0026-895X/92/040652-06$03.00/0

MOLECULAR PHARMACOLOGY, 41:652-659

Molecular Cloning and Expression of the Rhesus Macaque D1 Dopamine Receptor Gene

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Received June 14, 1991; Accepted January 18, 1992

SUMMARY

Using homologous probes for the cloning of related genes within the family of guanine nucleotide-binding protein-coupled receptors, we have cloned the gene for the rhesus macaque D1 dopamine receptor. By using the rat D1 receptor coding sequence as a probe under high stringency conditions, the rhesus D1 receptor gene was isolated from a λ EMBL3 rhesus genomic DNA library. The rhesus D1 dopamine receptor gene is intronless and encodes a 446-amino acid protein that contains two consensus sites for asparagine-linked glycosylation (Asn-5 and Asn-176) and two consensus sites for cAMP-dependent protein kinase phosphorylation (Thr-136 and Thr-268). The primary amino acid sequence of the rhesus D1 dopamine receptor shows an extremely high degree of similarity (99.6%) to the human D1 receptor. Genomic DNA analyses conducted with high and reduced stringency hybridizations indicate that the rhesus macaque D1 receptor is a member of a large multigene family. Like the human D1 receptor mRNA, the rhesus D1 receptor mRNA is approximately 4 kilobases in size and is localized predominantly in the caudate, with lesser amounts in the hippocampus and cortex. The rhesus D1 receptor coding region was inserted into the cytomegalovirus promoter-driven expression vector pcDNA-1, and the recombinant (pcDNA-D1) was cotransfected with the selectable marker pRSVneo, conferring G418 resistance, into D1 receptor-deficient C_6 glioma cells. Analyses of the selected transfectant demonstrate the expression of a high affinity, functional D1 dopamine receptor. The D1 receptor radioligand [^{3}H]SCH 23390 bound transfectant membranes with an affinity (K_d), of 0.3 nM; the D2-selective ligand spiperone, the dopamine receptor ligand clozapine, and the serotonin receptor antagonist ketanserin bound with considerably lower affinities (102, 80, and 95 nM, respectively). Both dopamine and the D1-selective agonist SKF 38393 inhibited the binding of [^{3}H]SCH 23390 to transfectant cell membranes; the binding of these agonists was sensitive to GTP. Dopamine potently stimulated the accumulation of cAMP in transfected C_6 cells, whereas SKF 38393 was a partial agonist in these cells. Also, the density of recombinant D1 receptors on the transfectant cells was decreased 40% upon treatment with 10 μM dopamine, indicating that occupation of recombinant D1 receptors by agonists alters surface expression of the receptors.

The dopamine receptors are members of a superfamily of neurotransmitter receptors that interact with G proteins to modulate second messenger systems (1, 2). Dopamine receptors serve as key elements in complex human behavior and are the primary targets for drugs used in the treatment of several psychomotor disorders, including Parkinson's disease, and schizophrenia (3, 4). Five subtypes of dopamine receptors (D1, D2, D3, D4, and D5) have been identified on the basis of their pharmacological properties, physiological effects, tissue and cell type specificity, and genetic structure (5–13). The D1 and D2 dopamine receptors have been traditionally classified according to pharmacological and biochemical criteria (1). Agonist binding to D1 receptors is linked to the stimulation of adenylyl cyclase activity; in contrast, activation of D2 receptors results in the inhibition of adenylyl cyclase activity, phosphatidylinositol turnover, and Ca^{2+} mobilization (1). The D3, D4, and D5 receptor genes have been recently isolated by homology screening (11–13). The D3 receptor is localized in limbic areas of the brain and has been postulated to serve as a potential target for neuroleptics (11). Of the five subtypes, the D4 receptor has the highest affinity for clozapine, an antipsychotic favored in the treatment of schizophrenia (12). The D5 receptor has an enhanced affinity for the endogenous neurotransmitter dopamine, compared with the D1 receptor, and may be involved in maintaining dopaminergic tone and arousal (13). All five dopamine receptor subtypes have conserved structural features, including hydrophobicity profiles consistent with seven transmembrane domains, sites of asparagine-linked glycosylation near the

C.A.M. is supported by National Institutes of Health Grants HL42358 and RR00163. K.A.N. is supported by the Veterans Affairs Merit Review Program and National Institutes of Health Grant MH45372. R.P.S. was supported by an American Heart Association, Oregon Affiliate Postdoctoral Fellowship.

ABBREVIATIONS: G protein, guanine nucleotide-binding protein; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; bp, base pairs; kb, kilobases; 5-HT, 5-hydroxytryptamine.

amino terminus, potential phosphorylation sites for protein kinases in presumed cytoplasmic domains, and highly conserved amino acid similarity within the transmembrane domains (5–13).

We report here the molecular cloning, nucleotide sequence, and expression of the rhesus macaque D1 dopamine receptor gene. The rhesus D1 dopamine receptor gene has been characterized on the basis of five criteria, 1) nucleotide sequence analyses of the rhesus D1 receptor gene and comparison with previously reported sequences of the human D1 receptor homologue and other dopamine receptor genes, 2) genomic DNA analyses conducted with high and reduced stringency hybridizations, which show that the rhesus D1 receptor gene is a member of a large multigene family, 3) demonstration of distinct of rhesus D1 receptor mRNA within selected tissues of the nervous system, paralleling the known distribution of D1 receptors, 4) transfection of the rhesus D1 receptor coding sequence under the control of the cytomegalovirus promoter into C_6 glioma cells and demonstration by receptor binding and second messenger analyses of high affinity, functional D1 dopamine receptors, and 5) demonstration of recombinant D1 receptor down-regulation in transfectant cells upon dopamine agonist stimulation.

Experimental Procedures

Materials. [^{3}H]SCH 23390, [α-^{32}P]dATP, [α-^{32}P]dCTP, and hybridization membranes (Colony/Plaque Screen) were obtained from DuPont-New England Nuclear. DNA-modifying enzymes were purchased from Boehringer Mannheim (Indianapolis, IN), New England Biolabs (Boston, MA), and Bethesda Research Laboratories (Gaithersburg, MD). Nylon hybridization membranes were obtained from New England Nuclear, Schleicher and Schuell, and Amersham. The Sequenase DNA sequencing kit was purchased from U.S. Biochemical Corporation (Cleveland, OH). Most of the drugs and reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Rhesus macaque tissues were obtained from the Oregon Regional Primate Research Center Tissue Distribution Program.

Cloning of the rhesus macaque D1 dopamine receptor gene. A λ EMBL3 genomic library (Clontech) containing *Bam*HI partial digests of rhesus macaque genomic DNA was transferred to nylon membranes (Colony/Plaque Screen, Dupont-New England Nuclear). The 770-bp *Pvu* II fragment containing rat D1 receptor coding sequence (generously provided by O. Civelli, Vollum Institute for Advanced Biomedical Research, Portland, OR) was labeled with [α-^{32}P]dCTP by the random primer method and used to screen the rhesus genomic library. Hybridizations were conducted in 50% formamide, 5× SSC (1× SSC is 0.15 M sodium chloride, 0.15 M sodium citrate), 5× Denhardt's solution (100Y Denhardt's solutin is 2% Ficoll (mol wt 400,000), 2% polyvinyl pyrolidone (mol wt 400,000), 2% bovine serum albumin), 0.1% sodium pyrophosphate, 1% SDS, 100 μg/ml denatured salmon sperm DNA, at 37° for 48 hr. Filters were washed at highest stringency in 0.2× SSC at 60°. DNA from positive isolates was digested with *Pvu*II and subjected to electrophoresis in agarose gels, before transfer onto nylon membranes and blot hybridization with the ^{32}P-labeled 770-bp *Pvu*II fragment. Prior blot analyses of rhesus macaque genomic DNA indicated that the two *Pvu*II restriction sites contained in the rat D1 receptor coding sequence were conserved. A 700–800-bp *Pvu*II restriction fragment that was recognized by the rat D1 receptor probe was identified in one set of genomic clones. The insert from one λ clone (λ 5–1) was subcloned into pGEM 3Zf(+) (Promega) and characterized extensively by restriction endonuclease mapping. An internal 2.1-kb *Eco*RI-*Sph*I fragment, containing the rhesus D1 receptor coding sequence, was targeted for DNA sequence analyses. DNA sequencing was performed on both strands by the Sanger dideoxynucleotide chain termination method (14), using Sequenase. Fragments for DNA sequencing were generated by use of the nested deletion approach described by Steggles (15) or by direct subcloning of identified restriction fragments. A 2.0-kb *Pst*I-*Sph*I fragment was subcloned into the polylinker of the cytomegalovirus promoter-driven expression vector pcDNA-1 (Invitrogen, San Diego, CA), and the recombinant pcDNA-D1 was used for expression analyses.

Nucleic acid isolation and blot analyses. Preparation and restriction endonuclease digestion of genomic, λ bacteriophage, or plasmid DNA, agarose gel electrophoresis, Southern transfer to nylon membranes (Nytran; Schleicher and Schuell), and generation of random-primed ^{32}P-labeled DNA probes (Boehringer Mannheim) have all been previously described (14). Southern blots were prehybridized with 30% (reduced stringency) or 50% (high stringency) formamide, 5× SSC, 5× Denhardt's solution, 1% SDS, 5 mM EDTA, 0.1% sodium pyrophosphate, 100 μg/ml denatured salmon sperm DNA, for 4 hr at 37°. ^{32}P-labeled probes were then added to the blots (5×10^6 dpm/ml) and allowed to hybridize for 48 hr at 37°. Southern blots were washed twice in 2× SSC, 1% SDS, at 55° for 10 min for reduced stringency analyses or twice in 2× SSC, 1% SDS, at 55° for 10 min and once in 0.1× SSC, 1% SDS, at 65° for 20 min for high stringency analyses.

RNA was extracted from brain tissues and prepared using the guanidinium isothiocyanate method (14). For Northern blot analysis, RNA was denatured using formaldehyde and was subjected to electrophoresis in 1.2% agarose gels (14). After electrophoresis, RNA was blotted onto nylon membranes (N-Bond; Amersham Corp.) and immobilized to the blots by UV cross-linking (1.2×10^5 μJ/cm^2) (Fisher Scientific). The membranes were prehybridized with 50% formamide, 0.2% polyvinylpyrrolidine (M_r 40,000), 0.2% Ficoll (M_r 400,000), 0.2% bovine serum albumin, 50 mM Tris·HCl (pH 7.5), 1 M sodium chloride, 0.1% sodium pyrophosphate, 1% SDS, 100 μg/ml denatured salmon sperm DNA, for 4 hr at 42°, and were subsequently hybridized with the ^{32}P-labeled 2.0-kb *Pst*I-*Sph*I fragment ($3–5 \times 10^6$ cpm/ml) for 48 hr at 42°. The blots were washed twice in 2× SSC, 1% SDS, at 55° for 10 min and once in 0.1× SSC, 1% SDS, at 65° for 15 min and were then exposed to X-ray film for 18 hr at −80°, with intensifying screens.

DNA transfection and cell culture. C_6 glioma cells were maintained in DMEM supplemented with 3% calf bovine serum and 2% fetal bovine serum. Transfection of DNA into C_6 cells was performed using the calcium phosphate precipitation method of Graham and van der Eb (16), with modifications as described by Chen and Okayama (17). Plasmid DNA (pRSVneo) conferring neomycin resistance (18) and pcDNA-D1 were mixed as a coprecipitate (15 μg total; molar ratio of pRSVneo to pcDNA-D1, 2:7) and applied to exponentially growing C_6 cells in a 100-mm culture dish for 24 hr. Drug selection began 72 hr later, with the addition of the neomycin analogue G418 (Sigma) (final concentration, 600 μg/ml) to the medium, and was maintained for approximately 2 weeks. Clonal G418-resistant cells were isolated using 10-μl cloning rings (Vangard International, Neptune, NJ) and were expanded into duplicate 60-mm dishes. Cell lines positive for D1 receptors, as determined by binding of [^{3}H]SCH 23390, were expanded into 100-mm dishes for further characterization. The apparent affinity of antagonist ligands was determined by inhibition of binding of [^{3}H]SCH 23390. Some of the antagonist competition experiments used pooled cells from several D1 receptor-positive transfectant clones. All other experiments used one clonal cell line.

Radioligand binding assays. Cells were lysed by replacing the growth medium with ice-cold hypotonic buffer (1 mM Na^+-HEPES, pH 7.4, 2 mM EDTA). After swelling for 10–15 min, the cells were scraped from the dish and centrifuged at 24,000 × *g* for 20 min. The resulting crude membrane fraction was resuspended with a Brinkmann Polytron homogenizer at setting 6, for 10 sec, in 50 mM HEPES (pH 8.0), for immediate use in radioligand binding assays. Aliquots of the membrane preparation were added to assay tubes containing (final concentrations) 50 mM HEPES, pH 8.0, 0.9% NaCl, 0.001% bovine serum albumin, [^{3}H]SCH 23390 (75 Ci/mmol), and appropriate drugs. (+)-Butaclamol

(2 μM for recombinant D1 receptors or 10 μM for rhesus caudate) was used to define nonspecific binding. Incubations were initiated by the addition of membranes, conducted at 30° for 60 min, and stopped by the addition of ice-cold Tris-buffered saline (10 mM Tris·HCl, pH 7.4, 0.9% NaCl) to each assay tube. The assays were filtered through glass fiber filters (Schleicher and Schuell no. 30) and washed with an additional 10 ml of wash buffer. The radioactivity retained on the filters was counted using a Beckman LS 1701 scintillation counter.

Membranes from rhesus macaque caudate tissue were prepared as described previously for preparation of human brain membranes (19). Tissue was homogenized in 40 volumes (w/v) of ice-cold Tris buffer (50 mM Tris·HCl, pH 7.4 at 25°) and centrifuged at 25,000 × *g* for 20 min. The resulting pellet was homogenized in 10 volumes (based on original wet weight) of ice-cold Tris buffer supplemented with 5 mM EDTA and was stored at −70°. Before use in radioligand binding assays, samples were thawed, diluted to 40 volumes with Tris-EDTA, and incubated at 30° for 30 min. The homogenate was then centrifuged again and resuspended in Tris-buffered saline for direct use in binding assays.

Saturation and competition binding experiments were conducted in volumes of 0.5 ml and 0.25 ml, respectively. IC_{50} values were determined by nonlinear regression analysis using the program GraphPAD. In competition experiments, the concentration of [^{3}H]SCH 23390 varied from 0.6 to 1.7 nM. K_i values were calculated from experimentally determined IC_{50} values as described by Munson and Rodbard (20). Averages for K_i and K_d values are expressed as the geometric means (the antilogarithm of mean logarithms) (21). Protein concentrations were determined by the method of Peterson (22). Experiments assessing the sensitivity of binding of agonists to 100 μM GTP were conducted as described above, with minor modifications. After centrifugation, the membrane pellet was resuspended in HEPES buffer containing 4 mM $MgCl_2$ and was incubated at 30° for 30 min before recentrifugation. The assay buffer in these experiments included 4 mM $MgCl_2$, 1 mM ascorbate, and 1 mM Na^+-EDTA.

cAMP accumulation. The accumulation of cAMP in intact cells was measured as described previously (23). Cells were seeded in six-well cluster dishes at a density of 18,000 cells/cm^2. On day 3, the growth medium was replaced by 1.5 ml of HEPES-buffered L15 medium, and the cells were incubated at 37° for 2 hr. [^{3}H]Adenine (1 μCi/well) was added to the incubation medium 15 min before addition of drug. Incubations with D1 receptor agonists were conducted for 7 min, in the presence of the β-adrenergic receptor antagonist propranolol, and were terminated by two rinses with ice-cold phosphate-buffered saline. [^{3}H]ATP and [^{3}H]cAMP were extracted in 3% trichloroacetic acid and separated using successive Dowex and alumina columns. Results are expressed as the percentage of [^{3}H]ATP converted to [^{3}H]cAMP.

Treatment of transfectant cells with dopamine. Cells were seeded in 15-cm-diameter tissue culture plates at a density of 18,000 cells/cm^2. On day 3, DMEM was replaced with fresh medium; treatment with dopamine began on day 5. Dopamine was dissolved in DMEM immediately before use, sterilized by filtration, and added to the plates at appropriate intervals. Dopamine treatment was terminated by rinsing cells with warm phosphate-buffered saline and incubating them for an additional 10 min in fresh DMEM. Cells were then lysed, membranes were prepared, and the density of receptors was determined by saturation binding analysis with [^{3}H]SCH 23390. GTP (100 μM) was added to these assays to inhibit binding of residual dopamine to the receptors.

Results

Isolation and characterization of the rhesus macaque D1 dopamine receptor gene. A rhesus macaque genomic library in λ EMBL3 was screened, under high stringency hybridization conditions, with a random-primed 770-bp *Pvu*II fragment containing coding sequences of the rat D1 receptor gene (6). Several clones were isolated under these conditions; one set of clones contained a 700–800-bp *Pvu*II fragment that hybridized to the rat D1 receptor probe upon Southern blot analyses. Sequence analyses of one of these genomic clones (clone 5–1) indicated that the 15-kb insert contained an intronless open reading frame encoding a 446-amino acid protein (M_r 48,304) (Fig. 1). The predicted amino acid sequence of this protein is 99.6% similar to the human D1 dopamine receptor. These results suggest that this clone contains sequences that encode the rhesus macaque homologue of the D1 dopamine receptor.

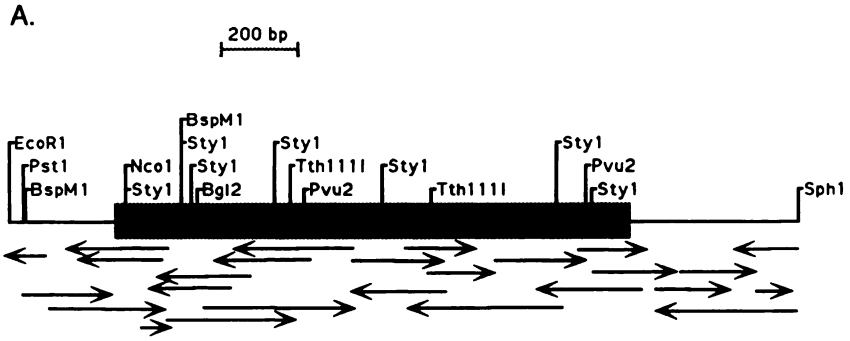
A comparison of the rhesus macaque (clone 5–1) and human D1 receptor sequences shows that they share nearly identical structural features. Both receptors possess two Asn-X-Ser(Thr) consensus sites for asparagine-linked glycosylation, one site located in the amino terminus at Asn-5 and the second site in the second extracellular loop at Asn-176, and two sites for cAMP-dependent protein kinase phosphorylation, at Thr-136 and Thr-268. The rhesus and human D1 receptors also share several common serine and threonine residues in the predicted cytoplasmic loops and in the carboxyl-terminal region that can serve as potential protein kinase C or receptor kinase phosphorylation sites (24, 25). Like other catecholamine receptors, the rhesus D1 receptor contains a conserved aspartate residue (Asp-103) in transmembrane domain III and a cluster of conserved serine residues (Ser-197, Ser-198, and Ser-202) in transmembrane domain V. These residues are postulated to serve as counterions in β-adrenergic receptor agonist binding (26, 27). In addition, the D1-like receptors (D1 and D5) contain a fourth conserved serine residue (Ser-199) not found in other catecholamine receptors. The contribution of each of these conserved serine residues to the binding of catecholamines to β-adrenergic receptors differs from their contribution to the binding of dopamine to D2 receptors[1]; it is not known which of these four serine residues are important determinants for dopamine binding to D1 receptors. In addition, the rhesus D1 receptor also contains a cysteine residue (Cys-347) in the carboxyl terminus that is conserved in many catecholamine receptors and may be palmitoylated (28). There are only two amino acid differences between the rhesus macaque and human D1 receptor sequences; Ile-311 and Ile-438 in the rhesus receptor are substituted with Asn-311 and Met-438, respectively, in the human receptor. The overall homology of the rhesus macaque D1 dopamine receptor to the human D2, rat D3, human D4, and human D5 receptors is 42%, 39%, 28%, and 50%, respectively.

Southern blot analyses of rhesus macaque genomic DNA digested with restriction endonucleases were performed using the rhesus 734-bp *Pvu*II fragment as a hybridization probe, under high and reduced stringency conditions (see Fig. 3). The rhesus 734-bp *Pvu*II fragment contains exclusively D1 receptor coding sequence (Fig. 1A) and is equivalent to the rat 770-bp *Pvu*II fragment. Under high stringency conditions (Fig. 2A), we observed patterns with multiple bands, suggesting that the rhesus D1 receptor gene has multiple copies or is a member of a highly related multigene family. When genomic Southern blot analyses were conducted under reduced stringency hybridization conditions (Fig. 2B), additional bands were observed, suggesting that the rhesus macaque D1 dopamine receptor gene is a potential member of an extended multigene family.

D1 dopamine receptor mRNA distribution within the

[1] K. A. Neve, unpublished observations.

A.



B.

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                                                                    TTAGGAACTTGAGGGGTGTCAGAGCCCCTGATGTGCTTTCCCTTAGGAAG   -1

ATG AGG ACT CTG AAC ACC TCT GCC ATG GAC GGG ACT GGG CTG GTG GTG GAG AGG GAC TTC TCT GTT CGT ATC CTC    75
Met Arg Thr Leu Asn Thr Ser Ala Met Asp Gly Thr Gly Leu Val Val Glu Arg Asp Phe Ser Val Arg Ile Leu

ACT GCC TGT TTC CTG TCG CTG CTC ATC CTG TCC ACG CTC CTG GGG AAC ACG CTG GTC TGT GCT GCC GTT ATC AGG   150
Thr Ala Cys Phe Leu Ser Leu Leu Ile Leu Ser Thr Leu Leu Gly Asn Thr Leu Val Cys Ala Ala Val Ile Arg

TTC CGA CAC CTG CGG TCC AAG GTT ACC AAC TTC TTT GTC ATC TCC TTG GCC GTG TCA GAT CTC TTG GTG GCC GTC   225
Phe Arg His Leu Arg Ser Lys Val Thr Asn Phe Phe Val Ile Ser Leu Ala Val Ser Asp Leu Leu Val Ala Val

TTG GTC ATG CCC TGG AAA GCA GTG GCT GAG ATT GCT GGC TTC TGG CCC TTT GGG TCC TTC TGT AAC ATC TGG GTG   300
Leu Val Met Pro Trp Lys Ala Val Ala Glu Ile Ala Gly Phe Trp Pro Phe Gly Ser Phe Cys Asn Ile Trp Val

GCC TTT GAC ATC ATG TGC TCC ACC GCG TCC ATC CTC AAC CTC TGT GTG ATC AGC GTG GAC AGG TAC TGG GCT ATC   375
Ala Phe Asp Ile Met Cys Ser Thr Ala Ser Ile Leu Asn Leu Cys Val Ile Ser Val Asp Arg Tyr Trp Ala Ile

TCC AGC CCT TTC CGG TAT GAG AGA AAG ATG ACC CCC AAG GCA GCC TTC ATC CTG ATC AGT GTG GCA TGG ACC TTG   450
Ser Ser Pro Phe Arg Tyr Glu Arg Lys Met Thr Pro Lys Ala Ala Phe Ile Leu Ile Ser Val Ala Trp Thr Leu

TCT GTA CTC ATC TCC TTC ATC CCA GTG CAG CTC AGC TGG CAC AAG GCA AAA CCC ACC AGC CCC TCT GAT GGG AAT   525
Ser Val Leu Ile Ser Phe Ile Pro Val Gln Leu Ser Trp His Lys Ala Lys Pro Thr Ser Pro Ser Asp Gly Asn

GCC ACT TCC CTG GCT GAG ACC ATA GAC AAC TGT GAT TCC AGC CTC AGC AGG ACA TAT GCC ATC TCA TCC TCT GTA   600
Ala Thr Ser Leu Ala Glu Thr Ile Asp Asn Cys Asp Ser Ser Leu Ser Arg Thr Tyr Ala Ile Ser Ser Ser Val

ATA AGT TTT TAC ATC CCT GTG GCC ATC ATG ATT GTC ACG TAC ACC AGG ATC TAC AGG ATT GCT CAG AAA CAA ATA   675
Ile Ser Phe Tyr Ile Pro Val Ala Ile Met Ile Val Thr Tyr Thr Arg Ile Tyr Arg Ile Ala Gln Lys Gln Ile

CGG CGC ATT GCG GCC TTG GAG AGG GCA GCA GTC CAT GCC AAG AAT TGC CAG ACC ACC ACA GGT AAT GGA AAG CCT   750
Arg Arg Ile Ala Ala Leu Glu Arg Ala Ala Val His Ala Lys Asn Cys Gln Thr Thr Thr Gly Asn Gly Lys Pro

GTC GAA TGT TCT CAA CCG GAA AGT TCT TTT AAG ATG TCC TTC AAA AGA GAA ACT AAA GTC CTG AAG ACT CTG TCA   825
Val Glu Cys Ser Gln Pro Glu Ser Ser Phe Lys Met Ser Phe Lys Arg Glu Thr Lys Val Leu Lys Thr Leu Ser

GTG ATC ATG GGC GTG TTT GTG TGC TGT TGG CTA CCT TTC TTC ATC CTG AAC TGC ATT TTG CCG TTC TGC GGG TCT   900
Val Ile Met Gly Val Phe Val Cys Cys Trp Leu Pro Phe Phe Ile Leu Asn Cys Ile Leu Pro Phe Cys Gly Ser

GGG GAG ACG CAG CCC TTC TGC ATC GAT TCC ATC ACC TTT GAC GTG TTT GTG TGG TTT GGG TGG GCT AAT TCA TCC   975
Gly Glu Thr Gln Pro Phe Cys Ile Asp Ser Ile Thr Phe Asp Val Phe Val Trp Phe Gly Trp Ala Asn Ser Ser

TTG AAC CCC ATC ATT TAT GCC TTT AAT GCT GAT TTT CGG AAG GCA TTT TCA ACC CTC TTA GGA TGC TAC AGA CTT 1050
Leu Asn Pro Ile Ile Tyr Ala Phe Asn Ala Asp Phe Arg Lys Ala Phe Ser Thr Leu Leu Gly Cys Tyr Arg Leu

TGC CCG GCG ACA AAT AAT GCC ATA GAG ACG GTG AGT ATC AAT AAC AAT GGG GCC GCG ATG TTT TCC AGC CAT CAC 1125
Cys Pro Ala Thr Asn Asn Ala Ile Glu Thr Val Ser Ile Asn Asn Asn Gly Ala Ala Met Phe Ser Ser His His

GAG CCA CGA GGT TCT ATC TCC AAG GAG TGC AAT CTG GTT TAC CTG ATC CCA CAT GCT GTG GGC TCC TCT GAG GAC 1200
Glu Pro Arg Gly Ser Ile Ser Lys Glu Cys Asn Leu Val Tyr Leu Ile Pro His Ala Val Gly Ser Ser Glu Asp

CTG AAA AAG GAG GAG GCA GCT GGA ATC GCC AGA CCC TTG GAG AAG CTG TCC CCA GCC CTA TCG GTC ATA TTG GAC 1275
Leu Lys Lys Glu Glu Ala Ala Gly Ile Ala Arg Pro Leu Glu Lys Leu Ser Pro Ala Leu Ser Val Ile Leu Asp

TAT GAC ACT GAC GTC TCT CTG GAG AAG ATC CAA CCC ATC ACA CAA AAC GGA CAG CAC CCA ACT TGA ACTCCCAGATG 1352
Tyr Asp Thr Asp Val Ser Leu Glu Lys Ile Gln Pro Ile Thr Gln Asn Gly Gln His Pro Thr

AATCCTG                                                                                              1359
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Fig. 1. Restriction map, nucleotide sequence, and deduced amino acid sequence of the rhesus macaque D1 dopamine receptor gene. A, Restriction map of the 2.1-kb *Eco*RI-*Sph*I fragment containing the rhesus D1 receptor coding sequence. *Arrows*, direction and extent of sequencing of individual subclones. B, Nucleotide and deduced amino acid sequences of the rhesus macaque D1 dopamine receptor. The potential asparagine-linked glycosylation sites and cAMP-dependent protein kinase phosphorylation sites are *underlined* or *bold*, respectively. *Numbers*, nucleotide sequence relative to the translational initiation site. Ile-311 and Ile-438 in the rhesus D1 receptor are substituted with Asn-311 and Met-438, respectively, in the human D1 receptor.

rhesus macaque nervous system. The tissue distribution of D1 dopamine receptor mRNA was examined by Northern blot analysis of selected brain regions (Fig. 3). RNA was isolated from rhesus macaque nervous system tissues (caudate, cerebellum, hippocampus, cortex, amygdala, thalamus, and midbrain) and subjected to formaldehyde-agarose gel electrophoresis and Northern blot analysis, using the 2.0-kb *Pst*I-*Sph*I fragment as hybridization probe. The rhesus macaque D1 dopamine receptor mRNA is approximately 4 kb and is localized predominantly in the caudate, with lesser amounts in cortex and hippocampus. This mRNA distribution parallels the distribution observed for human D1 dopamine receptor mRNAs (5–7) and is consistent with radioligand binding studies (1, 3).

Characterization of radioligand binding to recombinant rhesus macaque D1 dopamine receptors. The D1 receptor radioligand [^{3}H]SCH 23390 bound with high affinity (K_i = 0.3 nM) to membranes prepared from one clonal line (clone 23) of C_6 cells transfected with pcDNA-D1 (Fig. 4A) but not to membranes from nontransfected cells (data not shown). The density of binding sites on these transfected cells was 350 ± 103 fmol/mg of protein. The pharmacological profile of the recombinant receptors was that expected of a D1 receptor (Fig. 4B; Table 1). Thus, the affinity of the recombinant receptors

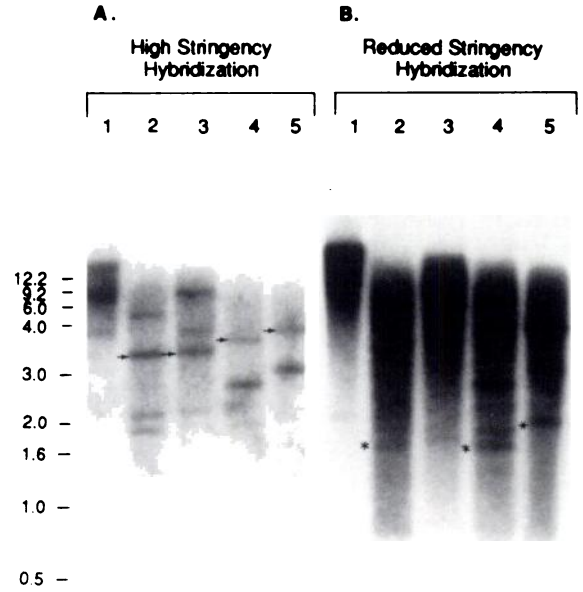

Fig. 2. Southern blot analyses of rhesus macaque genomic DNA conducted with high (A) or reduced (B) stringency hybridizations. Twenty micrograms of genomic DNA were digested with *Xba*I (*lane 1*), *Xba*I-*Pst*I (*lane 2*), *Xba*I-*Eco*RI (*lane 3*), *Xba*I-*Hind*III (*lane 4*), or *Xba*I-*Sac*I (*lane 5*) and were subjected to Southern blot analyses using the 734-bp rhesus *Pvu*II fragment (contains the rhesus D1 receptor coding sequence; see restriction map in Fig. 1) as hybridization probe. A and B contain identical DNA digests. High and reduced stringency hybridizations and washes were conducted as described in Experimental Procedures. Based on restriction mapping analyses of the genomic λ 5–1 clone, restriction enzymes for the Southern analyses were chosen to generate single fragments that contained the entire rhesus D1 receptor coding sequence. In all cases, the restriction enzymes chosen do not recognize sites within the 734-bp *Pvu*II fragment. *Arrows in A*, predicted restriction fragments. *Asterisks in B*, representative additional bands that appeared with reduced stringency hybridization and wash. *Ordinate*, migration of DNA molecular weight standards (Bethesda Research Laboratories), with weights in kb.

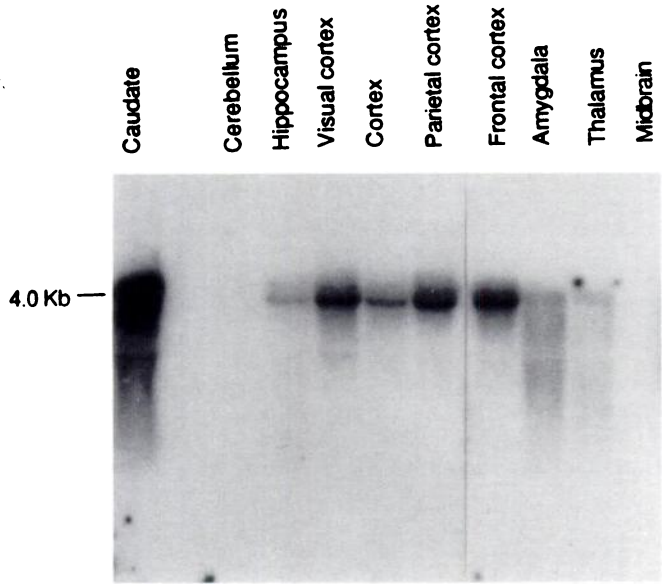

Fig. 3. Northern blot analyses of rhesus macaque D1 dopamine receptor mRNA in selected brain regions. Each lane contains 20 μg of total rhesus RNA. Northern blot analyses were conducted with the 2.0-kb *Pst*I-*Sph*I hybridization probe, as described in Experimental Procedures. *Ordinate*, size of the rhesus D1 receptor mRNA, in kb.

TABLE 1

Comparison of affinity values for binding of drugs to recombinant or native rhesus macaque D1 dopamine receptors

Mean affinity values, followed by the 95% confidence limit of the mean in parentheses, are given for inhibition of the binding of [^{3}H]SCH 23390 by the indicated drugs. Values for recombinant D1 receptors were determined in two to four independent experiments for each drug, whereas the data for native D1 receptors were determined in four experiments using rhesus caudate tissue preparations from two animals. Data for SCH 23390 are K_d values determined from saturation analysis of the binding of [^{3}H]SCH 23390 and were used to convert IC_{50} values to K_i values for all other drugs.

Drug	Affinity (K_i)	
	Native rhesus D1 receptor	Recombinant rhesus D1 receptor
SCH 23390	1.4 nM (1.1–1.8)	0.3 nM (0.2–0.4)
cis-Flupenthixol	12 nM (8–19)	2 nM (1–3)
(+)-Butaclamol	11 nM (8–2)	1.1 nM (0.8–2.1)
Spiperone	1.3 μM (0.9–1.8)	0.1 μM (0.05–0.2)
Clozapine	1.2 μM (0.5–2.8)	0.08 μM (0.06–0.11)
Ketanserin	1.1 μM (0.9–1.5)	0.09 μM (0.06–0.15)
Dopamine	24 μM (20–30)	12 μM (9–15)

for [^{3}H]SCH 23390 was greater than for (+)-butaclamol (K_i = 1.1 nM) or *cis*-flupenthixol (1.9 nM). In addition, the D2-selective ligand spiperone (102 nM), clozapine (81 nM), and the serotonin receptor antagonist ketanserin (95 nM) bound with considerably lower affinities. Radioligand binding to recombinant rhesus D1 receptors was stereoselectively inhibited; (+)-butaclamol was 6000 times more potent than (−)-butaclamol (6400 nM). Although the rank order of potency of ligands was similar in membranes prepared from transfected cells and from rhesus caudate, the affinity values obtained in the two tissues differed for all ligands except dopamine (Table 1), with higher affinity binding being observed in membranes from transfected cells. The discrepancy was not due to binding of [^{3}H]SCH 23390 to 5-HT_2 receptors, because under our assay conditions inhibition of radioligand binding by low nanomolar concentrations of ketanserin was not reliably observed.

Inhibition of the binding of [^{3}H]SCH 23390 by mianserin was performed to determine whether any binding of radioligand was to 5-HT_{1c} receptors. K_i values in transfected cells and rhesus caudate were 38 and 70 nM, respectively (K_i values are reproducible in two independent experiments). These values are close to the reported affinity of D1 receptors for mianserin and considerably higher than the K_i of mianserin at 5-HT_{1c} receptors (29). Hill coefficients for mianserin were close to unity for both transfected cells (1.06) and rhesus caudate (1.02), arguing against a measurable population of $5HT_{1c}$ receptors in either tissue.

To control for the possibility that a significant proportion of binding at higher concentrations of radioligand could be due to 5-HT_2 receptors, saturation analyses in rhesus caudate were carried out in the presence of varying concentrations of ketanserin, so that the ratio of the concentrations of ketanserin and radioligand was always 40. Thus, at a concentration of [^{3}H]SCH 23390 equal to its K_d value, the concentration of ketanserin in the assay was 50 nM, or one twentieth of the K_i of ketanserin in rhesus caudate. At this concentration, there should be little effect of ketanserin on either the K_d or B_{max} values for binding of [^{3}H]SCH 23390 to D1 receptors. If there was an effect, it would be to decrease both the apparent B_{max} and K_d for the radioligand; the apparent affinity of [^{3}H]SCH 23390 would be increased (30). Under these conditions, [^{3}H]SCH 23390 bound to D1 receptors in the rhesus caudate with

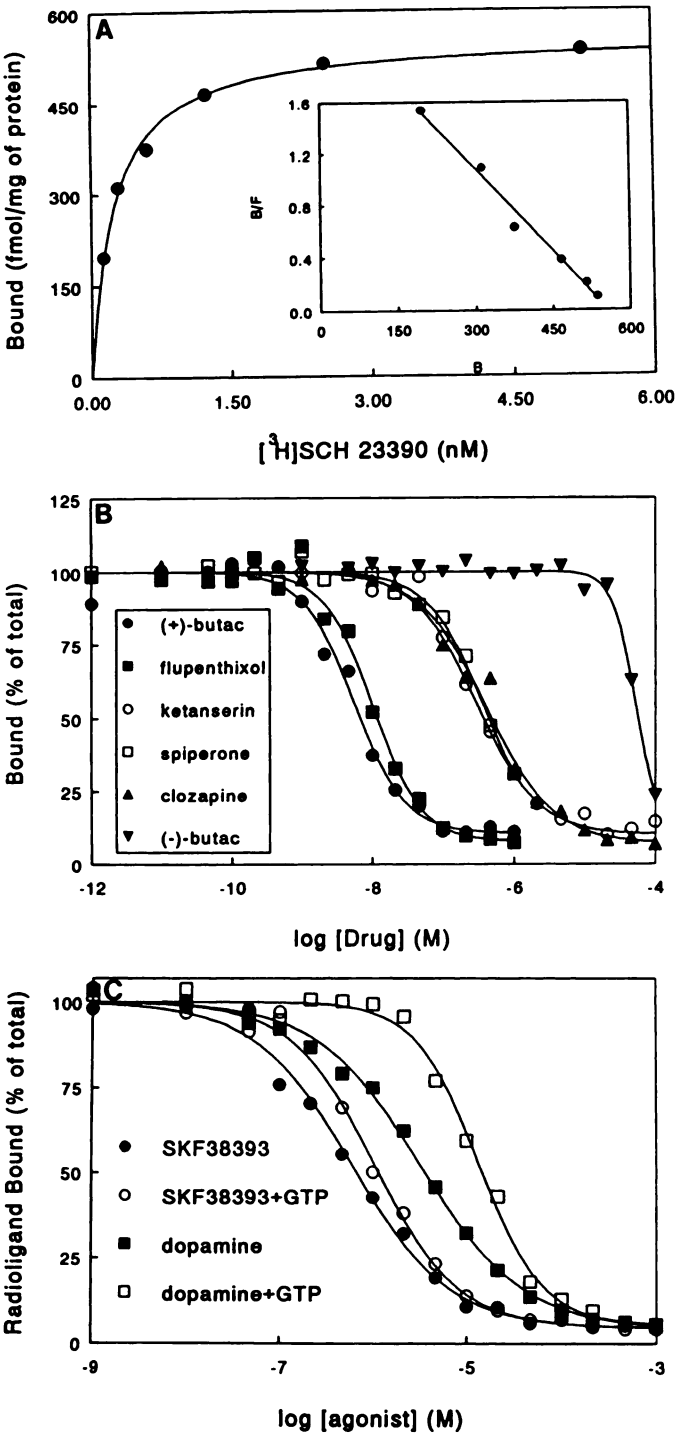

Fig. 4. Binding of [^{3}H]SCH 23390 to membranes prepared from cells transfected with the recombinant pcDNA-D1. A, A saturation isotherm for binding of [^{3}H]SCH 23390 in one of three independent experiments is shown. Results are plotted as radioligand specifically bound versus the free concentration of radioligand. *Inset*, data are transformed and plotted as bound radioligand/free radioligand $\times 10^{-3}$ (*B/F*) versus amount of radioligand bound (*B*). B and C, Results are shown from one experiment in which the binding of [^{3}H]SCH 23390 was inhibited by increasing concentrations of the indicated drugs. Each drug was tested in three independent experiments. Data are plotted as radioligand bound, expressed as the percentage of total binding, versus the logarithm of the drug concentration. IC_{50} values for the experiment shown in B (1 nM [^{3}H]SCH 23390) were as follows: (+)-butaclamol, 1.1 nM; *cis*-flupenthixol, 2.0 nM; ketanserin, 60 nM; spiperone, 78 nM; clozapine, 82 nM; and (−)-butaclamol, 11,000 nM. IC_{50} values for the experiment shown in C (1.7 nM [^{3}H]SCH 23390) were as follows: SKF 38393, 0.6 μM; SKF 38393 and 100 μM GTP, 1.1 μM; dopamine, 3.2 μM; and dopamine and 100 μM GTP, 13.4 μM.

a K_d value of 1.4 nM (Table 1). The mean density of binding sites was 467 and 871 pmol/mg of protein in caudate nuclei obtained from two rhesus macaques.

Both dopamine and the D1-selective agonist SKF 38393 inhibited the binding of [^{3}H]SCH 23390 to membranes prepared from the transfectant cells (Fig. 4C). The binding of the agonists was sensitive to GTP, in that competition curves generated with assays conducted in the presence of GTP were steeper and shifted to the right, compared with competition curves generated with assays conducted in the absence of GTP. Hill coefficients for inhibition of radioligand binding by dopamine were increased from 0.67 ± 0.06 in the absence of GTP to 1.04 ± 0.13 in the presence of GTP, and IC_{50} values were increased by GTP 4-fold, from 2 μM to 8 μM (K_i in the presence of GTP = 1.7 μM; three experiments). The binding of SKF 38393 was less sensitive to GTP. Hill coefficients and IC_{50} values were 0.87 ± 0.05 and 0.4 μM, respectively, in the absence of GTP and 0.95 ± 0.04 and 0.8 μM (K_i = 0.13 μM), respectively, in the presence of GTP (four experiments). Inhibition curves for dopamine in the absence of GTP were significantly ($p < 0.001$) improved by analysis of terms of two classes of binding sites, with 32% of the sites having high affinity for dopamine (K_i = 0.01 μM) and the remaining sites having lower affinity (K_i = 1 μM). In contrast, inhibition curves for SKF 38393 in the absence of GTP were significantly ($p < 0.05$) improved by assuming two classes of sites in only two of four experiments. K_i values determined for dopamine in these experiments were lower than values in Table 1, presumably reflecting the different preincubation and assay conditions.

Stimulation of adenylyl cyclase activity by recombinant rhesus macaque D1 dopamine receptors. Adenylyl cyclase activity was assessed by quantifying the conversion of [^{3}H]ATP to [^{3}H]cAMP in intact C_6 cells incubated with [^{3}H] adenine (Fig. 5). Dopamine potently stimulated the accumulation of cAMP in transfected C_6 cells. The mean EC_{50} value was 69 nM (three experiments). Maximal stimulation of enzyme activity by SKF 38393 was less than by dopamine. Dopamine did not stimulate enzyme activity in nontransfected C_6 cells, which do not express endogenous D1 receptors (data not shown).

Down-regulation of recombinant D1 receptors on C_6 cells. C_6 cells expressing recombinant rhesus macaque D1 receptors were treated with 10 μM dopamine for 2–8 hr (Fig. 6). Dopamine treatment decreased the density of receptors from a mean control of 649 fmol/mg of protein to 424 fmol/mg of protein after 2 hr of treatment, with no significant change in affinity of the receptors for [^{3}H]SCH 23390 (data not shown). Treatment of the transfectant cells for up to 8 hr with 10 μM dopamine caused little additional reduction in the density of receptors (mean B_{max} = 362 fmol/mg after 8 hr).

Discussion

The gene encoding the rhesus macaque D1 dopamine receptor has been isolated on the basis of analysis of the sequence and the properties of the clone expressed in eukaryotic cells. There is a remarkable degree of amino acid sequence conservation (99.6%) between the rhesus macaque and human D1 receptors.

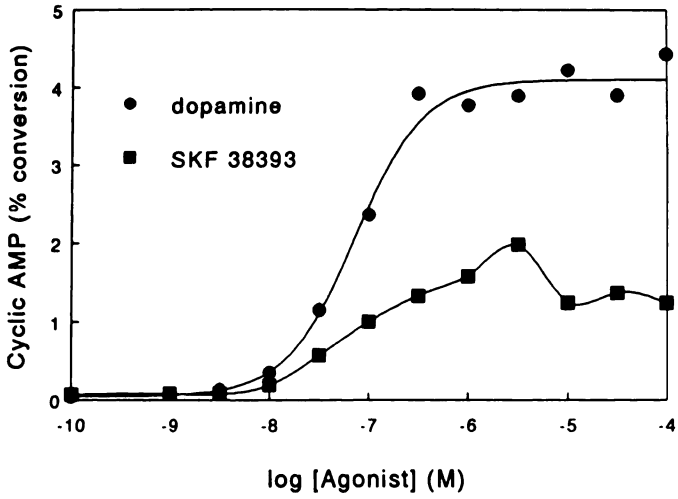

Fig. 5. Agonist-stimulated adenylyl cyclase activity in cells expressing the recombinant rhesus macaque D1 receptor. Results are shown from one of three (dopamine) or four (SKF 38393) independent experiments, in which the conversion of [^{3}H]ATP to [^{3}H]cAMP was determined in cells incubated with [^{3}H]adenine. Data are plotted as [^{3}H]cAMP/[^{3}H]ATP × 100 versus the logarithm of the concentration of agonist. EC_{50} and E_{max} values for dopamine in the experiment shown were 72 nM and 4.1%, respectively. The data for SKF 38393 were fit by a spline function, because the observed peak at an agonist concentration of 32 μM and the subsequent decrease in stimulation of enzyme activity were replicated in all four experiments. All experiments were carried out in the presence of 1 μM propranolol, to prevent stimulation of β-adrenergic receptors.

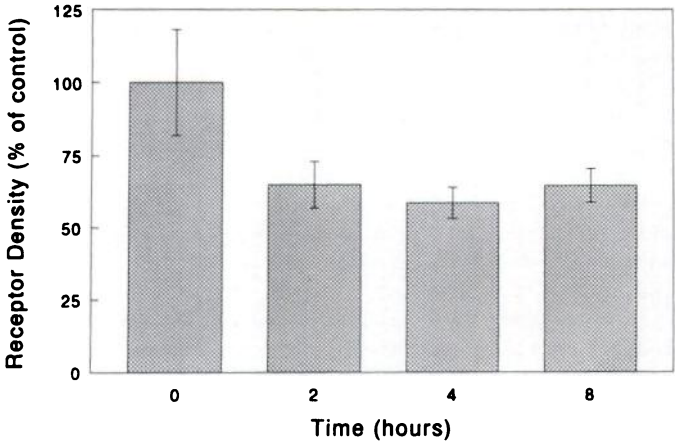

Fig. 6. Dopamine-induced down-regulation of recombinant D1 receptors. Data are shown from four independent experiments in which the effects of treatment of intact transfectant cells with 10 μM dopamine were determined. After incubation in the presence of dopamine for the indicated time, the density of D1 receptors on membranes prepared from the cells was determined by saturation analysis of the binding of [^{3}H] SCH 23390. Data shown are mean ± standard error, expressed as a percentage of the control receptor density at time 0.

There are only two amino acid differences between the human and rhesus receptors. Asn-311 and Met-438 in the human receptor are substituted by Ile-311 and Ile-438, respectively, in the rhesus receptor. These changes, one at the interface of cytoplasmic loop III and transmembrane domain VII and the other at the extreme end of the carboxyl terminus, represent nonconservative and conservative residue changes, respectively. The third cytoplasmic loop has been implicated in playing an important role in receptor-G protein coupling (31). The Asn-311 to Ile-311 conversion observed between the human and rhesus receptors does not appear to interfere with the ability of the recombinant rhesus D1 receptor to accumulate cAMP upon stimulation with dopamine in transfected cells.

Like the human D1 receptor homologue and D5 receptor genes (5–7, 13), the rhesus D1 receptor gene is intronless. Interestingly, the high stringency genomic Southern blot analyses (Fig. 2A) suggests that there are multiple copies of the rhesus macaque D1 dopamine receptor gene or that the probe detects other highly related sequences. Because the human D5 receptor gene was originally isolated by hybridization with portions of the human D1 receptor coding region (13), it appears likely that one of the bands in Fig. 2A could represent rhesus D5 receptor sequences. Furthermore, reduced stringency genomic Southern blot analyses detect restriction fragments from other related genes (Fig. 2B), possibly including genes from other members of the catecholamine receptor family (e.g., dopamine receptors or adrenergic receptors).

The distribution of rhesus macaque D1 dopamine receptor mRNA within the nervous system parallels the known tissue distribution for the human and rat D1 receptor mRNAs (5–8). Like the human D1 receptor mRNA, the rhesus D1 receptor transcript is approximately 4 kb and is localized predominantly in the caudate, with lesser amounts in cortex and hippocampus. Interestingly, multiple D1 receptor transcripts are observed in the caudate. It is unclear whether these multiple forms are due to transcription from highly related but distinct D1 receptor genes or due to transcription from alternate promoters located in a single D1 receptor gene. We are currently investigating these possibilities.

The recombinant rhesus macaque D1 dopamine receptor coding sequence, under the control of a cytomegalovirus promoter, was transfected into a mammalian cell line and shown to direct the expression of high affinity, functional D1 receptors. The ligand binding and stereoselectivity profile (Fig. 4) of the recombinant rhesus receptor parallels those of recombinant rat and human D1 receptors. Interestingly, affinity values determined using membranes from rhesus caudate differed from values determined using transfected cells. This discrepancy could reflect the presence in rhesus caudate of several "D1-like" receptors, with slightly to moderately varying affinities for [^{3}H]SCH 23390 and other ligands, or could reflect differences in membrane composition or levels of receptor expression. The sensitivity to GTP of the binding of agonists indicates that the recombinant rhesus macaque D1 receptor is capable of interacting with G proteins. Furthermore, the binding of dopamine receptor agonists to the expressed receptor stimulates the accumulation of cAMP (Fig. 5), as expected of D1 receptors. The relative lack of high affinity binding of SKF 38393 to recombinant receptors in the absence of GTP is similar to that reported for rat striatal D1 receptors (32) and may be a reflection of the partial agonist activity of SKF 38393 (33). The reduced efficacy of SKF 38393 for stimulation of adenylyl cyclase activity, compared with the efficacy of dopamine, confirms that the drug is a partial agonist at D1 receptors. In addition, the density of recombinant rhesus D1 receptors, like that of native D1 receptors on cultured cells (34), is regulated by stimulation of the receptors with dopamine, indicating that surface expression is altered by occupation of receptors with an agonist. This provides a model system for evaluation by site-directed mutagenesis of D1 receptor domains involved in ago-

nist-induced down-regulation of receptors. The cloning of the rhesus macaque D1 dopamine receptor gene and the development of a stable transfectant cell line provide valuable tools for the investigation of structure and regulation of expression of the D1 dopamine receptors.

Acknowledgments

The authors wish to express their thanks to Dr. Olivier Civelli and Q.-Y. Zhou (Vollum Institute for Advanced Biomedical Research) for their generous contribution of the rat D1 receptor *Pvu*II fragment and for invaluable discussions concerning the molecular biology of D1 dopamine receptors. We also wish to thank Dr. Richard Simerly for conducting the dissections of rhesus macaque brain tissue, Dr. Janice Thornton for her helpful discussions, and Ms. Lori Hennings for excellent secretarial assistance.

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