory bulb; MES, mesencephalon; RET, retina; PIT, pituitary; STR, striatum. (*Right*) Short (36-hr) exposure of RNA from striatum. This experiment was performed twice with similar results.

smaller open reading frame of unknown significance (nucleotides −259 to −154). Hydrophobicity analysis (data not shown) of the translated protein reveals seven clusters of ≈24 hydrophobic residues, predicted to represent membrane-spanning domains, connected by three extracellular and three intracellular loops. This pattern is similar to that observed for other cloned G-protein-coupled receptors where the NH_2 terminus is proposed to be extracellular and the COOH terminus projects into the cytoplasm (24). The NH_2 terminus contains one consensus site for N-linked glycosylation, while the predicted third cytoplasmic loop exhibits one consensus recognition site for phosphorylation by the cAMP-dependent protein kinase (Fig. 1*A*). In addition, the long COOH terminus contains several serine and threonine residues possibly representing additional sites for regulatory phosphorylation (25).

Comparison of the deduced amino acid sequence for the pB73D1 cDNA clone with the sequences of various catecholamine receptors (Fig. 1*B*) shows that the regions of highest identity occur within the predicted transmembrane domains. Within these regions, the pB73D1 protein exhibits sequence homologies of 44% with the rat D_2 dopamine receptor; 44%, 43%, and 40% with the human β_1-, β_2-, and β_3-adrenergic receptors, respectively; and 43% and 42% with the hamster α_{1B}- and human α_{2A}-adrenergic receptors, respectively. When compared with various serotonin receptors, the transmembrane regions of the pB73D1 protein exhibited homologies of 40% for $5HT_{1A}$ and 37% for both $5HT_{1C}$ and $5HT_2$ receptors (data not shown). The NH_2 and COOH termini and the extracellular and intracellular loops are significantly more divergent among these receptors. It is interesting that within the third putative transmembrane domain of pB73D1, there is a conserved aspartic residue that is common to all biogenic amine receptors that have been sequenced thus far (26). Moreover, the fifth transmembrane domain of pB73D1 also contains two serine residues that are conserved among catecholamine receptors and are critical for the recognition of agonist ligands possessing a catechol group

FIG. 1 (*on opposite page*). (*A*) Nucleotide sequence of pB73D1 along with the deduced amino acid sequence of the longest open reading frame. Nucleotide sequence is numbered from the putative initiator methionine and indicated at the left of each line; amino acid numbers are indicated at right. The postulated N-linked glycosylation site is indicated by an asterisk. The potential site for phosphorylation by the cAMP-dependent protein kinase is underlined. (*B*) Comparison of the rat D_1 dopamine receptor amino acid sequence with that of various other catecholamine receptors. Amino acid sequences of the human (HUM) β_1-, β_2-, β_3-, and α_{2A}-adrenergic receptors (AR) (21), the hamster (HAM) α_{1B}-adrenergic receptor (22), and the rat D_{2L} dopaminergic receptor (6) were aligned to optimize the homology with the rat D_1 dopamine receptor sequence. Amino acid identities between the D_1 receptor and at least two other catecholamine receptors are indicated by boxes. The putative transmembrane (TM) regions are indicated by the heavy lines.

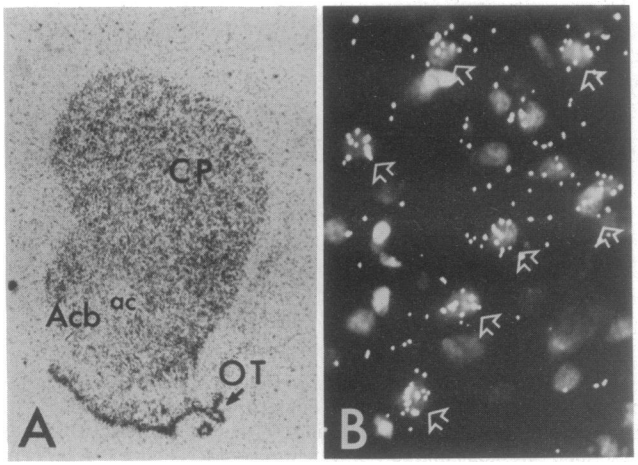

FIG. 3. *In situ* hybridization histochemical localization of D_1 receptor mRNA in a coronal section of rat brain that includes the striatum. *In situ* hybridization was performed using ^{35}S-labeled oligonucleotides as described (*Methods* and ref. 6). (*A*) Low magnification of autoradiographic film apposed to forebrain section, showing dense labeling within the striatum, including the caudate–putamen (CP) and nucleus accumbens (Acb). Labeling is also quite strong in the olfactory tubercle (OT). ac, Anterior commissure. (×6.5.) (*B*) High magnification darkfield photomicrograph showing silver grains (white dots) overlying medium-size neurons in the striatum generated by *in situ* hybridization labeling of D_1 receptor mRNA. About half of the medium-size neurons in this field of the striatum are labeled (arrows), which is typical of the striatum as a whole. (×150.)

(26). These observations suggest the pB73D1 clone encodes a receptor for an endogenous catecholamine ligand.

In an initial attempt to establish the identity of pB73D1, we analyzed the tissue distribution of its corresponding mRNA by Northern blot and *in situ* hybridization analyses. Northern blot analysis (Fig. 2) of various neural tissues reveals a transcript of ≈4 kb that is predominantly located in the striatum with lesser amounts in the cortex and retina. In contrast, no mRNA is observed in the cerebellum, hippocampus, olfactory bulb, mesencephalon, or pituitary. *In situ* hybridization also indicates a high abundance of mRNA in the striatum as well as in the olfactory tubercle (Fig. 3*A*). Approximately half of the medium-sized neurons in the striatum are identified by this technique (Fig. 3*B*). The tissue distribution of pB73D1 mRNA exhibited in Figs. 2 and 3 is remarkably similar to that of the D_1 dopamine receptor as demonstrated by receptor binding and autoradiography studies (1).

To definitively establish the identity of the receptor encoded by the pB73D1 clone, the cDNA insert was subcloned into the pCD-SRα vector (17) for expression in eukaryotic cells. The resulting plasmid, pSRα-D1, was used to transiently transfect COS-7 cells. Fig. 4*A* shows that [^{3}H]SCH-23390, a D_1-selective radiolabeled antagonist, binds to COS-7 membranes in a saturable fashion with high specific activity (≈400 fmol/mg of protein) and an affinity (0.3 ± 0.03 nM) in good agreement with that found in the rat striatum (1). No specific binding is detected in COS-7 cells that have not been transfected with pSRα-D1 or that have been transfected with the pCD-SRα vector alone (data not shown). Fig. 4*B* demonstrates the ability of a variety of dopaminergic ligands to compete for specific [^{3}H]SCH-23390 binding to transfected COS-7 cell membranes. (+)-SCH-23390 is the most potent agent (0.2 ± 0.01 nM) and is ≈200-fold more potent than its enantiomer, (−)-SCH-23388 (41 ± 1.2 nM). The nonselective dopaminergic antagonist (+)-butaclamol also exhibits high affinity (2.8 ± 0.2 nM) and is >4 orders of magnitude more potent than its inactive isomer, (−)-butaclamol (31 ± 0.8 μM). The D_2-selective antagonist spiperone exhibits relatively low affinity (290 ± 7 nM), as do the serotonin antagonists ketanserin (0.42 ± 0.031 μM) and mianserin (0.18 ± 0.042 μM). The endogenous agonist, dopamine, is also able to completely inhibit [^{3}H]SCH-23390 binding (0.64 ± 0.092 μM). This rank order of potency and the absolute affinities (K_i) of these compounds agree well with those previously demonstrated for striatal D_1 receptors (1).

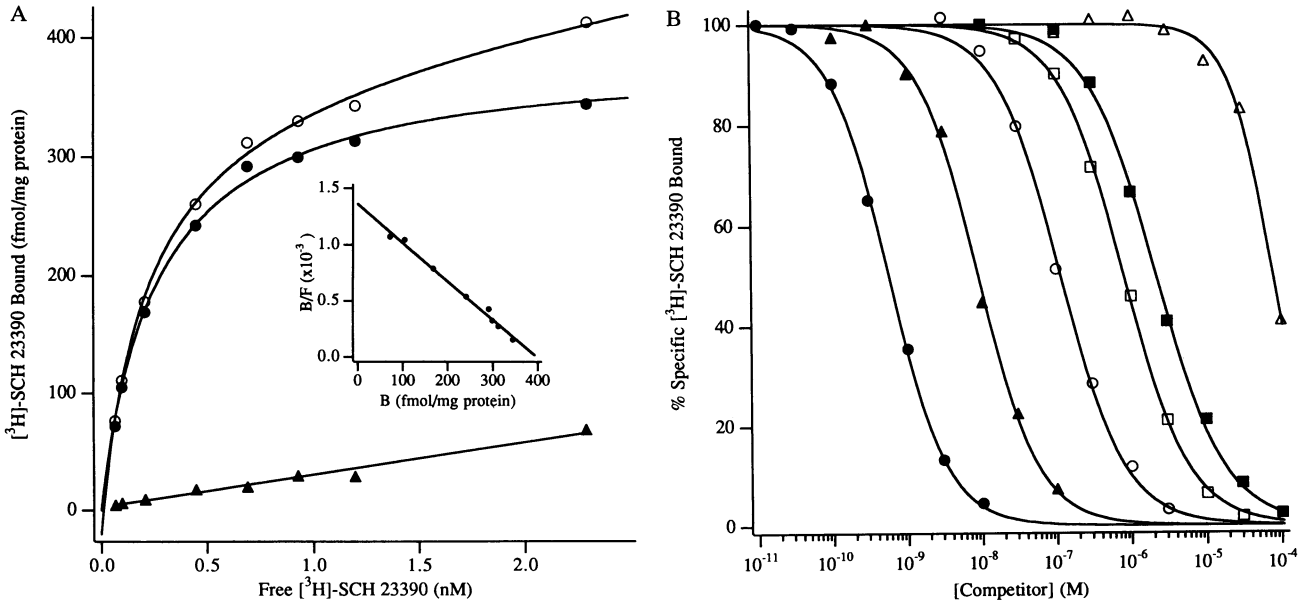

FIG. 4. Expression of the D_1 receptor cDNA in COS-7 cells assayed by [^{3}H]SCH-23390 binding. (*A*) Saturation isotherms of the total (○), nonspecific (▲), and specific (●) binding of [^{3}H]SCH-23390 to transfected COS-7 cell membranes. (*Inset*) Scatchard transformation of the specific binding data; B, bound; F, free. In this experiment, which was representative of three, the calculated K_D and B_{max} values were 0.3 nM and 400 fmol/mg of protein, respectively. (*B*) Competition analysis of various dopaminergic ligands for [^{3}H]SCH-23390 binding in COS-7 cell membranes. In this experiment, [^{3}H]SCH-23390 (0.5 nM) was incubated with various concentrations of the following ligands: (+)-SCH-23390 (●), (+)-butaclamol (▲), (−)-SCH-23388 (○), spiperone (□), dopamine plus guanosine 5′-[β,γ-imido]triphosphate (■), and (−)-butaclamol (△). Average K_i and SEM values from three experiments are given in the text.

pSRα-D1-transfected COS-7 cells also exhibit D_1 receptor-mediated stimulation of cAMP production. Dopamine stimulates cAMP production ≈2-fold in these transfected cells (Fig. 5). In contrast, no response to dopamine is observed in nontransfected cells (data not shown). The D_1-selective agonists (+)-SKF-38393 and (±)-SKF-82958 stimulate cAMP accumulation to a similar extent as dopamine. Although SKF-38393 has been reported to be a partial agonist at D_1 receptors, its fuller efficacy observed here is probably due to the presence of spare receptors resulting from overexpression of receptor protein. In addition, the stimulation by SKF-38393 exhibits appropriate stereoselectivity, with the (−) isomer exhibiting a lower potency (Fig. 5). Finally, the β-adrenergic agonist epinephrine also exhibits a low potency relative to dopamine, as expected for a D_1 receptor. In these experiments, the β-adrenergic antagonist propranolol was included in the assays to preclude stimulation of the endogenous COS-7 cell β-adrenergic receptor. These expression data thus confirm that the cDNA which we have cloned encodes a functional D_1 dopamine receptor protein.

It is important to emphasize that the D_1 receptor that we have cloned is functionally coupled to the stimulation of adenylyl cyclase. Recently, unique D_1 receptors have been described in kidney that stimulate phospholipase C activity independently from the activation of adenylyl cyclase (12–14). We previously showed, using *Xenopus* oocyte expression experiments, that rat striatum contains mRNA encoding D_1 receptors that can couple to phospholipase C, inositol phosphate production, and Ca^{2+} mobilization in a cAMP-independent fashion (15). It is interesting that the mRNA encoding this D_1 receptor-stimulated phospholipase C response is ≈2.5 kb long (15), in comparison with the ≈4-kb D_1 receptor mRNA observed here. Moreover, in preliminary experiments, we have found that when mRNA is transcribed from the pB73D1 D_1 receptor cDNA clone and injected into *Xenopus* oocytes, dopamine will stimulate cAMP accumulation ≈2-fold but is incapable of producing a Ca^{2+} mobilization response (unpublished observations). These findings suggest that the striatum contains two separate D_1 receptor proteins that are coupled to different signal-transduction pathways. Consequently, we propose that the D_1 receptor subtypes linked to the activation of adenylyl cyclase and phospholipase C be designated D_{1A} and D_{1B}, respectively. Further experimentation involving the cloning and expression of the D_{1B} receptor subtype will be required to confirm this hypothesis.

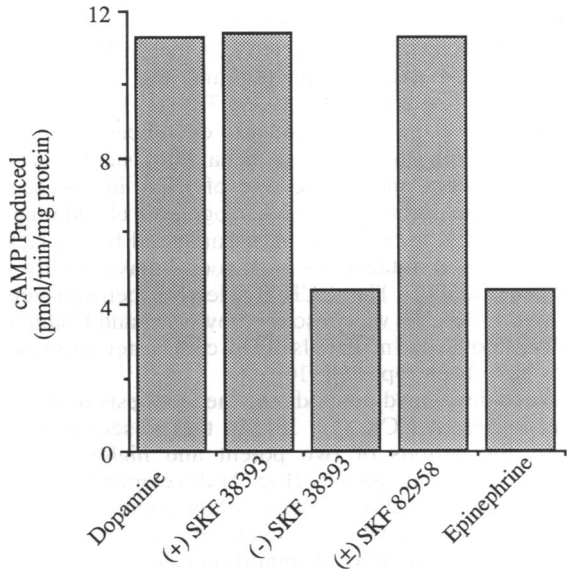

FIG. 5. D_1 receptor stimulation of cAMP production in COS-7 cells transfected with pSRα-D1. Each agonist was tested at 1 μM in the presence of 0.5 μM β-adrenergic antagonist (propranolol). Data are presented as amount of cAMP produced over the basal level, which corresponded to 17.7 pmol/min per mg of protein. The experiment shown is representative of two different transfection experiments.

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