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Paradoxical Regulation of Dopamine Receptors in Transfected 293 Cells

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SUMMARY

Selective expression of subtypes of receptors in mammalian cell lines permits the study of the regulation of receptors in a homogeneous population of cells growing under controlled conditions. cDNAs encoding the human $D2_L$ and $D2_S$ receptors were ligated into a eukaryotic expression vector, pRc/CMV. The resulting plasmid, which contains a cytomegalovirus promoter for high expression levels, was used for stable transfection of 293 cells, a human kidney cell line. Expression of $D2_L$ and $D2_S$ receptors in 293 cells was confirmed by radioligand binding assays with [^{125}I]NCQ 298. The pharmacological properties of the expressed receptors were comparable to those of receptors in rat striatal homogenates and in other transfected cell lines. $D2_L$ and $D2_S$ receptors were coupled to inhibition of cAMP accumulation in 293 cells. Incubation of 293-$D2_L$ cells with agonists resulted in an increase in the density of D2 receptors without a change in the affinity of the receptors for [^{125}I]NCQ 298. This effect was time dependent, with a $t_{1/2}$ of approximately 6 hr. The dose dependence of up-regulation followed the pharmacological profile expected of a D2 receptor, with an order of potency of *N*-propylnorapomorphine (NPA) > quinpirole > dopamine. The density of receptors was further increased by incubation of cells with agonist together with forskolin or 8-bromo-cAMP. $D2_S$ receptors responded similarly to $D2_L$ receptors to treatment with NPA and forskolin. Exposure of 293-$D2_L$ cells to the β-adrenergic receptor agonist isoproterenol did not change the density of $D2_L$ receptors. Similarly, NPA had no effect on levels of endogenously expressed β-adrenergic receptors in 293-$D2_L$ cells, as assayed by binding of [^{125}I]iodocyanopindolol. Levels of β-adrenergic receptors in transfected 293-β_2 or 293-$D2_L$ cells did not increase after exposure to NPA but decreased after exposure to isoproterenol. Cells expressing $D2_L$ receptors were incubated with antagonists, including SCH-23390, sulpiride, haloperidol, clozapine, and epidepride, alone or in combination with NPA. Incubation of cells with SCH-23390 had no effect on the density of D2 receptors, and SCH-23390 did not block the effect of NPA. D2-selective antagonists increased the density of receptors. $D2_L$ receptor mRNA levels were unchanged during agonist treatment. This suggests a role for translational or post-translational mechanisms in the regulation of D2 receptor levels in transfected cell lines.

The cloning of multiple subtypes of dopamine receptors has led to uncertainty as to the properties and distribution of each subtype. The D2-like receptor family now includes $D2_L$ (1–4), $D2_S$ (5), D3 (6), and D4 (7) receptors. $D2_L$ and $D2_S$ receptors differ by 87 bases, corresponding to a 29-amino acid insert in the putative third intracellular loop. Both $D2_L$ and $D2_S$ receptors are coupled to inhibition of cAMP accumulation (8). Thus far, no differences in pharmacological properties have been reported for $D2_L$ and $D2_S$ receptors. D3 receptors have been expressed in CHO cells but have not been shown to couple to any second messenger system (6). Comparison of the properties of D2 and D3 receptors showed that D3 receptors have a higher affinity for D2 agonists and autoreceptor antagonists than do D2 receptors. D4 receptors, expressed in COS-7 cells, were shown to have a higher affinity for the atypical neuroleptic clozapine than do D2 receptors (7). D4 receptors have not been shown to couple to any of the known second messenger systems, but agonist binding to the D4 receptors was sensitive to GTP. Levels of mRNA encoding D3 receptors were shown to be higher in limbic than in striatal areas, whereas mRNA for D2 receptors was highly expressed in the striatum. D4 mRNA was found at lower levels in striatum than in amygdala, midbrain, and frontal cortex. Due to their lower expression levels in the basal ganglia, D3 and/or D4 receptors may be important targets for neuroleptic drugs that cause fewer extrapyramidal side effects. Although some drugs show moderate selectivity for the known subtypes of D2-like receptors, radioligands have not been available that distinguish among co-localized D2-like re-

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ABBREVIATIONS: CHO, Chinese hamster ovary; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; 8-Br-cAMP, 8-bromo-cAMP; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; EMEM, Eagle's minimum essential medium; F12, Ham's F12 nutrient mixture; [^{125}I]ICYP, [^{125}I] iodocyanopindolol; [^{125}I]NCQ 298, (*S*)-3-[^{125}I]iodo-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-5,6-dimethoxysalicylamide; NPA, *N*-propylnorapomorphine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ceptors in heterogeneous tissues such as regions of the central nervous system.

Transformed or transfected cell lines that express a single subtype of dopamine receptor can be used to define the pharmacological specificities, effector systems, and regulatory responses associated with an individual subtype of receptor. In pursuit of such a system, a transformed cell line (SUP1) was derived from rat pituitary tumor 7315a. SUP1 cells expressed D2 receptors coupled to inhibition of cAMP accumulation and inhibition of prolactin release. Exposure of SUP1 cells to either dopamine or NPA did not result in desensitization of the response to dopamine. Furthermore, agonist exposure caused an unexpected increase in the density of receptors that was blocked by coincubation with the antagonist spiroperidol (9).

Although they provide an *in vitro* system with which to study receptor regulation, SUP1 cells express relatively low levels of $D2_L$ receptors. In addition, no cell lines have been described that endogenously express $D2_S$ receptors. 293 cells transfected with plasmid containing sequences for human $D2_L$ and $D2_S$ receptors expressed high levels of each receptor subtype. Although expression was under the control of a viral promoter in the transfected cells, exposure of 293-$D2_L$ and 293-$D2_S$ cells to dopamine or NPA resulted in an increase in the density of D2 receptors.

Experimental Procedures

Materials. [^{125}I]NCQ 298 (2.2 Ci/μmol) was initially provided as a gift from DuPont/NEN (Boston, MA) and was most recently synthesized in the laboratory of Dr. Hank Kung (Department of Radiology, University of Pennsylvania School of Medicine, Philadelphia, PA). [^{125}I]ICYP was synthesized as described previously (10). [^{3}H]Adenine and [α-^{32}P]CTP were purchased from DuPont/NEN. (+)-Butaclamol, (−)-butaclamol, NPA, quinpirole, SCH-23390, and sulpiride were purchased from Research Biochemicals Inc. (Natick, MA). Dopamine-HCl, GTP, bovine γ-globulin, Tris, isoproterenol, isobutylmethylxanthine, BSA, 8-Br-cAMP, and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). BES and forskolin [7β-deacetyl-7β-(γ-*N*-methylpiperazino)butyrylforskolin] were purchased from Calbiochem (La Jolla, CA). EDTA was purchased from Fisher Scientific (Fairlawn, NJ). EMEM, Dulbecco's modified Eagle's medium, F12, and G418 sulfate were purchased from GIBCO (Grand Island, NY). Fetal bovine serum was purchased from Hyclone Laboratories, Inc. (Logan, UT). *Thermus aquaticus* polymerase was purchased from Perkin Elmer Cetus (Norwalk, CT). The plasmid vectors pGEM 7Z(f)+, pRc/CMV, and pBS(+) were purchased from Promega (Madison, WI), Invitrogen (San Diego, CA), and Stratagene Cloning Systems (La Jolla, CA), respectively. pZ523 columns for DNA purification were obtained from 5 Prime-3 Prime Inc. (Boulder, CO). Sequenase was purchased from United States Biochemical (Cleveland, OH).

Tissue culture. 293 cells, a human embryonic kidney cell line (11), were grown in monolayer culture at 37° in 5% CO_2 and were fed with EMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were fed every fourth day and subcultured or used for assay on day 7. CHO cells were grown in monolayer culture at 37° in 5% CO_2 and were fed with F12 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. COS-7 cells, an SV40-transformed cell line from monkey kidney (12), were grown in the same manner except for the use of Dulbecco's modified Eagle's medium instead of EMEM or F12 as culture medium.

Transfection of mammalian cells. cDNAs encoding the human $D2_L$ and $D2_S$ receptors were obtained from Dr. Peter Seeburg (Laboratory of Molecular Neuroendocrinology, University of Heidelberg, Heidelberg, Germany) through Dr. Dolan Pritchett (Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, PA). A cDNA encoding the β_2-adrenergic receptor was obtained from Dr. Richard Dixon (Texas Biotechnology Corp., Houston, TX). The sequences were ligated into the multiple-cloning site of the eukaryotic expression vector pRc/CMV. This vector contains a cytomegalovirus promoter for high expression levels in mammalian cells and a neomycin resistance gene for continual selection of expressing cells. Cells were transfected by the calcium-phosphate precipitation method of Chen and Okayama (13). CHO, COS, or 293 cells were plated at 20% confluency in 10-cm^2 tissue culture plates and were allowed to attach overnight. pRc/CMV plasmid DNA (20 μg) containing receptor-coding sequence was incubated in BES buffer (25 mM BES, pH 6.95, 140 mM NaCl, 0.75 mM Na_2HPO_4, 125 mM $CaCl_2$) for 10 min at room temperature. The DNA mixture was added dropwise to cells, and the cells were incubated overnight at 35° in 3% CO_2. The cells were then washed twice with medium and grown for 48 hr before subculturing and selection with 500 μg/ml G418 sulfate (a neomycin-type antibiotic). Neomycin-resistant cells were cloned by isolation and expanded, and receptor expression was measured using radioligand binding assays.

Drug treatments of cells in culture. Wild-type or receptor-expressing 293 cells were grown to 80% confluency in six-well culture dishes containing 2 ml of medium/well. Drugs in distilled deionized water (or 1% DMSO for antagonists) were filtered through 0.2-μm syringe filters and added in 100-μl aliquots to cells at varying times before harvesting.

Radioligand binding. Membrane-bound receptors were measured with a filtration binding assay as described previously (14). Cells were harvested after the plates had been rinsed with isotonic buffer (138 mM NaCl, 4.1 mM KCl, 5.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 11.1 mM glucose, pH 7.4) and placed on ice for 5 min in hypotonic lysis buffer (10 mM Tris, pH 7.4, 5 mM EDTA). Cells were scraped from the plates, homogenized, and frozen at −70°. Upon thawing, membranes were centrifuged at 20,000 × *g* for 20 min, collected, and resuspended (0.1 mg of protein/ml) in binding buffer (50 mM Tris, pH 7.4, 10 mM EDTA, 150 mM NaCl, 0.3 mM GTP). Membranes from cells that had been previously treated with drug were preincubated in 10 ml of binding buffer for 30 min at 37° to remove residual drug and were then pelleted by centrifugation as described above.

Binding assays were initiated by addition of 50 μl of membranes (5–25 μg of protein/assay) to 50 μl of [^{125}I]NCQ 298 (25–500 pM) and were incubated at 37° for 1 hr. Nonspecific binding was defined with 1 μM (+)-butaclamol. Assays were terminated by the addition of 5 ml of ice-cold wash buffer (20 mM Tris, pH 7.4, 150 mM NaCl), filtered over glass fiber filters (Schleicher and Schuell no. 30), and washed with 10 ml of ice-cold wash buffer to separate bound from free ligand. Antagonists were prepared as stock solutions in 10% DMSO. Agonists for competition binding studies were prepared in 0.025% ascorbic acid containing BSA (1 mg/100 ml). A low-salt buffer of 50 mM Tris, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, and 0.3 mM GTP was used for competition binding assays with agonists. Proteins were determined by the method of Lowry *et al.* (15), using bovine γ-globulin as a standard.

Assays of β-adrenergic receptors were carried out with [^{125}I]ICYP (10–100 pM) in a final volume of 0.5 ml. Cells were washed with 10 ml of 50 mM Tris, pH 7.4, and centrifuged twice (20,000 × *g* for 10 min) to remove residual drug. Nonspecific binding was defined with 100 μM isoproterenol. Assays, incubated for 3 hr at 37° in a buffer containing 12 mM Tris, pH 7.4, 93 mM NaCl, 1.1 mM ascorbic acid, 0.4 mg/100 ml BSA, and 0.1 mM GTP, were terminated by the addition of ice-cold wash buffer.

B_{max} and K_d values were determined by Scatchard transformation (16) of saturation binding data using unweighted linear regression analysis. Competition curves for nonradioactive compounds were analyzed by nonlinear regression for a one-site or two-site fit by using the iterative curve-fitting program SigmaPlot 3.12a for Macintosh (Jandel Scientific, Corte Madera, CA). IC_{50} values were converted to K_i values by the method of Cheng and Prusoff (17).

cAMP accumulation. cAMP accumulation was determined from

the conversion of [^{3}H]ATP to [^{3}H]cAMP, as described by Ivins *et al.* (9). Cells were grown to 80% confluency in 12-well tissue culture plates and incubated for 18 hr with [^{3}H]adenine (1 μCi/ml of medium). Cells were rinsed twice with 0.5 ml of isotonic buffer at 37° and incubated for 10 min at 37° in 400 μl/well of EMEM containing 1 mM isobutylmethylxanthine (a phosphodiesterase inhibitor) and 50 mM HEPES, pH 7.4. Drug (100 μl in EMEM) was then added to wells for 7 min at 37°. Reactions were stopped by the addition of 1 ml of 7.5% trichloroacetic acid at 4°, and plates were cooled on ice. [^{3}H]cAMP was separated from [^{3}H]ATP and [^{3}H]ADP by sequential chromatography over Dowex and alumina columns. cAMP accumulation was expressed as the percentage of conversion of radioactivity in the ATP eluate to radioactivity in the cAMP fraction.

Development of riboprobes. cDNAs coding for portions of the third intracellular loop of the human $D2_L$ receptor and for human cyclophilin were amplified by polymerase chain reaction. Oligonucleotide primers were designed using published sequence data for each gene (8, 18) and were synthesized by the DNA Synthesis Service, Department of Chemistry, University of Pennsylvania. Templates for polymerase chain reactions consisted of cDNA coding for the full length human $D2_L$ receptor and human genomic DNA for cyclophilin. Fragment amplification was carried out using *T. aquaticus* polymerase in an automated temperature cycler (Coy Instruments, Ann Arbor, MI).

Amplified sequences were digested with restriction endonucleases, purified by agarose gel electrophoresis, and ligated into pGEM 7Z(f)+ for the $D2_L$ probe or pBS(+) for the cyclophilin probe. These constructs, which contain SP6 or T3 RNA polymerase promoters for riboprobe production, were used to transform *Escherichia coli* (DH5α), which was grown on Luria-Bertani medium agar plates with ampicillin. Single colonies were selected for expansion. Plasmid DNA, isolated from bacteria by alkaline lysis and ethanol precipitation (19), was purified by centrifugation through pZ523 columns. Segments coding for the D2 receptor were sequenced using Sequenase and the dideoxy random termination method of Sanger *et al.* (20).

Extraction of RNA. Cells were harvested for quantitation of mRNA by rinsing of the plates in isotonic buffer to remove excess medium. Cells, removed from plates by agitation in 2 ml of buffer, were then centrifuged (400 × *g* for 5 min at 4°). Buffer was decanted and pellets were homogenized in 5 M guanidine isothiocyanate, 50 mM Tris, 10 mM EDTA. RNA was isolated from tissue samples by the LiCl precipitation method of Cathala *et al.* (21).

Solution hybridization. D2 receptor and cyclophilin cDNA sequences cloned into plasmid vectors were used as templates for the production of radiolabeled riboprobes by incorporation of [α-^{32}P]CTP. A solution hybridization/nuclease protection assay was carried out as described previously (22). This assay yielded a protected fragment of 291 bases for $D2_L$ mRNA and a protected fragment of 145 bases for cyclophilin mRNA, both of which were measured concurrently in each sample. After hybridization to radiolabeled riboprobes, samples were dissolved in formamide loading buffer and size-fractionated on an urea/acrylamide gel (7.6 M urea, 6% acrylamide in 90 mM Tris, 90 mM borate, 2 mM EDTA). Autoradiographs were generated after exposure of dried urea/acrylamide gels to a storage phosphor screen. The latent image generated on the phosphor screen was then laser-scanned, digitized, and quantitated using a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The amount of radioactivity in the $D2_L$ fragment was normalized to the radioactivity in the cyclophilin fragment to correct for intersample variability.

Results

Properties of dopamine receptors expressed in 293 cells. 293 cells transfected with cDNAs encoding human $D2_L$ and $D2_S$ receptors were cloned and screened for binding of [^{125}I] NCQ 298, a benzamide-type D2 receptor antagonist (23). No specific binding was observed in wild-type 293 cells. Clones expressing each receptor subtype were characterized by saturation binding. The density of receptors varied among clonal lines (20–1000 fmol/mg of protein). For some clones the density of receptors declined as a function of time in culture, but K_d values remained constant as long as receptors were detectable. No pharmacological differences were found between $D2_S$ and $D2_L$ receptors. In the presence of GTP the affinity of the receptors for agonists was decreased 2–3-fold (data not shown).

In 293-$D2_L$ cells the affinities of the receptors for agonists and antagonists were similar to those reported for D2 receptors

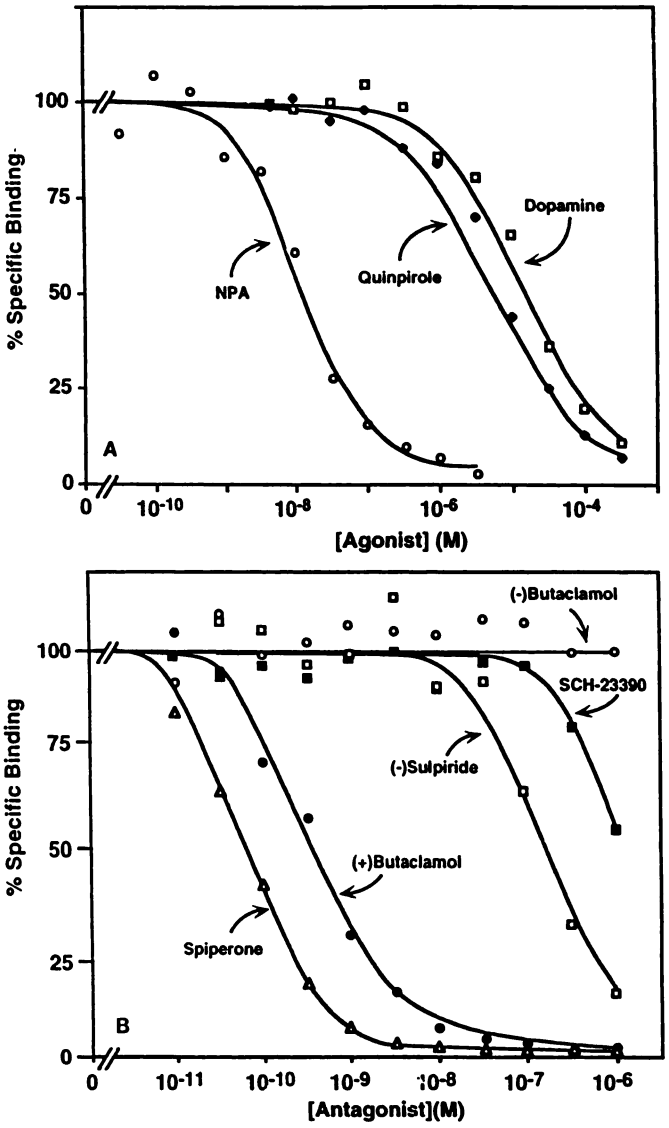

Fig. 1. Agonist and antagonist inhibition of the binding of [^{125}I]NCQ 298 to membranes from 293-$D2_L$ cells. Cells were grown and membranes were harvested as described. A, Inhibition of [^{125}I]NCQ 298 binding by agonists was measured in the presence of 300 μM GTP in a buffer containing 50 mM NaCl) and 10 mM $MgCl_2$. B, Inhibition of [^{125}I]NCQ 298 binding by antagonists was measured in isotonic buffer without $MgCl_2$ or GTP. Saturation binding to 293-$D2_L$ membranes under the same buffer conditions was used to provide K_d values for [^{125}I]NCQ 298, to permit conversion of IC_{50} values to K_i values. K_i values for agonists were as follows: NPA, 4.2 ± 1.9 nM; dopamine, 10.5 ± 6.3 μM; and quinpirole, 3.9 ± 0.81 μM. K_i values for antagonists were as follows: spiperone, 51.1 ± 14.7 pM; (+)-butaclamol, 336 ± 75 pM; (−)- sulpiride, 31.3 ± 3.9 nM; SCH-23390, 796 ± 48 nM; and (−)-butaclamol, >1 μM. *Curves* shown are representative of one of two or three independent experiments performed in triplicate.

in SUP1 cells (9) and transfected CHO cells (6). The order of potency for agonists was NPA > quinpirole > dopamine (Fig. 1A) and for antagonists was spiperone > (+)-butaclamol > (−)-sulpiride > SCH-23390 > (−)-butaclamol (Fig. 1B).

To study receptor coupling to adenylyl cyclase activity in 293 cells, inhibition of cAMP accumulation by 1 μM NPA was measured in the absence and presence of 10 μM forskolin. NPA had no effect on cAMP accumulation in wild-type 293 cells but inhibited both basal (Fig. 2, *inset*) and forskolin-stimulated (Fig. 2) cAMP accumulation in 293-$D2_L$ and 293-$D2_S$ cells. No differences were observed between $D2_S$ and $D2_L$ receptor coupling to inhibition of cAMP accumulation. The difference shown between 293-$D2_L$ and 293-$D2_S$ cells in the ability of forskolin to stimulate cAMP accumulation was not found consistently across clones. Forskolin stimulation of cAMP accumulation in multiple lines of 293-$D2_L$ cells ranged from 4.5- to 11.25-fold above basal levels. Forskolin was able to stimulate cAMP accumulation 2.4–12.2-fold above basal levels in several 293-$D2_S$ cell lines (data not shown). The order of potency of agonists for inhibition of cAMP accumulation was quinpirole > NPA > dopamine (Fig. 3). All three drugs were equally effective as full agonists; quinpirole and dopamine were more potent as inhibitors of cAMP accumulation than as inhibitors of the binding of [^{125}I]NCQ 298 (Table 1). As expected, sulpiride, a D2 receptor antagonist, had no effect by itself on cAMP accumulation, at concentrations up to 100 μM (data not shown).

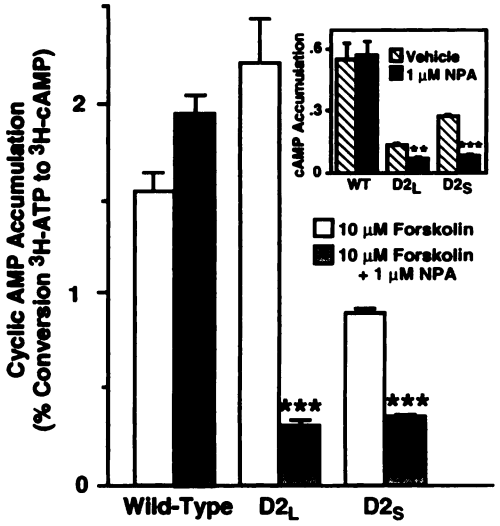

Fig. 2. Inhibition by NPA of cAMP accumulation in 293 cells. Wild-type 293, 293-$D2_L$, and 293-$D2_S$ cells were grown in 12-well plates until 80% confluent, and cAMP accumulation was measured as described. Cells were treated for 7 min with either 10 μM forskolin (□) or 10 μM forskolin plus 1 μM NPA (■). ***, $p \leq 0.001$ for NPA plus forskolin-treated, compared with forskolin-treated, samples. Data shown are mean ± standard error of three independent experiments performed in triplicate. Experiments were performed on a single clonal line of each cell type. *Inset*, data from wild-type (*WT*), 293-$D2_L$, or 293-$D2_S$ cells treated with vehicle (■) or 2.5 μM NPA (■) in the absence of forskolin. ***, $p \leq 0.001$; **, $p \leq 0.01$ for NPA-treated, compared with vehicle-treated, samples.

Characterization of $D2_L$ receptor regulation by agonists in transfected 293 cells. To study agonist-induced regulation of receptors, 293-$D2_L$ cells were incubated with 2.5 μM NPA for times ranging from 30 min to 30 hr. A time-dependent increase in the density of receptors was observed, with a $t_{1/2}$ of approximately 6 hr. A 3–4-fold increase in the density of receptors above basal levels was observed within 12–15 hr (Fig. 4). K_d values for the binding of [^{125}I]NCQ 298 in NPA-treated samples were unchanged from those in vehicle-treated controls (Fig. 4, *inset*). In another transfected cell line, COS-$D2_L$ cells, exposure to NPA also resulted in an increase in the density of $D2_L$ receptors, as seen in studies with 293 cells (data not shown).

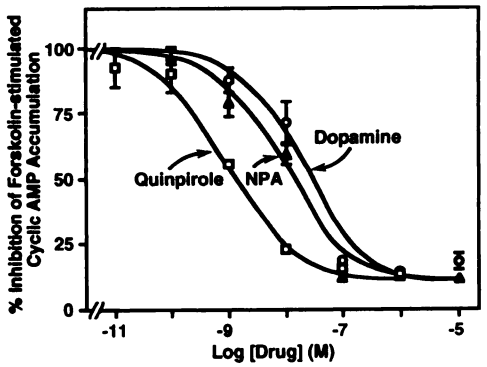

Fig. 3. Dose-response curves of agonists and antagonists for inhibition of cAMP accumulation in 293-$D2_L$ cells. Cells were grown to 80% confluency in 12-well culture plates and assays were performed as described. Forskolin (10 μM) was added to each well to stimulate cAMP accumulation. Increasing concentrations of quinpirole, dopamine, or NPA were incubated with the cells for 7 min. As shown, IC_{50} values for inhibition of cAMP accumulation were as follows: NPA, 13.4 nM; dopamine, 17.5 nM; and quinpirole, 1.1 nM. Shown are representative data from one of three independent experiments performed in quintuplicate with a single clonal line of 293-$D2_L$ cells.

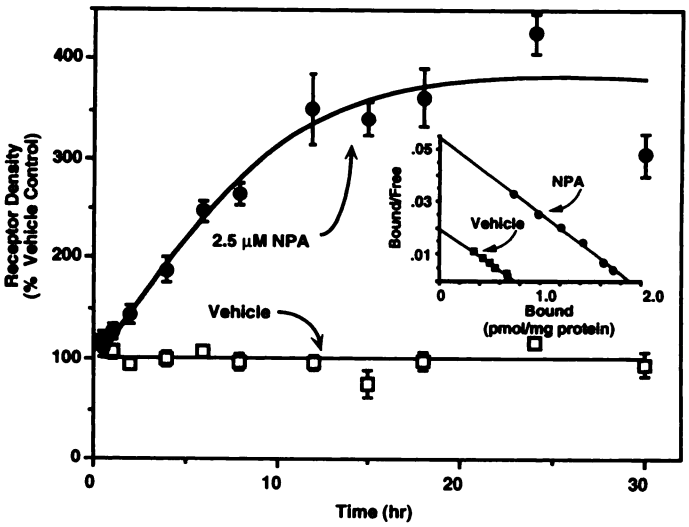

Fig. 4. Time course of the effect of NPA on 293-$D2_L$ cells. Cells were grown in six-well plates as described and were treated for 30 min to 30 hr with 100 μl of 2.5 μM NPA or vehicle. Saturation binding assays with [^{125}I]NCQ 298 were performed on each sample and B_{max} values were determined from Scatchard transformations. Results are expressed as a percentage of the average density in vehicle-treated controls. Data shown are mean ± standard error of three separate experiments performed in triplicate. Data are from experiments performed on two clonal lines of 293-$D2_L$ cells. The $t_{1/2}$ value for up-regulation was approximately 6 hr, with a maximal increase at 24 hr of 375%. *Inset*, Scatchard transformation of representative data from cells treated for 18 hr with 2.5 μM NPA or vehicle.

increases in the density of receptors (Fig. 5). The pharmacological profile for up-regulation was similar to that seen for inhibition of the binding of [^{125}I]NCQ 298. The concentrations of agonists required for up-regulation were, however, greater than those required for inhibition of radioligand binding for all three agonists (Table 1).

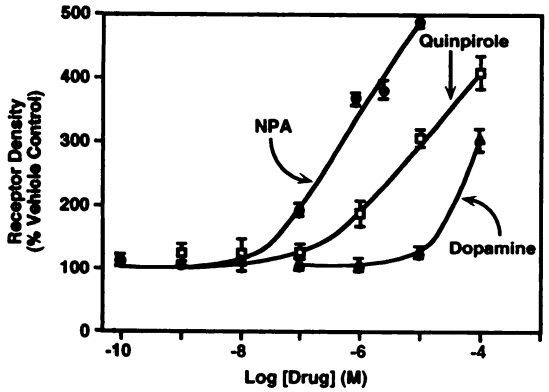

Fig. 5. Effect of treatment of 293-D2$_L$ cells with agonists. Cells were grown in six-well plates as described and were exposed to the indicated concentrations of NPA, dopamine, or quinpirole 24 hr before harvesting. Plates of cells were harvested and membranes were prepared. Assays with [^{125}I]NCQ 298 were performed and B_{max} values were determined from Scatchard transformation of saturation binding data. Results are expressed as a percentage of the average density in vehicle-treated controls. Data shown are mean ± standard error of three independent determinations for each concentration, assayed in triplicate. Experiments were performed on a single clonal line of 293-D2$_L$ cells.

TABLE 1

Agonist effects on D2$_L$ receptors expressed in 293-D2$_L$ cells

Cells were grown in 12-well, six-well, or 10-cm culture plates and harvested or treated as described in Experimental Procedures. IC_{50} values are from inhibition by agonists of cAMP accumulation, as described in Fig. 3. K_i values were determined from [^{125}I]NCQ 298 competition experiments, as described in Fig. 1. E_{200} values represent the concentration of agonist required to induce a doubling of the density of D2$_L$ receptors, as measured by [^{125}I]NCQ 298 binding and described in Fig. 5. Values are mean ± standard error, with the number of repetitions shown in parentheses.

	NPA	Dopamine	Quinpirole
K_i	4.2 ± 1.9 nM (3)	11 ± 6.3 μM (3)	3.9 ± 0.81 μM (3)
IC_{50}	8.7 ± 0.77 nM (10)	16 ± 1.8 nM (6)	1.2 ± 0.15 nM (5)
E_{200}	65 ± 4.8 nM (3)	48 ± 3.3 μM (3)	830 ± 147 nM (3)

Effects of isoproterenol and NPA on endogenous β receptors and transfected D2$_L$ receptors in 293-D2$_L$ cells and on transfected β_2 receptors in 293-β_2 cells. To investigate the specificity of up-regulation, the effect of NPA on β-adrenergic receptors endogenously expressed in 293 cells, at a density of 20–50 fmol/mg of protein, and on β_2-adrenergic receptors transfected into 293 cells, at a density of approximately 1000 fmol/mg of protein, was determined. Isoproterenol stimulated cAMP accumulation in both β_2- and D2$_L$-transfected 293 cells (Fig. 6). Basal levels of cAMP accumulation were comparable in the two types of cells, but the effect of isoproterenol was markedly increased in cells expressing a high density of β_2 receptors. Isoproterenol (1 μM) stimulated cAMP accumulation (4-fold) to a lesser degree than did forskolin (10-fold) in 293-D2$_L$ cells but to a greater degree (20-fold) than did forskolin in 293-β_2 cells.

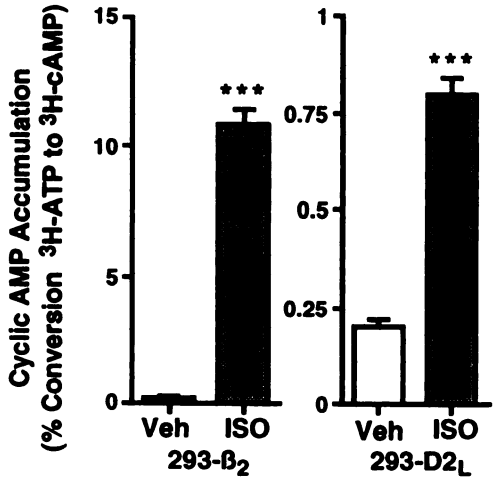

Fig. 6. Stimulation by isoproterenol (*ISO*) of cAMP accumulation in 293 cells. 293-D2$_L$ and 293-β_2 cells were grown as described in Experimental Procedures and were treated with 1 μM isoproterenol or vehicle (*Veh*). ***, $p < 0.001$ for isoproterenol, compared with vehicle control. Data are mean ± range for two independent experiments performed in quadruplicate. Experiments were performed on a single clone of each transfected cell line.

NPA had no effect on the density of endogenously expressed β-adrenergic receptors in 293-D2$_L$ cells or on the density of transfected β_2 receptors in 293-β_2 cells, as assessed by saturation binding of [^{125}I]ICYP. In both 293-D2$_L$ and 293-β_2 cells, incubation with isoproterenol reduced the level of [^{125}I]ICYP binding significantly (Fig. 7, A and B). Conversely, isoproterenol had no effect on the density of D2$_L$ receptors, as assessed by [^{125}I]NCQ 298 binding, in 293-D2$_L$ cells (Fig. 7C).

D2$_L$ receptor levels in 293-D2$_L$ cells after exposure to 8-Br-cAMP or forskolin. 293-D2$_L$ cells were treated for 24 hr with 2.5 μM NPA, 10 μM forskolin, 100 μM 8-Br-cAMP, or combinations thereof. Although forskolin and 8-Br-cAMP did not cause significant increases in the density of receptors, they were synergistic with NPA (Fig. 8).

Regulation of D2$_S$ receptors in transfected cells. Treatment of 293-D2$_S$ cells with 2.5 μM NPA resulted in up-regulation of receptors to 234 ± 17% of basal levels (n = 6). The use of NPA in combination with forskolin resulted in an increase in the density of receptors to 424 ± 56% of basal levels (n = 6). In another stably transfected cell line being studied, CHO-D2$_S$ cells, exposure to 2.5 μM NPA also resulted in an increase in the density of D2$_S$ receptors to 334 ± 63% of basal levels (n = 3).

Receptor levels after treatment with antagonists. To assess the pharmacological specificity of the effect of NPA on 293-D2$_L$ cells, NPA (2.5 μM) was added to cells in the presence or absence of D2-selective (sulpiride) or D1-selective (SCH-23390) antagonists. Neither of the antagonists, alone or together, was able to inhibit up-regulation of D2$_L$ receptors by NPA (Fig. 9). However, sulpiride at the same concentration was able to partially block (40 ± 4%) the inhibition of cAMP accumulation produced by 2.5 μM NPA in 293-D2$_L$ cells. SCH-23390 had no effect on levels of D2$_L$ receptors or on cAMP accumulation. Exposure to sulpiride alone led to up-regulation of receptors, an effect not blocked by SCH-23390. Other selective or nonselective D2 antagonists, including a butyrophenone (haloperidol), clozapine, and epidepride, were also able to up-

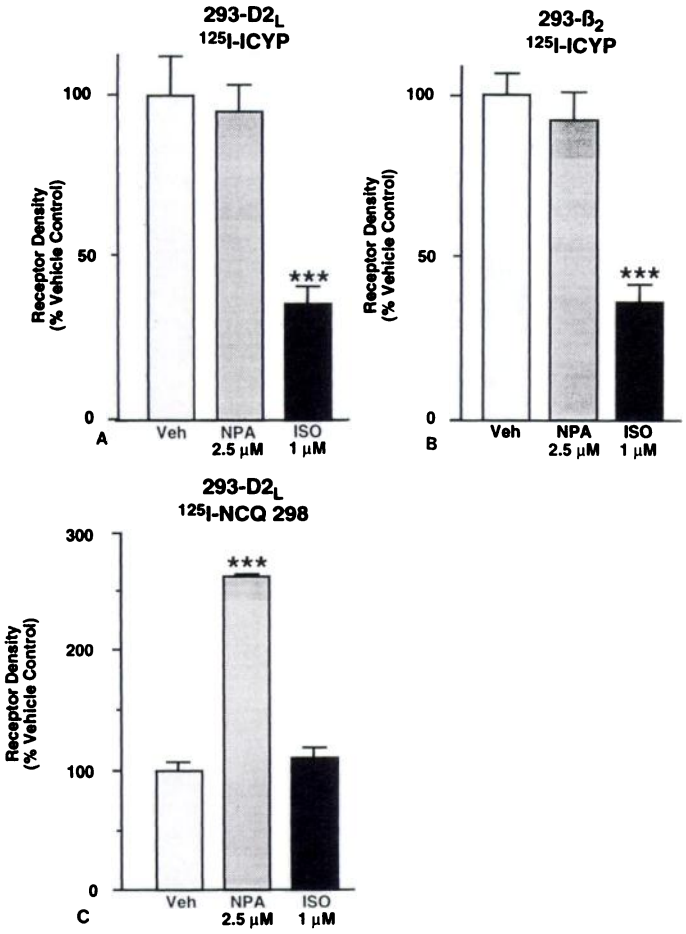

Fig. 7. Effects of isoproterenol and NPA on receptors in 293 cells. 293-$D2_L$ cells (A and C) and 293-β_2 cells (B) were grown in 10-cm^2 plates as described and were treated 24 hr before harvesting with 100 μl of 2.5 μM NPA (▨), 1 μM isoproterenol (■), or vehicle (□). Cells were harvested and membranes were prepared as described. Assays with [^{125}I]NCQ 298 (C) or [^{125}I]ICYP (A and B) were performed and B_{max} values were determined by Scatchard transformations. Data are expressed as a percentage of the average vehicle control from each experiment. The density of β-adrenergic receptors was 15–50 fmol/mg of protein in 293-$D2_L$ cells (A) and 800–1000 fmol/mg of protein in 293-β_2 cells (B). The density of $D2_L$ receptors was 500–1000 fmol/mg of protein in 293-$D2_L$ cells (C). Data shown are mean ± standard error of three independent experiments performed in triplicate. ***, $p \leq 0.001$, compared with vehicle. Experiments were performed on two clones of each transfected cell type.

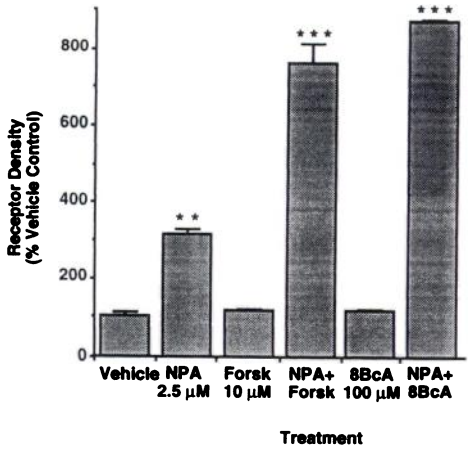

Fig. 8. Effect of 8-Br-cAMP and forskolin on 293-$D2_L$ cells. Cells were treated for 24 hr with 2.5 μM NPA, 10 μM forskolin (*Forsk*), 2.5 μM NPA plus 10 μM forskolin, 100 μM 8-Br-cAMP (*8BcA*), or 2.5 μM NPA plus 100 μM 8-Br-cAMP, and receptors were assayed with [^{125}I]NCQ 298 as described. Basal $D2_L$ receptor levels were 500–1000 fmol/mg of protein. Data shown are mean ± standard error of three to six independent determinations assayed in triplicate. **, $p \leq 0.01$; ***, $p \leq 0.001$, compared with vehicle controls. Experiments were performed on two clonal lines of 293-$D2_L$ cells.

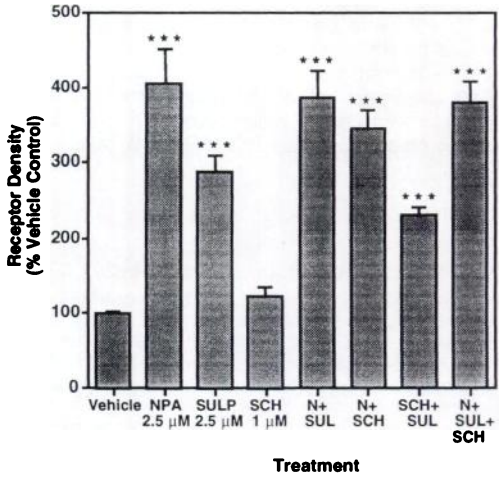

Fig. 9. Receptor levels after treatment with antagonists. 293-$D2_L$ cells were grown in six-well plates and were treated for 24 hr with 100 μl of 2.5 μM NPA, 2.5 μM sulpiride (*SULP*), 1 μM SCH-23390 (*SCH*), 2.5 μM NPA plus 2.5 μM sulpiride (*N+SUL*), 2.5 μM NPA plus 1 μM SCH-23390 (*N+SCH*), 1 μM SCH-23390 plus 2.5 μM sulpiride (*SCH+SUL*), 2.5 μM NPA plus 1 μM SCH-23390 plus 2.5 μM sulpiride (*N+SUL+SCH*), or 10% DMSO (*Vehicle*). Cells were harvested as described and receptors were assayed with [^{125}I]NCQ 298. The density of $D2_L$ receptors in vehicle-treated cells was 250–800 fmol/mg of protein. Data shown are mean ± standard error of six to eight independent determinations. ***, $p \leq 0.001$, compared with vehicle. Experiments were performed on two or three clonal lines of 293-$D2_L$ cells.

regulate $D2_L$ receptors to at least 200% of basal levels at concentrations of 1 or 10 μM.

$D2_L$ mRNA levels after treatment with NPA. To study agonist-induced regulation of $D2_L$ mRNA levels, 293-$D2_L$ cells were incubated with 2.5 μM NPA for times ranging from 30 min to 30 hr (Fig. 10). No significant change in levels of $D2_L$ mRNA or cyclophilin mRNA, as assessed by solution hybridization analysis, was observed at any time. [^{125}I]NCQ 298 binding was assayed in a portion of tissue from the samples used for mRNA analysis. $D2_L$ receptor levels increased to 400% of vehicle-treated controls over 30 hr. For mRNA analysis, each time point included paired agonist- and vehicle-treated samples, which were normalized to a cyclophilin standard assayed concurrently on each lane of the gel. NPA treatment did not affect the amount of cyclophilin mRNA in the samples. No systematic change in cyclophilin mRNA levels was observed as a function of time or treatment in either vehicle- or NPA-treated samples. Cyclophilin mRNA levels have been shown to be invariant after a variety of drug treatments and provide a convenient standard for normalization.[1]

Discussion

The goal of the present studies was to characterize the regulation of dopamine receptors in homogeneous cell popula-

[1] Unpublished observations.

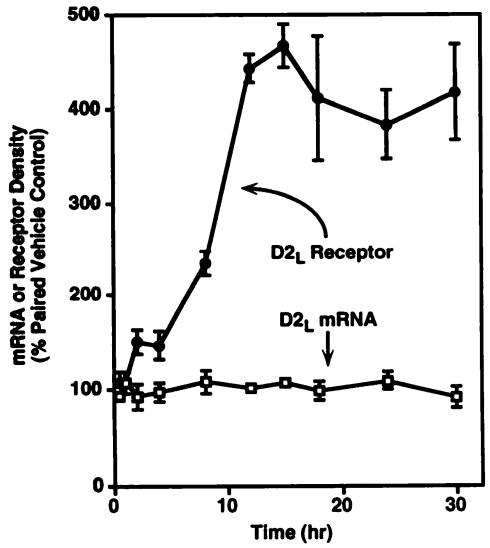

Fig. 10. Effect of NPA on $D2_L$ mRNA levels in 293-$D2_L$ cells. Cells were grown in six-well plates as described and quadruplicate samples were treated for 30 min to 30 hr with 100 μl of 2.5 μM NPA or vehicle. Treated cells were harvested into isotonic buffer and each sample was then divided for radioligand binding and solution hybridization analyses, as described in Experimental Procedures. Results from NPA-treated samples are expressed as a percentage of the $D2_L$ mRNA level (□) or $D2_L$ receptor level (●) in paired vehicle-treated controls. Data shown are mean ± standard error of a representative experiment assayed in quadruplicate. Similar results were seen in one other experiment.

tions expressing single receptor isoforms. The pharmacological properties of D2 receptors in transfected 293 cells were similar to those reported for transfected CHO cells (6). Receptor properties remained consistent over time in culture, whereas the density of receptors varied among several clonal cell lines expressing either $D2_L$ or $D2_S$ receptors. In 293-$D2_L$ cells, the affinity of $D2_L$ receptors for both dopamine and quinpirole was much lower than the potency of dopamine or quinpirole in inhibiting cAMP accumulation. NPA inhibited cAMP accumulation at roughly the same concentrations as those needed to inhibit binding of [^{125}I]NCQ 298. These differences between potency and affinity could result from a larger number of spare receptors being available to dopamine and quinpirole than to NPA in 293-$D2_L$ cells. Although all three compounds appear to be full agonists at the $D2_L$ receptor, levels of receptor reserve may be different for each agonist. However, the discrepancies between binding affinity and apparent potency have yet to be fully explained. The IC_{50} value for inhibition of cAMP accumulation by quinpirole in 293-$D2_L$ cells (1.2 nM) was 100-fold lower than that reported for $D2_S$-transfected Ltk$^-$ cells by Neve *et al.* (24), which may reflect spare receptor differences between cell types or D2 isoforms.

In transfected 293 cells increases in receptor density were seen after exposure to agonists. The K_d values of treated and control cell membranes were the same, suggesting that up-regulation was the result of an increase in the density of binding sites and not the result of a change in the properties of the receptor. The time dependence of the effect ($t_{1/2} \approx 6$ hr) suggested that the agonist was not acting to protect the receptor from degradation occurring during harvest or assay. In addition, 293-$D2_L$ cells have a doubling time in culture of approximately 48 hr. If NPA were acting nonspecifically as a trophic factor, one would expect up-regulation to occur over a much longer time ($t_{1/2} > 24$ hr). β-Adrenergic receptors endogenously expressed in 293 cells were unaffected by NPA treatment, showing that protein biosynthesis was not affected by NPA.

NPA was not acting directly at the promoter region of the transfection plasmid to increase receptor expression, because it caused no change in the density of transfected β_2 receptors (expressed in the same cell line and under control of the same promoter) or in the density of $D2_L$ mRNA. In addition, exposure to NPA resulted in an increase in the densities of both isoforms of D2 receptors in C6 glioma cells in which transcription was under the control of a Rous sarcoma virus promoter.[2] In our studies, NPA also caused an increase in the density of D2 receptors in 293-$D2_S$, CHO-$D2_S$, and COS-$D2_L$ cells. These findings indicate that the regulatory response of D2 receptors to agonist treatment is not dependent on the D2 receptor isoform, the transcriptional promoter, or the host cell.

Because D2-selective antagonists and agonists acted indistinguishably to induce up-regulation in 293-$D2_L$ cells, methods other than blockade by antagonist were necessary to show specificity. Only D2-selective drugs, not isoproterenol (a β receptor agonist) or SCH-23390 (a D1 receptor antagonist), caused up-regulation. The relative concentrations of agonists required to increase receptor density (NPA < quinpirole < dopamine) suggested that up-regulation occurred through occupancy of the D2 receptor (binding affinities, NPA > quinpirole > dopamine). However, for all three drugs the absolute concentrations of agonists required to increase receptor density were greater than the concentrations required to inhibit [^{125}I] NCQ 298 binding. The up-regulation seen with sulpiride, which does not affect cAMP accumulation, may indicate that receptor occupancy, whether by an agonist or an antagonist, is sufficient to induce an increase in receptor density.

The signal transduction mechanisms involved in dopamine receptor regulation are not known. Treatment of SUP1 cells, which endogenously express D2 receptors, with dopamine or NPA triggered compensatory changes over time, leading to an increase in basal and forskolin-stimulated cAMP formation (9). Quinpirole, when added to Ltk$^-$ cells transfected with $D2_S$ receptors, caused a long-term increase in basal levels of cAMP (25). No changes in the density of $D2_S$ receptors were observed when transfected Ltk$^-$ cells were treated with 1 μM quinpirole. In our studies, consistent changes in receptor expression required concentrations of quinpirole above 3 μM. The finding that isoproterenol, 8-Br-cAMP, and forskolin did not cause increases in the density of $D2_L$ receptors suggests that increased levels of cAMP are not sufficient to cause up-regulation of $D2_L$ receptors. However, forskolin and 8-Br-cAMP potentiated the effect of NPA, leading to marked increases in receptor levels. The synergistic effect of NPA and 8-Br-cAMP or forskolin suggests that up-regulation may be supplemented by increases in intracellular cAMP when accompanied by agonist occupancy of the receptor.

We have shown that $D2_L$ receptor mRNA levels do not increase during exposure of 293-$D2_L$ cells to NPA, which suggests that transcriptional regulation does not account for receptor up-regulation in the transfected cells. This finding prompted us to consider translational or post-translational mechanisms. Regulation of β-adrenergic receptors by agonists involves multiple mechanisms. Exposure to an agonist leads to phosphorylation of the receptor, followed by its uncoupling from G_s and sequestration within the cell. Transient increases

[2] K. Neve, personal communication.

in mRNA levels are followed by decreases in mRNA and receptor levels (26, 27). The decrease in mRNA levels is thought to be due to a decrease in mRNA stability. It is interesting that the $D2_L$ and $D2_S$ receptors lack the multiple serine and threonine phosphorylation sites in the carboxyl termini that are phosphorylated on β-adrenergic receptors after exposure to agonists. Given the fact that $D2_L$ receptor expression in transfected 293 cells is under the control of a constitutively active promoter, it is not surprising that levels of $D2_L$ mRNA did not change. Our observations further suggest that changes in mRNA stability do not occur.

Increased levels of cAMP have been associated with increases in the rate of transcription of the α_{2A}-adrenergic receptor gene in HT-29 cells, which leads to increased α_{2A} receptor expression (28). Increases in β_3-adrenergic receptor levels and β_3 mRNA were induced by chronic exposure of 3T3-F442A cells to agonists (29). In these cells, β_1 receptors were expressed in the undifferentiated phenotype and were down-regulated upon exposure to agonists. Upon differentiation, the cells expressed β_3-adrenergic receptors, whose density increased upon exposure to isoproterenol. The up-regulation was a result of increased cAMP levels interacting with multiple cAMP-responsive elements in the 5′-flanking region of the gene to increase transcription. A mechanism involving increases in mRNA has been described for the agonist-induced up-regulation of 5-hydroxytryptamine$_2$ receptors coupled to phosphoinositide turnover in cerebellar granule cells (30). Although receptor regulation in transfected cell lines (such as 293-$D2_L$ cells) is likely to occur at the translational or post-translational level, cAMP may be involved in regulation of protein synthesis, protein degradation, or receptor modification.

Compensatory changes in the density of dopamine receptors may be important in understanding the mechanism of action of neuroleptics. The density of D2 receptors has been reported to increase after treatment with antagonists *in vivo* (31, 32). An up-regulation of D2-like receptors after treatment with antagonists *in vivo* could explain the appearance of tardive dyskinesias during treatment with neuroleptics. On the other hand, directly and indirectly acting dopamine receptor agonists have been shown to lead to sensitization to several of the behavioral effects of the drugs, an effect called reverse tolerance (33). Receptor up-regulation after agonist treatment *in vivo* could explain these observations. Increases in the density of D2 receptors, measured by quantitative autoradiography, have been seen in the nucleus accumbens, although not in striatum, after chronic administration of cocaine to rats (34). Tolerance to some of the behavioral effects of cocaine and amphetamine also occurs after chronic administration of these drugs. Up-regulation of subtypes of dopamine receptors, including inhibitory D2-like autoreceptors, may offer an explanation of these findings. Functional assays of the ability of dopamine to inhibit [^{3}H]dopamine release from preloaded slices showed that animals treated with cocaine for 8 days or longer were supersensitive (35). Sensitization of inhibitory D2-like autoreceptors after agonist administration would limit dopamine release and may provide a model for tolerance.

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