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Regulation of Responsiveness at D_2 Dopamine Receptors by Receptor Desensitization and Adenylyl Cyclase Sensitization

MICHAEL D. BATES, SUSAN E. SENOGLES,[1] JAMES R. BUNZOW, STEPHEN B. LIGGETT, OLIVIER CIVELLI, and MARC G. CARON

Departments of Cell Biology (M.D.B., S.E.S., M.G.C.), Pharmacology (S.B.L.), and Medicine (S.B.L., M.G.C.) and the Howard Hughes Medical Institute (M.G.C.), Duke University Medical Center, Durham, North Carolina 27710, and the Vollum Institute for Advanced Biochemical Research, Oregon Health Sciences University, Portland, Oregon 97201 (J.R.B., O.C.)

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SUMMARY

The regulation of cellular responsiveness to dopamine via the D_2 dopamine receptor was investigated in mouse fibroblast Ltk$^-$ cells stably expressing the rat $D_{2\text{-short}}$ receptor [*Nature (Lond.)* **336**:783–787 (1988)]. Dopamine inhibited forskolin-stimulated cAMP levels in these cells (half-maximal inhibition at 3.9 ± 1.1 nM), and the inhibition by dopamine was blocked by D_2 antagonists and was pertussis toxin sensitive. Treatment of these cells with the D_2 agonist quinpirole (1 μM) resulted in desensitization of dopaminergic inhibition of forskolin-stimulated cAMP accumulation, with a ~4-fold decrease in the potency of dopamine after 1 hr of treatment. No significant changes in total cellular D_2 receptor concentrations were observed, even after prolonged agonist treatment. At longer time points, basal and forskolin-stimulated cellular cAMP levels were increased in treated cells. The effect of D_2 agonist treatment on membrane adenylyl cyclase (EC 4.6.1.1) activity was examined. Basal and forskolin- and prostaglandin E_1-stimulated adenylyl cyclase activities were increased by quinpirole treatment for 24 hr. This sensitization of adenylyl cyclase was blocked by the presence of a D_2 antagonist. Pertussis toxin pretreatment blocked the sensitization of adenylyl cyclase by quinpirole, although pertussis toxin also caused increased adenylyl cyclase activity on its own. Sensitization was not dependent upon dopaminergic inhibition of intracellular cAMP levels, because quinpirole treatment in the presence of membrane-permeable cAMP analogs or 3-isobutyl-1-methylxanthine (an inhibitor of cAMP phosphodiesterase) resulted in greater sensitization of adenylyl cyclase activity than quinpirole treatment alone. These results suggest that, in this model system, responsiveness to dopamine via the D_2 receptor is regulated by both desensitization of receptor function and sensitization of the stimulatory adenylyl cyclase pathway.

Dopamine is a hormone/neurotransmitter involved in a wide variety of physiological processes in the central nervous system as well as in the periphery. The central receptors for dopamine have been divided into two groups, on the basis of their interaction with the effector enzyme adenylyl cyclase (EC 4.6.1.1) (1, 2); D_1 receptors stimulate adenylyl cyclase via the stimulatory G protein G_s, whereas D_2 receptors inhibit or have no effect on this enzyme. Besides the inhibition of adenylyl cyclase, D_2 receptors have been shown to interact with other signaling pathways, including potassium channels, calcium channels, and phosphatidylinositol metabolism (for review, see Ref. 3). The G proteins involved in the coupling of the D_2 receptor to these effectors have not been specifically identified. However, these effects of dopamine are PTX sensitive, implicating one of the G_i proteins or G_o. Moreover, purified bovine

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[1]Present address: Department of Biochemistry, University of Tennessee-Memphis, Memphis, TN 38163.

ABBREVIATIONS: G protein, guanine nucleotide-binding protein; SRIF, somatostatin; NPA, (*R*)-(−)-propylnorapomorphine; Gpp(NH)p, guanylimidodiphosphate; IBMX, 3-isobutyl-1-methylxanthine; 8Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; diBu-cAMP, N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate; ScAMPTME, 2′-*O*-monosuccinyladenosine 3′,5′-cyclic monophosphate tyrosyl methyl ester; PGE_1, prostaglandin E_1; FSK, forskolin {[(3*R*)-(3α,4aβ,5β,6β,6aα,10α,10aβ,10bα)]-5-(acetyloxy)-3-ethenyldodecahydro-6,10,10b-trihydroxy-3,4a,7,7,10a-pentamethyl-1*H*-naphtho(2,1-*b*)pyran-1-one}; spiperone, 8-[4-(4-fluorophenyl)-4-oxobutyl]-1-phenyl-1,3,8-triazaspiro[4,5]decan-4-one; PTX, pertussis toxin; DMEM, Dulbecco's modified Eagle medium; (+)-butaclamol, [(3*S*)-(3α,4aα,13bβ)]-3-(1,1-dimethylethyl)-2,3,4,4a,8,9,13b,14-octahydro-1*H*-benzo(6,7)cyclohepta(1,2,3-*de*)pyrido(2,1-*a*)isoquinolin-3-ol; quinpirole (LY 171555), (*trans*)-(−)-(4a*R*)-4,4a,5,6,7,8,8a,9-octahydro-5-propyl-1*H*-pyrazolo[3,4-*g*]quinoline; RIA, radioimmunoassay; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); IC_{50}, concentration giving half-maximal inhibition; sulpiride, 5-(aminosulfonyl)-*N*-[(1-ethyl-2-pyrrolodinyl)-methyl]-2-methoxybenzamide; SCH 23390, (*R*)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; pindolol, 1-(1*H*-indol-4-yloxy)-3-[(1-methylethyl)amino]-2-propanol; yohimbine, 17-hydroxyyohimban-16-carboxylic acid methyl ester; raclopride, (*S*)-3,5-dichloro-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-2-hydroxy-6-methoxybenzamide.

anterior pituitary D_2 receptors couple most effectively with purified G_{i2} in a reconstituted system (4).

A general characteristic of hormone-receptor-effector interactions is the tendency for the responsiveness of the system to diminish following agonist exposure, termed desensitization. Desensitization may involve a decrease in the potency or efficacy of an agonist. Among the G protein-coupled receptors, the β-adrenergic receptor, which is stimulatory to adenylyl cyclase, has provided perhaps the most widely studied model of receptor desensitization (for review, see Ref. 5). However, less is known about the mechanisms by which receptors inhibitory to adenylyl cyclase are regulated. Conceptually, the loss of receptor function (i.e., desensitization) could be caused by a loss of functional receptors (i.e., down-regulation), an uncoupling of receptors from their signal transduction pathways, or an alteration of distal components of these pathways. Results on studies of inhibitory receptor regulation have been variable, depending upon the system studied.

Decreased inhibition of adenylyl cyclase following agonist exposure has been shown for opiate (6–8), muscarinic (9), and α_2-adrenergic (10) receptors in neuroblastoma × glioma NG108-15 cells. Similar reports have been made for SRIF receptors in S49 lymphoma cells (11) and A_1 adenosine receptors in rat adipocytes (12). Receptor down-regulation following agonist treatment has been shown for opiate (13), muscarinic (10), α_2-adrenergic (10), SRIF (14), and A_1 adenosine receptors (12). On the other hand, exposure of GH_4C_1 rat pituitary cells to SRIF (15) or of platelets to α_2 agonists (16) induces no loss of receptor-mediated adenylyl cyclase inhibition. Besides alterations of receptor concentration and coupling, regulation also occurs by increases in membrane adenylyl cyclase activity (or basal and stimulated cellular cAMP levels) following chronic (days) agonist exposure. Such increases were first reported by Sharma *et al.* (6), studying the responsiveness of NG108-15 cells to opiate agonists. Similar sensitizations have been observed for the other receptors mentioned above as well (9, 14, 17–20).

Studies of the cellular regulation of D_2 dopaminergic responsiveness have been difficult to perform due to the lack of cell lines expressing D_2 receptors. The recent cloning of two D_2 dopamine receptor isoforms has allowed the construction of cell lines expressing the receptor, for use in the study of its function and regulation in an isolated cell system. For the present study, the rat D_2 receptor (21) was used. This isoform is now called the $D_{2\text{-short}}$ receptor, following the cloning of an alternatively spliced isoform, called the $D_{2\text{-long}}$ receptor [which contains an additional 29 amino acids in the putative third intracellular loop; no functional differences between the isoforms have yet been described (22–25)]. Expression of the $D_{2\text{-short}}$ receptor in mouse fibroblast Ltk^- cells confers dopaminergic inhibition of adenylyl cyclase on these cells (26). In this study, we examine the effects of D_2 dopamine agonist treatment on cellular responsiveness to dopamine and other regulators of adenylyl cyclase activity. Regulation of responsiveness by this inhibitory receptor occurs in two ways, first, by desensitization of receptor function and, second, by sensitization of adenylyl cyclase and the stimulatory effector pathway.

Experimental Procedures

Materials. ATP, GTP, cAMP, IBMX, 8Br-cAMP, diBu-cAMP, ScAMPTME, phospho(enol)pyruvate, PGE_1, and myokinase (adenylate kinase, EC 2.7.4.3) were obtained from Sigma Chemical Co. (St. Louis, MO). Gpp(NH)p was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Pyruvate kinase (EC 2.7.1.40) was obtained from Calbiochem (La Jolla, CA), and FSK was from Hoechst-Roussel (Somerville, NJ). [^{3}H]Spiperone, [α^{32}P]ATP, [^{3}H]cAMP, and $Na^{125}I$ were obtained from New England Nuclear (Boston, MA). Media, sera, and other tissue culture reagents were obtained from GIBCO Laboratories (Grand Island, NY). Raclopride and (−)- and (+)-sulpiride were gifts of Dr. Peter Andersen (Novo-Nordisk Co., Copenhagen, Denmark); other drugs were obtained from Research Biochemicals, Inc. (Natick, MA). PTX was obtained from List Biochemicals (Campbell, CA). All other materials were obtained from commercial sources and were of the highest quality available.

Cells. Transfection and selection procedures used were as described by Neve *et al.* (26). Mouse fibroblast Ltk^- cells expressing the D_2 dopamine receptor were grown as monolayers in 75-cm^2 tissue culture flasks or 24-well plates, in DMEM supplemented with 10% fetal bovine serum (which was treated with activated charcoal and dextran to remove endogenous catecholamines), in a 37° incubator with an atmosphere of 95% O_2/5% CO_2. Experiments were performed when cell cultures were confluent.

Membrane preparation. Crude membranes from control and treated cells were prepared as follows. The cells (grown in 75-cm^2 flasks) were washed twice with 10 ml of ice-cold phosphate-buffered saline. Ten milliliters of ice-cold lysis buffer (5 mM Tris, 2 mM EDTA, pH 7.4) were added, and the cells were scraped from the flask. After further washing of the flask with 5 ml of lysis buffer, the cells were homogenized using a Polytron cell disrupter (Brinkmann), with a 15-sec burst on setting 7. The membranes were pelleted by centrifugation at 43,000 × *g* at 4° for 20 min, and the pellets were resuspended in 10 ml of ice-cold lysis buffer and recentrifuged. The resulting pellets were then resuspended at 1 ml/flask, in the buffers appropriate for the ligand binding assay or the adenylyl cyclase assay (see below), and were used immediately. Final protein concentrations were ~1 mg/ml, as determined by the method of Bradford (27), using bovine serum albumin as the standard.

Ligand binding assay. Membranes (above) were resuspended in ligand binding assay buffer (50 mM Tris, 100 mM NaCl, 12.5 mM $MgCl_2$, 2 mM EDTA, pH 7.4). Fifty microliters of membranes were incubated with ~1 nM [^{3}H]spiperone (K_D = 50–60 pM) (26),[2] in the absence or presence of 1 μM (+)-butaclamol (to define nonspecific binding), in ligand binding assay buffer at a final volume of 2 ml. After incubation at 25° for 1 hr, the assay was terminated by rapid filtration through GF/C filters (Whatman), using a Brandel cell harvester, and rapid washing with 20 ml/tube ice-cold wash buffer (50 mM Tris, 100 mM NaCl, pH 7.4). Filters containing bound radioligand were then counted by liquid scintillation counting.

Agonist competition assay. Agonist competition curves versus [^{3}H]spiperone (~1 nM) were performed to measure the interaction of D_2 receptors with G proteins under equilibrium conditions. The dopaminergic agonist NPA was used because of the large differences in the affinity binding constants for this drug for high and low affinity binding. The conditions of the ligand binding assay described above were used, except that the ligand binding buffer also contained 5 mM sodium ascorbate. The assays were carried out in the absence or presence of the nonhydrolyzable GTP analog Gpp(NH)p. The competition curves in the presence or absence of Gpp(NH)p were modeled for two-site binding using a least-squares fitting program (28); in the presence of Gpp(NH)p only low affinity binding of NPA to the D_2 receptor is observed.

Desensitization and whole-cell cAMP assay. Cellular cAMP levels in response to challenge with drugs were measured by a modification of the method of Liggett *et al.* (29), with cells grown to confluence (~5 × 10^5 cells/well) in 24-well culture plates. Cells were incubated with DMEM plus serum at 37°, in the presence or absence of the full

[2] M. D. Bates, Unpublished observations.

D_2 agonist quinpirole (1 μM) or dopamine (1 μM), for the indicated times. To facilitate the removal of agonist, the medium was decanted and the cells were incubated for 20 min in DMEM plus 5 mM sodium ascorbate (without serum or quinpirole). Each well was then washed twice with 500 μl of warm phosphate-buffered saline. Cells were then incubated in DMEM, containing the indicated drugs plus 5 mM sodium ascorbate, at 37° for 5 min, at which time stimulated cAMP levels are maximal. Three wells were used for each assay condition. The medium was aspirated, and perchloric acid (6.6 M, 50 μl/well) was added to rapidly kill the cells. DMEM (500 μl/well) was added, the cells were scraped from the wells, and the suspensions were placed in 2-ml microfuge tubes. Potassium bicarbonate (3 M, 125 μl) was then added to neutralize each sample. Samples were stored at 4° overnight or at −135° for up to 2 weeks before RIA.

cAMP RIA. To increase the sensitivity of the cAMP RIA, samples were acetylated before assay, as described (30). Whole-cell cAMP assay samples were microfuged, and 150 μl of each supernatant were combined with 50 μl of 20 mM MES, pH 6.2 (MES buffer). Five microliters of triethylamine/acetic anhydride (2:1, v/v, freshly prepared) were added to each tube, which was immediately vortexed. Each sample was then brought up to a volume of 1 ml with MES buffer. Acetylated samples were assayed for cAMP content by RIA by the method of Steiner *et al.* (31), with the modifications described. Samples (50 μl) were assayed versus [^{125}I]iodo-ScAMPTME (~12,000 cpm/tube; prepared using chloramine T), using rabbit antiserum raised against succinyl-cAMP, at a total volume of 500 μl in MES buffer. For standards (5–200 fmol/tube), cAMP in MES buffer was acetylated in parallel with the samples. The assay was incubated at 4° overnight. Bound ligand was pelleted by the addition of 100 μl of bovine γ-globulin (1 mg/ml in MES buffer), followed by 2.5 ml of 60% saturated ammonium sulfate and centrifugation at 4° for 20 min. Supernatants were decanted, the tubes were counted, and the cAMP concentrations were calculated using an LKB RIAGamma γ counter.

Adenylyl cyclase assay. Adenylyl cyclase activity in membranes was measured by the method of Salomon *et al.* (32), with the modifications of Katada *et al.* (33). Assay mixtures contained 20 μl of membranes, 100 mM HEPES, 100 mM NaCl, 4 mM $MgCl_2$, 2 mM EDTA, 220 μM ATP with 1 μCi of [α-^{32}P]ATP/tube, 53 μM GTP, 100 μM cAMP, 2.7 mM phospho(enol)pyruvate, 0.2 IU of pyruvate kinase, 1 IU of myokinase, 0.02% ascorbate, and varying concentrations of drugs of interest, with a total assay volume of 50 μl. Assays, performed in duplicate, were incubated at 37° for 30 min and were terminated by the addition of 1 ml of ice-cold stop solution (400 μM ATP, 300 μM cAMP, and ~25,000 cpm of [^{3}H]cAMP). Under such conditions, the appearance of [^{32}P]cAMP is linear with time. cAMP was isolated from ATP by chromatography over 1-ml Dowex and 1-ml alumina columns. Double-labeled samples were counted by liquid scintillation counting and the amount of [^{32}P]cAMP produced was calculated.

Data analysis. Dose-response curves from whole-cell cAMP and membrane adenylyl cyclase assays were modeled using the least-squares fitting program ALLFIT (34). Statistical significance indicates that Student's *t* test gave a *p* value of less than 0.05, for three or more experiments.

Results

The rat brain D_2 dopamine receptor has been shown to inhibit adenylyl cyclase activity in membranes when expressed in Ltk$^-$ cells (26). Fig. 1 shows that activation of this receptor resulted in the inhibition of FSK-stimulated cAMP levels in whole cells. Dopamine blocks cAMP accumulation with an IC_{50} of 3.9 ± 1.1 nM (see Fig. 3). Agonist and antagonist pharmacology were appropriate for a D_2 receptor. Antagonists blocked the inhibitory effect of dopamine in a stereoselective manner [(−)- > (+)-sulpiride]. Antagonists inactive at D_2 receptors and selective for D_1 dopamine (SCH 23390), 5-hydroxytryptamine$_{1A}$ (pindolol), or α_2-adrenergic (yohimbine) receptors did not affect

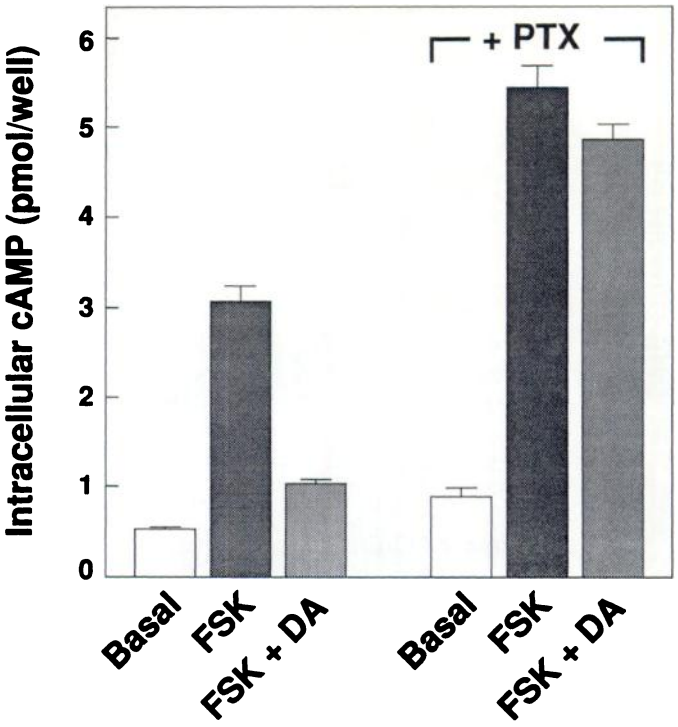

Fig. 1. Ability of dopamine to inhibit intracellular cAMP levels and its sensitivity to PTX. Whole-cell cAMP assays were performed as described in Experimental Procedures. Concentrations of FSK and dopamine (*DA*) were each 1 μM. PTX-treated cells were incubated for 24 hr with 10 ng/ml PTX before assay. The means ± standard errors from three wells in one representative experiment are shown.

the inhibition of cAMP levels by dopamine (data not shown). Finally, as shown in Fig. 1, pretreatment of the cells with PTX abolished dopaminergic inhibition of FSK-stimulated intracellular cAMP levels. In addition, basal and FSK-stimulated cAMP levels were both significantly increased in PTX-treated as compared with control cells (by 31% and 77%, respectively).

To assess the effect of dopaminergic agonist treatment on D_2 receptor function, cells were treated with the D_2-selective agonist quinpirole. Quinpirole was used because it has affinities similar to dopamine for both the high and low affinity states of the D_2 dopamine receptor (35) but is not subject to oxidation like dopamine. A concentration of 1 μM, which maximally activates the D_2 receptor (data not shown), was routinely used for pretreatments. Control cells contained 1.6 ± 0.1 pmol of D_2 receptors/mg of protein (as defined by [^{3}H]spiperone binding). Fig. 2 shows that treatment with quinpirole for up to 60 hr had a small but statistically insignificant ($p > 0.05$) effect on the total cellular D_2 receptor density.

Although no changes in D_2 receptor number were observed, D_2 receptor function, as assayed by measurement of the ability of dopamine to inhibit FSK (1 μM)-stimulated cAMP levels, was impaired by D_2 agonist exposure. The effect of treatment with quinpirole for 1–24 hr is shown in Fig. 3 and Table 1. The potency of dopamine was decreased ~4-fold after 1 hr of treatment with quinpirole, after which time the potency did not change significantly. In addition, the efficacy of dopamine in inhibiting FSK-stimulated cAMP levels remained greater than 90% and did not change significantly ($p > 0.05$) with up to 24 hr of quinpirole treatment. Interestingly, FSK-stimulated cAMP levels were increased by 2.3-fold in quinpirole-treated cells, from 2.37 ± 0.25 pmol of cAMP/well in control cells to

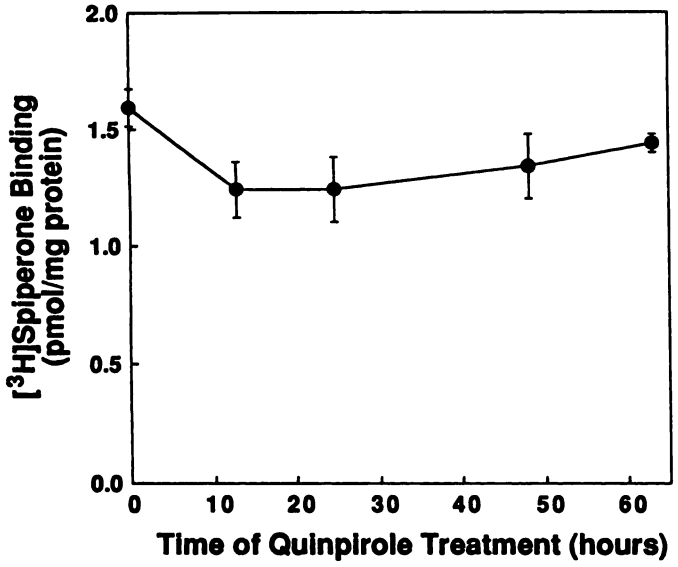

Fig. 2. Lack of effect of quinpirole treatment on cellular D_2 dopamine receptor density. Membranes were prepared from cells incubated in the presence or absence of quinpirole (1 μM) for the indicated periods of time, and D_2 receptor concentration was determined by radioligand binding assay using [^{3}H]spiperone, as described in Experimental Procedures. Data shown represent the means ± standard errors from three experiments. No statistically significant change in D_2 receptor concentration was observed.

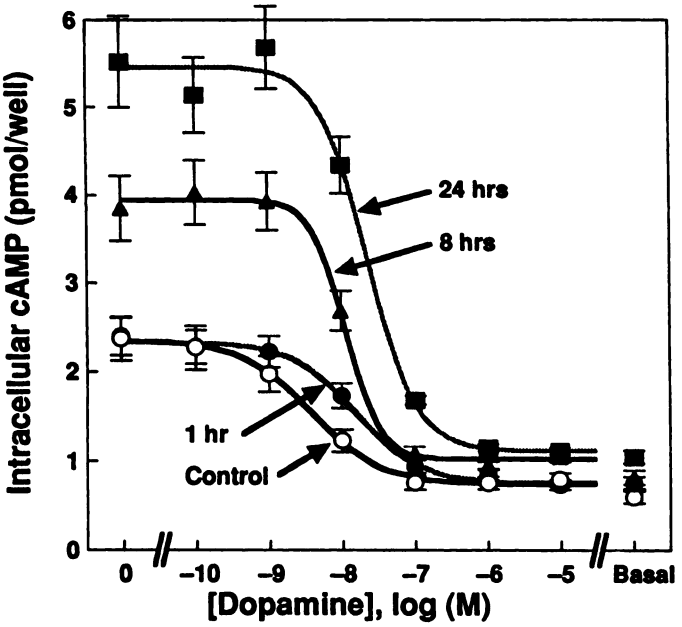

Fig. 3. Desensitization of D_2 dopamine receptor function. Cells were incubated in the presence or absence of quinpirole (1 μM), and whole-cell cAMP assays were performed as described in Experimental Procedures. Data from three representative experiments (mean ± standard error) were modeled as described; computer-generated fits are shown. Results from the fits are given in Table 1.

5.53 ± 0.53 pmol/well after 24 hr of quinpirole treatment ($p < 0.005$). This increase occurred in a time-dependent manner after a lag of at least 1 hr. Basal cAMP levels were also increased significantly after 24 hr of quinpirole treatment (control, 0.60 ± 0.05 pmol/well; quinpirole-treated, 1.05 ± 0.08 pmol/well; $p < 0.005$).

The proposition that these effects of quinpirole are dependent upon activation of D_2 dopamine receptors can be demonstrated several ways. First, exposure of the cells to the natural agonist dopamine (1 μM) for 1 hr resulted in a decrease in dopaminergic potency identical to that induced by quinpirole. Second, the effects of quinpirole were blocked by the presence of the selective D_2 antagonist raclopride (1 μM); exposure of the cells to raclopride alone for 24 hr had no effect on either the potency or the efficacy of dopamine. Finally, similar effects were also observed in Ltk$^-$ cells expressing a lower level of D_2 receptors (~200 fmol/mg of protein).

The increase in FSK-stimulated cAMP levels after 8–24 hr of quinpirole treatment (Fig. 3) suggested that long term treatment of the cells with a dopaminergic agonist might increase adenylyl cyclase activity. To examine directly the possibility of increased adenylyl cyclase activity in quinpirole-treated cells, basal and FSK- and PGE_1-stimulated adenylyl cyclase activities were measured in membranes prepared from cells treated with either vehicle or quinpirole (1 μM) for 24 hr (Fig. 4 and Table 2). Basal adenylyl cyclase activity was increased by 36% in membranes from quinpirole-treated cells. Maximal stimulations of adenylyl cyclase by FSK (+40%) and PGE_1 (+38%) were increased to a similar extent. Maximal fold stimulations for FSK and PGE_1 were unchanged. FSK was 2-fold more potent in quinpirole-treated cells, whereas no statistically significant change in the potency of PGE_1 was observed. With long term treatment, basal and stimulated (100 μM FSK or PGE_1) adenylyl cyclase activities increased as much as 60–70%, with a $t_{1/2}$ of approximately 20 hr (Fig. 5).

To demonstrate that the sensitization of adenylyl cyclase activity was mediated by D_2 receptor activation, control and quinpirole treatments were carried out in the presence or absence of 1 μM raclopride, a D_2-selective antagonist. Fig. 6 shows that the sensitization of basal and FSK-stimulated adenylyl cyclase activity by quinpirole did not occur in the presence of raclopride. Raclopride similarly blocked quinpirole sensitization of PGE_1-stimulated adenylyl cyclase activity (data not shown). It should be noted that, in untransfected Ltk$^-$ cells, basal and stimulated adenylyl cyclase activities were not affected by quinpirole treatment (data not shown).

The possible role of altered coupling of the D_2 receptor to G proteins in desensitization induced by quinpirole was examined by agonist competition binding. Quinpirole treatment induced a small but statistically insignificant decrease in the fraction of D_2 receptors in the high affinity state [34.8 ± 5.9% for control versus 32.9 ± 1.9% ($p > 0.05$) after 1 hr and 28.1 ± 1.7% ($p > 0.05$) after 24 hr of quinpirole exposure], but it did not affect the K_H or K_L of NPA.

The mechanism by which D_2 receptor activation caused the sensitization of adenylyl cyclase was investigated by examination of the effect of PTX pretreatment (Fig. 7). Cells were incubated for 12 hr with or without PTX (50 ng/ml) and then for 24 hr longer, with or without PTX, in the presence of either vehicle or quinpirole (1 μM). PTX treatment alone caused an increase in basal and FSK-stimulated adenylyl cyclase activity of about 2–3-fold (Fig. 7). Cells treated with both PTX and quinpirole had adenylyl cyclase activities identical to those of cells treated with PTX alone. Results for PGE_1-stimulated adenylyl cyclase activity were similar (data not shown). In addition, the potency of FSK was increased by either PTX or quinpirole treatment (control, 3.30 ± 0.41 μM in these experi-

TABLE 1

Quinpirole-induced desensitization of D_2 dopamine receptor function

Cells were incubated for the indicated times with 1 μM quinpirole or vehicle and were assayed for dopaminergic inhibition of FSK-stimulated intracellular cAMP, as described in Experimental Procedures. Means ± standard errors of data and ALLFIT results from three experiments (performed in triplicate) are shown. Maximum inhibitions (in percent) were calculated for each experiment using the basal cAMP levels and the top and bottom of the dopamine dose-response curves determined by ALLFIT.

Treatment	Time	IC_{50}	Maximal inhibition	Cellular cAMP	
				Basal	FSK-stimulated
	hr	*nM*	%	*pmol/well*	
Control		3.9 ± 1.1	90.8 ± 3.2	0.60 ± 0.07	2.37 ± 0.25
Quinpirole	1	15.7 ± 2.9[a]	100.1 ± 3.6	0.76 ± 0.07	2.40 ± 0.21
	8	11.3 ± 1.1[a]	93.7 ± 3.8	0.82 ± 0.08	3.86 ± 0.37[b]
	24	23.5 ± 2.7[b]	99.1 ± 3.6	1.05 ± 0.07[b]	5.53 ± 0.53[b]

[a] $p < 0.05$ versus control.
[b] $p < 0.005$ versus control.

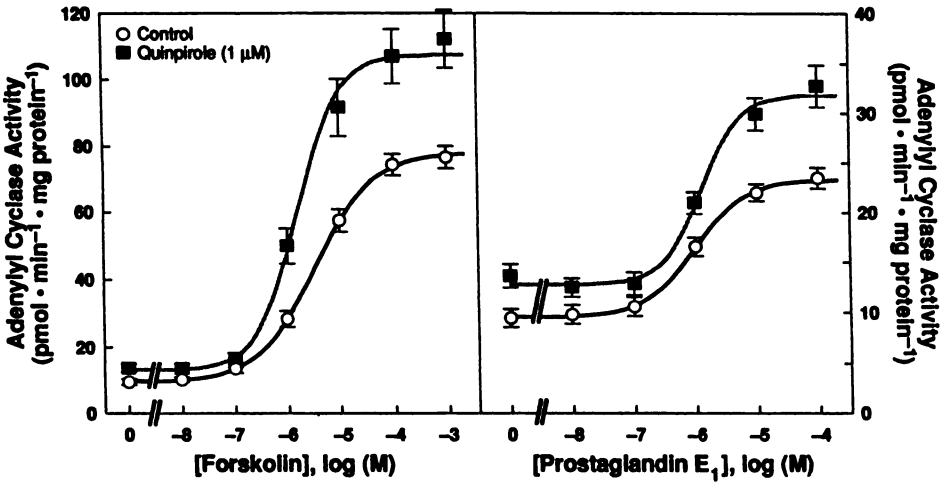

Fig. 4. Quinpirole-induced sensitization of adenylyl cyclase activity. Cells were incubated for 24 hr in the presence of 1 μM quinpiroleor vehicle. Membranes were prepared and adenylyl cyclase activity was measured as described in Experimental Procedures. Data points shown represent the mean ± standard error (FSK, eight experiments; PGE_1, seven experiments). Curves shown were obtained by modeling of these mean values. Results from the fits are given in Table 2.

TABLE 2

Quinpirole-induced sensitization of adenylyl cyclase activity

Cells were incubated for 24 hr in the presence of 1 μM quinpirole or vehicle. Membranes were prepared, adenylyl cyclase activity was measured, and data from each experiment were modeled using ALLFIT, as described in Experimental Procedures. Fit parameters from each experiment were then tabulated. The means ± standard errors from eight (for forskolin) or seven (for PGE_1) experiments are shown.

Stimulator	Pretreatment	Adenylyl cyclase activity		Fold stimulation	EC_{50} (FSK or PGE_1)
		Basal	Maximal		
		pmol · min⁻¹ · mg protein⁻¹			*μM*
Forskolin	Control	9.46 ± 0.82	78.4 ± 2.90	8.3	3.87 ± 0.79
	Quinpirole	13.0 ± 1.30[a]	110 ± 8.70[b]	8.3	1.85 ± 0.16[a]
PGE_1	Control	9.25 ± 0.89	23.8 ± 1.15	2.6	1.19 ± 0.20
	Quinpirole	12.5 ± 0.98[a]	32.8 ± 2.12[b]	2.5	1.80 ± 0.44

[a] $p < 0.05$ versus control.
[b] $p < 0.005$ versus control.

ments; quinpirole-treated, 1.65 ± 0.19 μM, $p < 0.05$ versus control; PTX-treated, 0.88 ± 0.09 μM, $p < 0.01$ versus control).

The role of direct D_2 receptor inhibition of adenylyl cyclase activity and lowering of cAMP levels in the long term sensitization of adenylyl cyclase was assessed using control or quinpirole incubations in the presence or absence of agents that increase intracellular cAMP levels, the membrane-permeable cAMP analogs 8Br-cAMP and diBu-cAMP and the cAMP phosphodiesterase inhibitor IBMX, each at a concentration of 1 mM. Results for 8Br-cAMP are shown in Fig. 8. Treatment of cells with 8Br-cAMP alone for 24 hr resulted in a slight decrease in basal and FSK- or PGE_1-stimulated adenylyl cyclase activities. However, treatment of cells with quinpirole plus 8Br-cAMP caused an even greater sensitization of adenylyl cyclase than that caused by the D_2 agonist alone. Experiments performed using diBu-cAMP or IBMX (which causes a steady increase in cellular cAMP levels over the incubation period) instead of 8Br-cAMP gave similar results (data not shown).

Discussion

The adenylyl cyclase system of mammalian cells involves both stimulatory and inhibitory inputs (36). Activation of a stimulatory receptor leads to stimulation of adenylyl cyclase via G_s, whereas activation of an inhibitory receptor (such as the D_2 dopamine receptor) leads to inhibition of the effector enzyme via one or more of the G_i proteins. The dependence of overall adenylyl cyclase activity of a cell on the stimulatory and inhibitory inputs means that the agonist-induced regulation of the activity of a receptor coupled to adenylyl cyclase activity could occur at several sites on either the stimulatory or inhib-

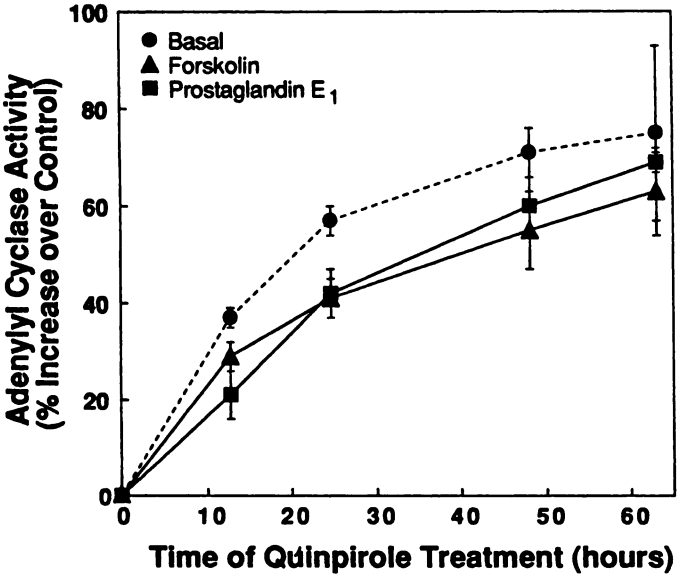

Fig. 5. Time course of quinpirole-induced adenylyl cyclase sensitization. Cells were incubated in the presence or absence of quinpirole (1 μM) for the indicated times; membranes were prepared and basal and FSK (10 μM)- and PGE_1 (10 μM)-stimulated adenylyl cyclase activities were measured as described in Experimental Procedures. Data are expressed as percentage increases in adenylyl cyclase activities over control, where control represents the basal and FSK- and PGE_1-stimulated activities in membranes from cells incubated in the absence of quinpirole. Data shown are the mean ± standard error from three experiments.

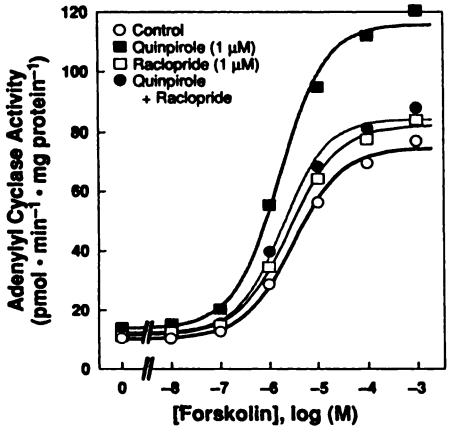

Fig. 6. D_2 dopaminergic specificity of quinpirole-induced sensitization of adenylyl cyclase. Cells were incubated in the absence or presence of quinpirole (1 μM) and/or raclopride (1 μM) for 24 hr. Membranes were prepared and adenylyl cyclase activity was measured as described in Experimental Procedures. Data shown are from one of three representative experiments.

itory side. The expression of the cDNA for the D_2 dopamine receptor in a cell line has allowed the study of the regulatory properties of this receptor in a cellular model system. We have examined the effect of D_2 agonist exposure on the D_2 receptor, with regard to the total cellular receptor concentration and the ability of this inhibitory receptor to regulate intracellular cAMP concentrations. The salient finding of this work is that, although exposure of cells to an inhibitory agonist causes relatively minor changes in the individual properties of the receptor and adenylyl cyclase, the overall effect of the changes to these two components is a dramatic desensitization of the responsiveness of the cells to dopamine.

Quinpirole treatment of Ltk^- cells expressing the D_2 receptor causes a decrease of ~3–6-fold in the potency of dopamine in inhibiting FSK-stimulated cAMP levels, without a change in the efficacy of dopamine. The decrease in dopaminergic function cannot be explained by a loss of total cellular receptors, although the possibility of a compartmentalization, or sequestration, of D_2 receptors from the adenylyl cyclase pathway cannot be ruled out. Agonist-induced sequestration has been described for the β_2-adrenergic receptors, although the contribution of this process to the overall desensitization appears to be minimal (37).

In addition to the decrease in potency of dopamine at D_2 receptors, D_2 agonist treatment also results in a dramatic increase in basal and stimulated cAMP levels. This increase is due to an increase in adenylyl cyclase activity and occurs over a slower time course than the shift in potency of dopamine in inhibiting cAMP accumulation. Both the shift in potency and the increase in adenylyl cyclase activity are due to activation of the D_2 receptor.

Physiologically, then, what would be the overall result of these observations on the ability of dopamine to regulate cAMP levels in whole cells or tissues? An examination of the dynamics of the system reveal that the relatively small changes observed can be translated into marked effects on the responsiveness of the system to dopamine (see Fig. 3). Furthermore, at later time points, sensitization of adenylyl cyclase appears to be the dominant component in the decrease in cellular responsiveness to dopamine. For example, at a concentration of 10 nM, dopamine inhibits FSK-stimulated cAMP levels in whole cells 64% relative to basal levels in control cells. This inhibition is decreased to 41% after 1 hr of agonist (quinpirole) exposure and to 26% after 24 hr. These percentage changes result from the decreased potency of dopamine in agonist-treated cells. However, with the increases in basal and FSK-stimulated cAMP levels, the absolute concentrations of cAMP in the cells at this dose of dopamine also increase dramatically with time of pretreatment, from 1.23 ± 0.13 pmol of cAMP/well in control cells to 4.34 ± 0.32 pmol/well in cells treated with quinpirole for 24 hr, an increase of 3.5-fold. Another way of describing the combined effects of the decreased potency of dopamine and the increased adenylyl cyclase activity in quinpirole-treated cells is to determine the dose of dopamine in the cAMP assay required to inhibit the FSK-stimulated cAMP level in the cells to a particular concentration.[3] In control cells, dopamine inhibits cAMP levels to 2 pmol/well at a concentration of 0.96 nM. The concentration required to reduce cellular cAMP to this level increases to 64 nM dopamine (~66-fold increase over control) after 24 hr of quinpirole treatment. At intermediate times of quinpirole treatment, the concentration of dopamine needed to achieve 2 pmol of cAMP/well is increased 4.3-fold (1 hr) and 17-fold (8 hr). Note that the concentrations of dopamine considered here, in the nanomolar range, are physiologically relevant concentrations of dopamine. For example, the concentration of dopamine in rat hypophysial portal plasma (to regulate prolactin secretion by anterior pituitary lactotropes via D_2 receptors) varies between 3 and 100 nM, depending upon the sex and endocrine status of the animal (38), and the basal concentration of extracellular dopamine in rat striatum (a

[3]These concentrations were determined by interpolation of the curve fits modeled using ALLFIT.

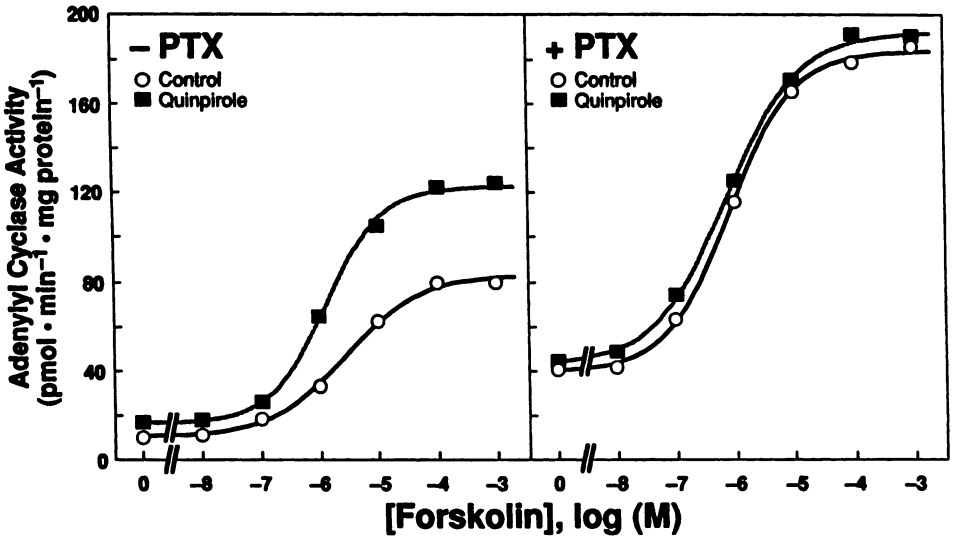

Fig. 7. Effect of PTX treatment on quinpirole-induced sensitization of adenylyl cyclase. Cells were preincubated for 12 hr with or without PTX and then incubated for 24 hr with or without PTX in the presence or absence of quinpirole. Membranes were prepared and adenylyl cyclase activity was measured as described in Experimental Procedures. Data shown are from one of three representative experiments.

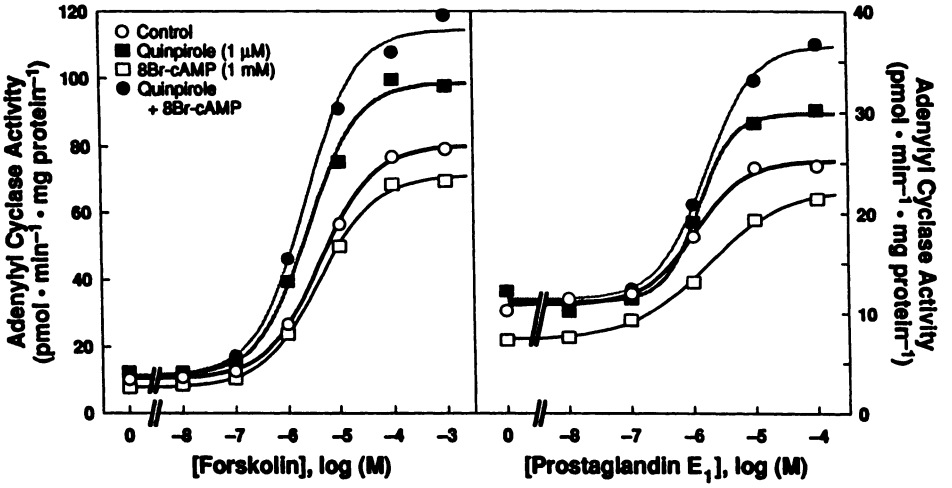

Fig. 8. Dependence or independence of quinpirole-induced adenylyl cyclase sensitization on cellular cAMP levels. Cells were incubated in the absence or presence of quinpirole (1 μM) and/or 8Br-cAMP (1 mM) for 24 hr. Membranes were prepared and adenylyl cyclase activity was measured as described in Experimental Procedures. Data shown are from one of three representative experiments.

prominent site of D_2 receptors in the brain) is about 20 nM (39). Thus, the combined effects of the loss of dopaminergic potency and the increased adenylyl cyclase activity may markedly influence cellular responsiveness to dopamine (at physiologically relevant concentrations), with the latter becoming predominant after hours of exposure to agonist.

Although the desensitization of inhibitory receptor function has not been observed in all systems, sensitization of adenylyl cyclase and its stimulatory pathways has been reported in a variety of systems for many *endogenous* inhibitory receptors (see Introduction). For the present study, a rat D_2 dopamine receptor has been expressed in a mouse fibroblast cell line. Although these cells are not known to contain any endogenous inhibitory receptors, they appear to contain the cellular machinery to allow desensitization of receptor function and sensitization of adenylyl cyclase activity when an inhibitory receptor is expressed in these cells. Thus, the sensitization of adenylyl cyclase caused by chronic activation of inhibitory receptors appears to be a general phenomenon and may play a key role in regulating the responsiveness of cells to inhibitory substances, as illustrated above.

Two questions may be asked regarding the receptor desensitization and adenylyl cyclase sensitization described. First, how does activation of an inhibitory receptor provide a signal for modulation of effector activity? We have attempted to address this question by asking whether either G protein function or the second messenger itself, in this case cAMP, might be involved in the sensitization process. The sensitization of adenylyl cyclase activity by D_2 agonists appears to be dependent upon G protein activation, because prior treatment of cells with PTX abolishes further sensitization of adenylyl cyclase by quinpirole, although PTX treatment itself increases basal and stimulated adenylyl cyclase activity. It is interesting to note that the potencies and efficacies of FSK and PGE_1 in stimulating adenylyl cyclase are increased by either PTX or quinpirole treatment.

To examine distal components of the adenylyl cyclase pathway in the onset of sensitization, the effect of raising intracellular cAMP levels, by using membrane-permeable cAMP analogs or by blocking the degradation of cAMP, was studied. Incubation of cells with quinpirole in combination with any of these agents resulted in a greater sensitization of adenylyl cyclase activity than that caused by quinpirole alone, suggesting that decreases in cAMP levels caused by quinpirole cannot account for adenylyl cyclase sensitization. Rather, other mechanisms must be at work. One possibility is that a signal transduction pathway in addition to adenylyl cyclase, such as polyphosphoinositide metabolism, is regulated concomitantly by

the D_2 receptor. Indeed, D_2 agonists cause slight increases in phospholipase C activity in the cells used in this study (40).[4] However, D_2 agonists also sensitize adenylyl cyclase when the receptor is expressed in GH_4C_1 cells (data not shown), a cell line in which D_2 receptor activation does not increase phospholipase C activity (40).[4]

A second question regarding the receptor desensitization and effector sensitization is, what is the alteration in the signaling pathways of the inhibitory agonist-treated cells? Several groups have attempted to address the latter question by examining the effect of inhibitory agonist treatment on the levels of G proteins, using immunochemical techniques. These studies have yielded conflicting results (41–43), although alterations in G protein content [in particular, decreases in G_i protein(s) and increases in G_s] may be involved in the development of adenylyl cyclase sensitization in cells exposed to an inhibitory agonist.

The processes that we have described here allow the adenylyl cyclase system and its inhibition by dopamine to be regulated over a broad range through time. Such regulation likely has important physiological ramifications. For example, dopamine released by tuberoinfundibular neurons tonically inhibits the synthesis and secretion of prolactin in the anterior pituitary by cAMP-dependent and -independent mechanisms. Removal of dopaminergic tone results in increased prolactin secretion, as well as increases in the sensitivity of the pituitary to dopamine (for review, see Ref. 44). Given that activation of the D_2 receptor can result in regulation of both stimulatory and inhibitory pathways, it is possible that slight alterations in tuberoinfundibular dopamine (in conjunction with alterations in the release of hypothalamic stimulatory hormones) could result in very sensitive and time-dependent regulation of prolactin secretion. The actions of dopamine in inhibiting the release of dopamine (45), acetylcholine (46), and glutamate (47) in the corpus striatum could also be regulated in such a complex manner. These possibilities bear scrutiny in light of our results in this model system.

To summarize, the rat D_2 dopamine receptor, when expressed in mouse fibroblast Ltk^- cells, inhibits the formation of cAMP stimulated by FSK, and its activity is dynamically regulated. Treatment of the cells with the D_2 agonist quinpirole results in a short term (minutes) desensitization of receptor function and a longer term (hours/days) sensitization of the stimulatory adenylyl cyclase pathway. This sensitization is not dependent upon decreases in intracellular cAMP levels induced by D_2 receptor activation.

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References

1. Spano, P. F., S. Govani, and M. Trabucchi. Studies on the pharmacological properties of dopamine receptors in various areas of the central nervous system. *Adv. Biochem. Psychopharmacol.* **19**:155–165 (1978).
2. Kebabian, J. W., and D. B. Calne. Multiple receptors for dopamine. *Nature (Lond.)* **277**:93–96 (1979).
3. Vallar, L., and J. Meldolesi. Mechanisms of signal transduction at the dopamine D_2 receptor. *Trends Pharmacol. Sci.* **10**:74–77 (1989).
4. Senogles, S. E., A. M. Spiegel, E. Padrell, R. Iyengar, and M. G. Caron. Specificity of receptor-G protein interactions: discrimination of G_i subtypes by the D_2 dopamine receptor in a reconstituted system. *J. Biol. Chem.* **265**:4507–4514 (1990).
5. Benovic, J. L., M. Bouvier, M. G. Caron, and R. J. Lefkowitz. Regulation of adenylyl cyclase-coupled β-adrenergic receptors. *Annu. Rev. Cell Biol.* **4**:405–428 (1988).
6. Sharma, S. K., W. A. Klee, and M. Nirenberg. Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc. Natl. Acad. Sci. USA* **72**:3092–3096 (1975).
7. Law, P. Y., D. S. Hom, and H. H. Loh. Loss of opiate receptor activity in neuroblastoma × glioma NG108-15 hybrid cells after chronic opiate treatment: a multiple-step process. *Mol. Pharmacol.* **22**:1–4 (1982).
8. Griffin, M. T., P.-Y. Law, and H. H. Loh. Involvement of both inhibitory and stimulatory guanine nucleotide binding proteins in the expression of chronic opiate regulation of adenylate cyclase activity in NG108-15 cells. *J. Neurochem.* **45**:1585–1589 (1985).
9. Nathanson, N. M., W. L. Klein, and M. Nirenberg. Regulation of adenylate cyclase activity mediated by muscarinic acetylcholine receptors. *Proc. Natl. Acad. Sci. USA* **75**:1788–1791 (1978).
10. Thomas, J. M., and B. B. Hoffman. Agonist-induced down-regulation of muscarinic cholinergic and α_2-adrenergic receptors after inactivation of N_i by pertussis toxin. *Endocrinology* **119**:1305–1314 (1986).
11. Mayor, F., J. L. Benovic, M. G. Caron, and R. J. Lefkowitz. Somatostatin induces translocation of the β-adrenergic receptor kinase and desensitizes somatostatin receptors in S49 lymphoma cells. *J. Biol. Chem.* **262**:6468–6471 (1987).
12. Parsons, W. J., and G. L. Stiles. Heterologous desensitization of the inhibitory A_1 adenosine receptor-adenylate cyclase system in rat adipocytes: regulation of both N_s and N_i. *J. Biol. Chem.* **262**:841–847 (1987).
13. Chang, K.-J., R. W. Eckel, and S. G. Blanchard. Opioid peptides induce reduction of enkephalin receptors in cultured neuroblastoma cells. *Nature (Lond.)* **296**:446–448 (1982).
14. Heisler, S., and C. B. Srikant. Somatostatin-14 and somatostatin-28 pretreatment down-regulate somatostatin-14 receptors and have biphasic effects on forskolin-stimulated cyclic adenosine, 3′,5′-monophosphate synthesis and adrenocorticotropin secretion in mouse anterior pituitary tumor cells. *Endocrinology* **117**:217–225 (1985).
15. Presky, D. H., and A. Schonbrunn. Somatostatin pretreatment increases the number of somatostatin receptors in GH_4C_1 pituitary cells and does not reduce cellular responsiveness to somatostatin. *J. Biol. Chem.* **263**:714–721 (1988).
16. Motulsky, H. J., S. J. Shattil, N. Ferry, D. Rozansky, and P. A. Insel. Desensitization of epinepherine-initiated platelet aggregation does not alter binding to the α_2-adrenergic receptor or receptor coupling to adenylate cyclase. *Mol. Pharmacol.* **29**:1–6 (1986).
17. Sabol, S. L., and M. Nirenberg. Regulation of adenylate cyclase of neuroblastoma × glioma hybrid cells by α-adrenergic receptors. II. Long lived increase of adenylate cyclase activity mediated by α receptors. *J. Biol. Chem.* **254**:1921–1926 (1979).
18. Jones, S. B., M. L. Toews, J. T. Turner, and D. B. Bylund. α_2-Adrenergic receptor-mediated sensitization of forskolin-stimulated cAMP production. *Proc. Natl. Acad. Sci. USA* **84**:1294–1298 (1987).
19. Reisine, T. D., and J. S. Takahashi. Somatostatin pretreatment desensitizes somatostatin receptors linked to adenylate cyclase and facilitates the stimulation of cyclic adenosine 3′:5′-monophosphate accumulation in anterior pituitary tumor cells. *J. Neurosci.* **4**:812–819 (1984).
20. Hoffman, B. B., H. Chang, E. Dall'Aglio, and G. M. Reaven. Desensitization of adenosine receptor-mediated inhibition of lipolysis: the mechanism involves the development of enhanced cyclic adenosine monophosphate accumulation in tolerant adipocytes. *J. Clin. Invest.* **78**:185–190 (1986).
21. Bunzow, J. R., H. H. M. Van Tol, D. K. Grandy, P. Albert, J. Salon, M. Christie, C. A. Machida, K. A. Neve, and O. Civelli. Cloning and expression of a rat D_2 dopamine receptor cDNA. *Nature (Lond.)* **336**:783–787 (1988).
22. Giros, B., P. Sokoloff, M.-P. Martres, J.-F. Riou, L. J. Emorine, and J.-C. Schwartz. Alternative splicing directs the expression of two D_2 dopamine receptor isoforms. *Nature (Lond.)* **342**:923–926 (1989).
23. Selbie, L. A., G. Hayes, and J. Shine. The major dopamine D2 receptor: molecular analysis of the human $D2_A$ subtype *DNA* **8**:683–689 (1989).
24. Grandy, D. K., M. A. Marchionni, H. Makam, R. E. Stofko, M. Alfano, L. Frothingham, J. B. Fischer, K. J. Burke-Howie, J. R. Bunzow, A. C. Server, and O. Civelli. Cloning of the cDNA and gene for a human D_2 dopamine receptor. *Proc. Natl. Acad. Sci. USA* **86**:9762–9766 (1989).
25. Monsma, F. J., L. D. McVittie, C. R. Gerfen, L. C. Mahan, and D. R. Sibley. Multiple D_2 dopamine receptors produced by alternative mRNA splicing. *Nature (Lond.)* **342**:926–929 (1989).
26. Neve, K. A., R. A. Henningsen, J. R. Bunzow, and O. Civelli. Functional characterization of a rat dopamine D-2 receptor cDNA expressed in a mammalian cell line. *Mol. Pharmacol.* **36**:446–451 (1989).
27. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
28. DeLean, A., J. M. Stadel, and R. J. Lefkowitz. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J. Biol. Chem.* **255**:7108–7117 (1980).
29. Liggett, S. B., S. D. Shah, and P. E. Cryer. Increased fat and skeletal muscle β-adrenergic receptors but unaltered metabolic and hemodynamic sensitivity to epinepherine in vivo in experimental human thyrotoxicosis. *J. Clin. Invest.* **83**:803–809 (1989).

[4] M. D. Bates, unpublished observations.

30. Harper, J. F., and G. Brooker. Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'O acetylation by acetic anhydride in aqueous solution. *J. Cyclic Nucleotide Res.* **1**:207–218 (1975).
31. Steiner, A. L., C. W. Parker, and D. M. Kipnis. Radioimmunoassay for cyclic nucleotides: I. Preparation of antibodies and iodinated cyclic nucleotides. *J. Biol. Chem.* **247**:1106–1113 (1972).
32. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
33. Katada, T., G. M. Bokoch, J. K. Northup, M. Ui, and A. G. Gilman. The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase: properties and function of the purified protein. *J. Biol. Chem.* **259**:3568–3577 (1984).
34. DeLean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.* **235**:E97–E102 (1978).
35. Seeman, P., and H. B. Niznik. Dopamine D_1 receptor pharmacology. *ISI Atlas Sci. Pharmacol.* **2**:161–170 (1988).
36. Gilman, A. G. G proteins and dual control of adenylate cyclase. *Cell* **36**:577–579 (1984).
37. Lefkowitz, R. J., W. P. Hausdorff, and M. G. Caron. Role of phosphorylation in desensitization of the β-adrenoceptor. *Trends Pharmacol. Sci.* **11**:190–194 (1990).
38. Ben-Jonathan, N., C. Oliver, H. J. Weiner, R. S. Mical, and J. C. Porter. Dopamine in hypophysial portal plasma of the rat during the estrous cycle and throughout pregnancy. *Endocrinology* **100**:452–458 (1977).
39. Church, W. H., J. B. Justice, and L. D. Byrd. Extracellular dopamine in rat striatum following uptake inhibition by cocaine, nomifensine, and benztropine. *Eur. J. Pharmacol.* **139**:345–348 (1987).
40. Vallar, L., C. Muca, M. Magni, P. Albert, J. Bunzow, J. Meldolesi, and O. Civelli. Differential coupling of dopaminergic D_2 receptors expressed in different cell types: stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis in Ltk^- fibroblasts, hyperpolarization, and cytosolic-free Ca^{2+} concentration decrease in GH_4C_1 cells. *J. Biol. Chem.* **265**:10320–10326 (1990).
41. Lang, J., and T. Costa. Chronic exposure of NG 108-15 cells to opiate agonists does not alter the amount of the guanine nucleotide-binding proteins G_i and G_o. *J. Neurochem.* **53**:1500–1506 (1989).
42. Attali, B., and Z. Vogel. Long-term opiate exposure leads to reduction of the αi-1 subunit of GTP-binding proteins. *J. Neurochem.* **53**:1636–1639 (1989).
43. Longabaugh, J. P., J. Didsbury, A. Spiegel, and G. L. Stiles. Modification of the rat adipocyte A_1 adenosine receptor-adenylate cyclase system during chronic exposure to an A_1 adenosine receptor agonist: alterations in the quantity of $G_{s\alpha}$ and $G_{i\alpha}$ are not associated with changes in their mRNAs. *Mol. Pharmacol.* **36**:681–688 (1989).
44. Ben-Jonathan, N. Dopamine: a prolactin-inhibiting hormone. *Endocrine Rev.* **6**:564–589 (1985).
45. Starke, K., L. Späth, J. D. Lang, and C. Adelung. Further functional in vitro comparison of pre- and postsynaptic dopamine receptors in rabbit caudate nucleus. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **323**:298–306 (1983).
46. Sethy, V. H. Regulation of striatal acetylcholine concentration by D_2-dopamine receptors. *Eur. J. Pharmacol.* **60**:397–398 (1979).
47. Mitchell, P. R., and N. S. Doggett. Modulation of striatal [^{3}H]-glutamic acid release by dopaminergic drugs. *Life Sci.* **26**:2073–2081 (1980).

Send reprint requests to: Dr. Marc G. Caron, Box 3287, Duke University Medical Center, Durham, NC 27710.