0026-895X/90/040531-11$03.00/0

MOLECULAR PHARMACOLOGY, 38:531-541



# Agonist-Induced Desensitization of $D_1$-Dopamine Receptors Linked to Adenylyl Cyclase Activity in Cultured NS20Y Neuroblastoma Cells

ANNE C. BARTON and DAVID R. SIBLEY

*Experimental Therapeutics Branch, National Institute for Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland*

Received May 4, 1990; Accepted July 12, 1990

**SUMMARY**

NS20Y neuroblastoma cells expressing a homogeneous population of $D_1$-dopamine receptors were used in the present study as a model system to investigate the mechanisms of agonist-induced stimulation and desensitization of $D_1$ receptor-coupled adenylyl cyclase activity. Membranes prepared from NS20Y cells showed a pharmacologically specific, dose-dependent increase in cAMP production in response to various dopaminergic agonists. Dopamine exhibited an $EC_{50}$ of 5 $\mu$M, and at 100 $\mu$M a maximal stimulation of 3–4-fold over basal enzyme activity was observed, which could be selectively antagonized by the active stereoisomers of SCH-23390 and butaclamol. Preincubation of NS20Y cells with dopamine induced homologous desensitization of $D_1$ receptor-coupled adenylyl cyclase activity, decreasing dopamine- but not prostaglandin-, adenosine-, or forskolin-stimulated cAMP production. Desensitization did not affect the $EC_{50}$ for dopamine but resulted in an 85–90% reduction in the maximal response. Dopamine-induced desensitization of adenylyl cyclase activity was found to be both dose and time dependent. As early as 5 min after preincubation with dopamine, cAMP production was decreased by 45–50%, with maximal desensitization occurring by 90 min. Preincubation of NS20Y cells with dopamine also induced a decrease in $D_1$ receptor ligand binding activity, as assessed with the radiolabeled antagonist [$^3$H]SCH-23390. This decrease in binding activity occurred more slowly than the loss of enzyme activity, not achieving maximal levels until after 3 hr. [$^3$H]SCH-23390 saturation binding isotherms in control and maximally desensitized NS20Y cell membranes revealed no change in affinity ($K_D$); however, a 65–70% decrease in receptor number ($B_{max}$) was observed. Because the maximal and temporal decrease in $D_1$ receptors does not correlate with the decrease in dopamine-stimulated enzyme activity, the desensitization may involve a functional uncoupling of the $D_1$ receptor in addition to receptor down-regulation. This is further suggested by a loss in high affinity agonist binding observed in agonist/[$^3$H]SCH-23390 competition experiments after desensitization. Removal of dopamine after maximal desensitization/down-regulation results in recovery to control values by 24 hr. This recovery is mostly, but not completely, blocked by protein synthesis inhibitors, suggesting an involvement of receptor degradation in the desensitization process.

Dopamine receptors are among the most intensively studied and physiologically important neurotransmitter receptor systems in the brain. Pharmacological, biochemical, and physiological criteria have been used to define two subcategories of dopamine receptors, referred to as $D_1$ and $D_2$ (1). Both of these receptor subtypes are members of a broad family of receptors that are coupled to their specific effector functions via G proteins (1, 2). $D_1$ receptors have been classically defined as being linked to the stimulation of adenylyl cyclase activity (3, 4), although recent evidence has suggested that some $D_1$ receptors may activate phospholipase C (5, 6). In contrast, $D_2$ receptor activation results in various responses including inhibition of adenylyl cyclase activity (7), inhibition of phosphatidylinositol turnover (8), increase in $K^+$ channel activity (9), and inhibition of $Ca^{2+}$ mobilization (10). Both $D_1$ and $D_2$ receptors are present predominantly in the central nervous system, where they are critically involved in regulating cognitive function as well as motor control.

Altered dopamine receptor function has been implicated in numerous neurological and endocrine disorders including schizophrenia, Parkinson's disease, Tourette's syndrome, tardive dyskinesia, Huntington's chorea, and hyperprolactinemia (11). In many instances, these disorders and/or their therapy have been suggested to involve aberrant regulatory mechanisms of the dopaminergic receptor systems (12). A wide variety of *in vivo* investigations have, in fact, documented that dopamine receptors are subject to dynamic regulation in both a positive and a negative fashion (13–15). Thus far, however, these regulatory phenomena have not been investigated in detail and the underlying biochemical mechanisms have not yet been addressed. In large part, this is due to the inherent limitations of *in vivo* experimentation and lack of suitable simple model

**ABBREVIATIONS:** G protein, guanine nucleotide-binding regulatory protein, NPA, *N*-propylnorapomorphine; 6,7-ADTN, (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; $PGE_2$, prostaglandin $E_2$; Gpp(NH)p, guanylyl-5′-imidodiphosphate.

systems for *in vitro* investigations of dopamine receptor function and regulation. An optimal *in vitro* model system would be a dopamine receptor-expressing neuronal cell line that could be treated under precisely defined conditions and grown in large quantities for biochemical experimentation.

Recently, we reported that the NS20Y neuroblastoma cell line expresses $D_1$-dopamine receptor binding sites, as assessed using radioligand binding techniques (16). These saturable receptor sites were shown to be pharmacologically specific, inasmuch as dopaminergic antagonists and agonists exhibited the appropriate rank order of potency and absolute molar affinities expected for a $D_1$-dopamine receptor (16). In addition, the presence of high affinity, guanine nucleotide-sensitive agonist binding suggested that these receptors were capable of functional G protein coupling. In the present investigation, we demonstrate that the $D_1$ receptors expressed by NS20Y cells are indeed functionally coupled to the stimulation of adenylyl cyclase activity, with the appropriate pharmacology. More importantly, we find that acute treatment of the cells with dopamine or other dopaminergic agonists results in a profound homologous desensitization of the adenylyl cyclase response to dopamine as well as a down-regulation of the $D_1$ receptors. Our data suggest that multiple biochemical mechanisms, including functional uncoupling and receptor sequestration and degradation, are involved in promoting these regulatory events.

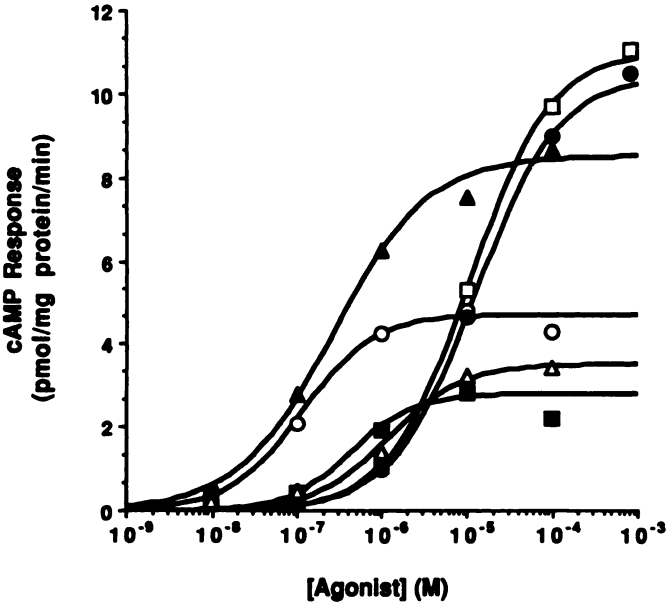


**Fig. 1.** Dose-response curves for dopaminergic agonist stimulation of adenylyl cyclase activity in NS20Y cell membranes. Membranes were prepared from NS20Y cells and incubated with increasing concentrations of dopamine (□), epinine (●), (±)-SKF-82526 (▲), (+)-SKF-38393 (○), (−)-NPA (△), or (−)-apomorphine (■) for 10 min at 37°, as described in Experimental Procedures. The data represent the amount of cAMP produced over basal levels of enzyme activity. The curves shown are representative of three independent experiments for each agonist. Averaged $EC_{50}$ values are presented in Table 1.

TABLE 1

**Pharmacology of $D_1$ receptor-stimulated adenylyl cyclase activity in NS20Y cell membranes**

Data presented are $EC_{50}$ values for agonist stimulation and $K_i$ values for antagonist inhibition of dopamine-stimulated adenylyl cyclase activity in NS20Y cell membranes. Experiments were performed as described for Figs. 1 and 2. $EC_{50}$ values for agonists were graphically determined, whereas antagonist dissociation constants ($K_i$) were determined by Cheng and Prusoff (40) correction of graphically determined $IC_{50}$ values, using the equation $K_i = IC_{50}/(1 + [\text{dopamine}]/\text{dopamine } EC_{50})$, where [dopamine] = 100 μM and the dopamine $EC_{50}$ = 5.83 μM. Values represent the means of two or three independent experiments (SE values were ≤15% of the means).

| Agonist | $EC_{50}$ | Antagonist | $K_i$ |
|---|---|---|---|
| | μM | | nM |
| (+)-SKF-38393 | 0.17 | (+)-SCH-23390 | 0.49 |
| (±)-SKF-82526 | 0.27 | (+)-Butaclamol | 4.57 |
| (−)-Apomorphine | 0.55 | (−)-SCH-23390 | 182 |
| (−)-NPA | 1.30 | (−)-Butaclamol | >100,000 |
| Dopamine | 5.83 | | |
| 6,7-ADTN | 7.0 | | |
| Epinine | 12.0 | | |
| Serotonin | — | | |
| Isoproterenol | — | | |
| Quinpirole | — | | |

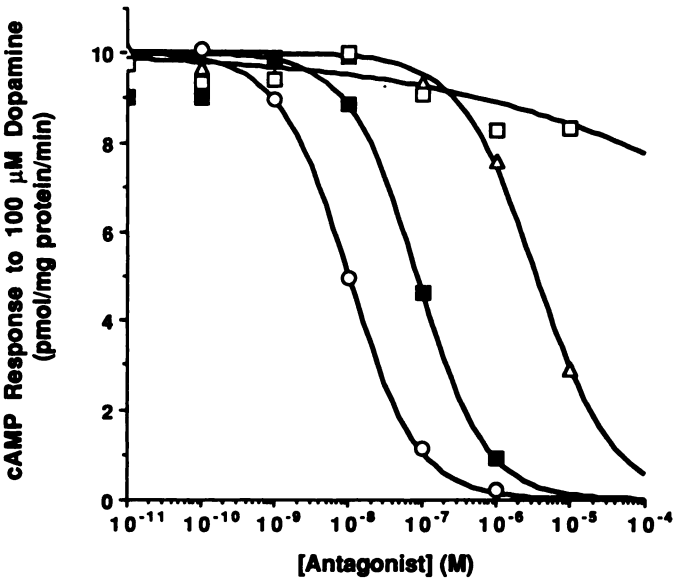


**Fig. 2.** Effect of dopaminergic antagonists on inhibition of dopamine-stimulated adenylyl cyclase activity in NS20Y cell membranes. Membranes prepared from NS20Y cells were incubated with 100 μM dopamine and increasing concentrations of (+)-SCH-23390 (○), (−)-SCH-23388 (△), (+)-butaclamol (■), or (−) butaclamol (□) for 10 min at 37°, as described in Experimental Procedures. The data represent the amount of cAMP produced over basal levels of enzyme activity. The curves shown are representative of two independent experiments for each antagonist. Averaged $K_i$ values are presented in Table 1.

## Experimental Procedures

**Materials.** [$^3$H]SCH-23390 [(*R*)-(+)-9-[$^3$H]8-Chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepine-7-ol] (60 Ci/mmol) was purchased from DuPont/NEN (Boston, MA). [$^3$H]cAMP (45 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). (+)-SCH-23390, (−)-SCH-23388, (+)-butaclamol, (−)-butaclamol, (+)-SKF-38393, (−)-SKF-38393, (−)-apomorphine, (−)-NPA, and 6,7-ADTN were purchased from Research Biochemicals, Inc. (Natick, MA). Dopamine was purchased from Calbiochem (La Jolla, CA). Epinine, adenosine, $PGE_2$, cycloheximide, serotonin, (−)-isoproterenol, and Gpp(NH)p were purchased from Sigma Chemical Co. (St. Louis, MO). RO-20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] was purchased from Biomol (Plymouth Meeting, PA). The following drugs were gifts from the respective pharmaceutical companies: (±)-SKF-82526 (fenoldopam), Smith, Kline & French Laboratories (Swedeland, PA); and quinpirole (LY-171555), Lilly Laboratories (Indianapolis, IN). Cell culture media, reagents, and fetal bovine serum were obtained from GIBCO Laboratories (Grand Island, NY). All other chemicals were purchased from commercial suppliers and were of the highest quality available.

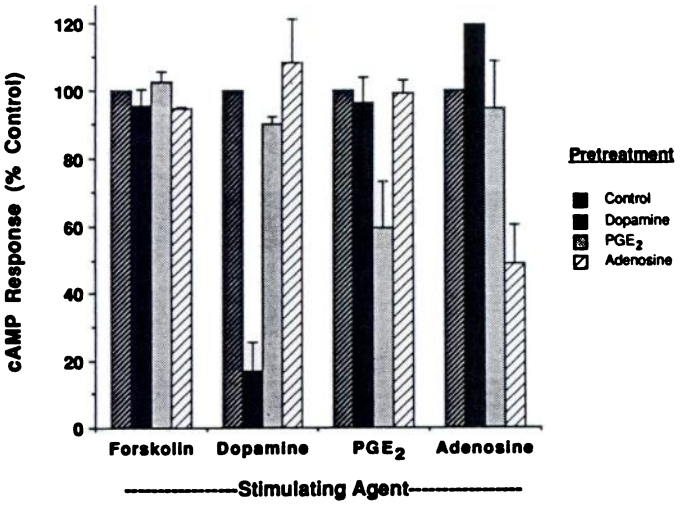


**Fig. 3.** Effect of pretreating NS20Y cells with dopamine, $PGE_2$, or adenosine on agonist- and forskolin-stimulated adenylyl cyclase activity. NS20Y cells were incubated with 200 $\mu$M sodium metabisulfite in Earle's balanced salt solution (control) or 200 $\mu$M sodium metabisulfite in Earle's balanced salt solution containing either 100 $\mu$M dopamine, 10 $\mu$M $PGE_2$, or 100 $\mu$M adenosine, for 1 hr at 37°. The cells were then washed extensively and membranes were prepared as described in Experimental Procedures. Adenylyl cyclase activities were then evaluated using the following stimulating agents: 50 $\mu$M forskolin, 100 $\mu$M dopamine, 10 $\mu$M $PGE_2$, or 100 $\mu$M adenosine. The data are representative of two similar experiments and are presented as a percentage of the enzyme activities from the control (untreated) cells, which are as follows (mean ± SE): 50 $\mu$M forskolin, 74.3 ± 8.0 pmol/mg of protein/min; 100 $\mu$M dopamine, 13.8 ± 1.6 pmol/mg of protein/min; 10 $\mu$M $PGE_2$ 34.4 ± 6.5 pmol/mg of protein/min; and 100 $\mu$M adenosine, 12.7 ± 1.7 pmol/mg of protein/min. In some data sets, the standard error values are too small to be observable. The basal enzyme activities were subtracted from the above values and were not affected by any agonist pretreatment.

**Cell culture.** NS20Y cells were cultured in Dulbecco's modified essential medium containing high glucose (4500 mg/liter), 1 mM sodium pyruvate, and 10% fetal bovine serum, in a humidified atmosphere of 10% $CO_2$ in air at 37°. Cells were grown in 150-$cm^2$ flasks and used at a density of $3.5 \times 10^7$ cells/flask for desensitization experiments.

**Membrane preparation.** NS20Y cells were detached from 150-$cm^2$ flasks with 1 mM EDTA in $Ca^{2+}$/$Mg^{2+}$-free Earle's balanced salt solution and were washed by centrifugation at 300 × *g* and resuspension with cold Earle's balanced salt solution (complete). The cells were then suspended in ice-cold lysis buffer (5 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$), transferred to a Dounce homogenizer on ice, and homogenized using 15 strokes with an A pestle. The homogenate was diluted with 1 volume of AC buffer (75 mM Tris·HCl, pH 7.4, 250 mM sucrose, 12.5 mM $MgCl_2$, 1.5 mM EDTA) and centrifuged for 10 min at 43,500 × *g*. The upper half of the resulting two-layer pellet was carefully removed and resuspended in the appropriate assay buffer (see below). For some experiments, the resulting membrane suspension was further divided, to generate two membrane fractions (one for adenylyl cyclase activity determination and one for [$^3$H]SCH-23390 binding), followed by recentrifugation and resuspension.

**Determination of cAMP production.** Fifty microliters of NS20Y cell membranes, (50 $\mu$g of protein) suspended in AC buffer containing 1 mM dithiothreitol and 200 $\mu$M sodium metabisulfite and supplemented with 2.75 mM phosphoenolpyruvate, 53 $\mu$M GTP, 0.12 mM ATP, 1.0 unit of myokinase, 0.2 unit of pyruvate kinase, and 100 $\mu$M RO-20-1724 (a phosphodiesterase inhibitor), were added to tubes on ice containing 10 $\mu$l of $H_2O$ or 10 $\mu$l of appropriate test compounds. The membranes were incubated for 10 min at 37° to generate cAMP and the reaction was stopped by a 3-min incubation in boiling $H_2O$. The cAMP thus generated was assayed by the method of Brown *et al.* (17), by incubation with cAMP-binding protein (prepared from bovine adrenal gland) in the presence of [$^3$H]cAMP at 4° for 2–16 hr, as previously described (18). Following incubation with cAMP-binding protein, free [$^3$H]cAMP was removed by treatment with charcoal/bovine serum albumin, and the bound [$^3$H]cAMP remaining in the supernatant was quantitated by liquid scintillation counting. cAMP concentrations were determined by comparison with standards, the curve of which was linear in the range of 1–30 pmol of cAMP/assay tube. All assays were performed in triplicate. A typical basal value for the adenylyl cyclase activity of the NS20Y cell membranes using this assay procedure was approximately 2.5 pmol of cAMP/mg of protein/min. In each experiment described, the basal adenylyl cyclase activities have been subtracted from the effector-stimulated enzyme activities.

**Radioligand binding assays.** Radioligand binding assays were performed as previously described (16). Briefly, 100 $\mu$l of NS20Y cell membranes (1.25 mg of protein/ml), suspended in binding buffer (50 mM Tris·HCl, pH 7.4, 1 mM EDTA, 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, 120 mM NaCl), were added to a final volume of 1 ml of binding buffer containing 0.05 to 2 nM [$^3$H]SCH-23390 for saturation analyses or 0.5 nM [$^3$H]SCH-23390 for single-point binding determinations. Nonspecific binding was determined in the presence of 1 $\mu$M (+)-butaclamol. Competition analysis of agonist binding was carried out using a high magnesium, low ionic strength buffer consisting of 50 mM Tris·HCl, pH 7.4 at 22°, 1.2 mM EDTA, 10 mM $MgSO_4$, and 0.1% sodium ascorbate. Incubations were carried out for 1 to 1.5 hr at room temperature and were terminated by rapid filtration under vacuum through Whatman GF/C filters (pretreated with 0.3% polyethyleneimine). The filters were washed with 5 × 4 ml of ice-cold 50 mM Tris·

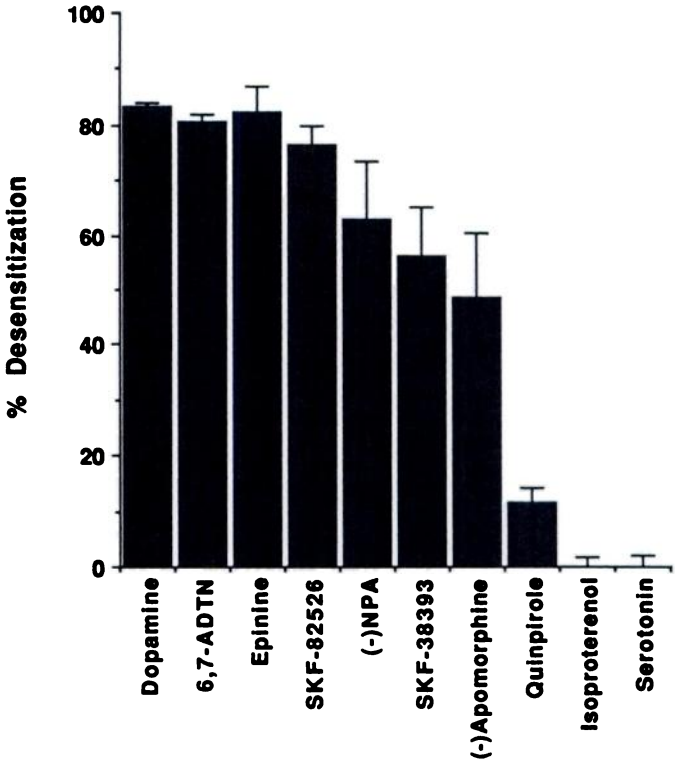


**Fig. 4.** Agonist-induced desensitization of dopamine-stimulated adenylyl cyclase activity in NS20Y cells. NS20Y cells were preincubated, as described in the legend to Fig. 3, with 100 $\mu$M concentrations of dopamine, 6,7-ADTN, epinine, quinpirole, isoproterenol, or serotonin or 10 $\mu$M concentrations of (±)-SKF-82526, (+)-SKF-38393, (−)-NPA, or (−)-apomorphine, for 2 hr at 37°. The cells were then washed extensively and the membranes prepared from each treatment group were assayed for dopamine-stimulated adenylyl cyclase activity. The percentage of desensitization refers to the percentage of decrease observed in the adenylyl cyclase response to 100 $\mu$M dopamine relative to that observed in control (untreated) cells. The data represent the means ± standard errors from two independent experiments.

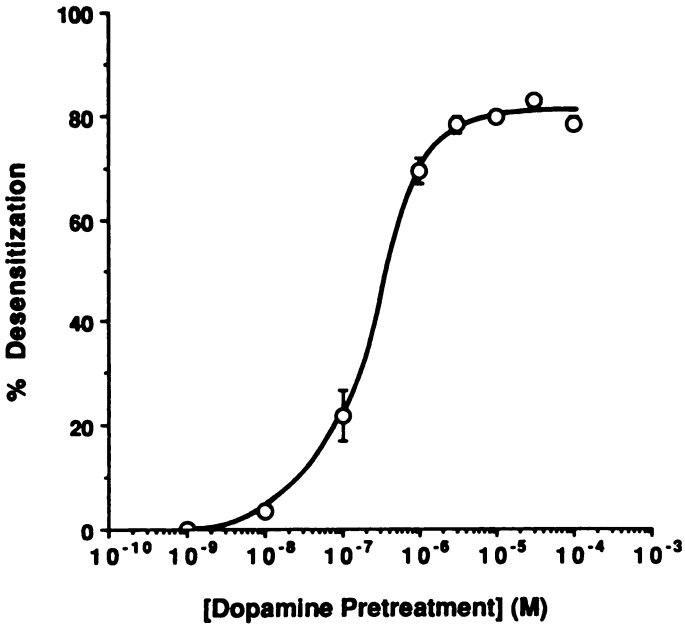


**Fig. 5.** Dose response for dopamine-induced desensitization of $D_1$ receptor-coupled adenylyl cyclase activity in NS20Y cell membranes. NS20Y cells were pretreated with 200 μM sodium metabisulfite in Earle's balanced salt solution (control) or the indicated concentrations of dopamine in 200 μM sodium metabisulfite in Earle's balanced salt solution for 1 hr at 37°. The adenylyl cyclase response to 100 μM dopamine was subsequently tested in membranes prepared from each treatment group. The data are presented as described in the legend to Fig. 4 and are representative of two independent experiments.

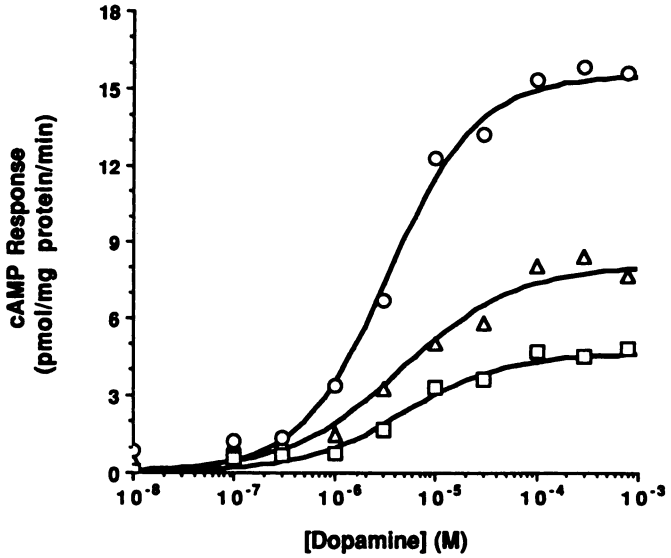


**Fig. 6.** Dopamine dose response for adenylyl cyclase stimulation in control and desensitized NS20Y cell membranes. NS20Y cells were preincubated in the absence (○) or presence of 100 μM dopamine for 5 min (△) or 20 min (□) at 37°, as described in the legends to Figs. 3–5. Membranes prepared from each group were subsequently assayed for adenylyl cyclase activity by measurement of cAMP production in response to the indicated concentrations of dopamine. Preincubation with 100 μM dopamine for 5 and 20 min results in a 50% and 70% decrease in the maximal cAMP response, respectively, but little alteration in the potency of dopamine ($EC_{50}$ for control, 3.6 μM; 5-min preincubation, 4.8 μM; 20-min preincubation, 4.9 μM). The data shown are representative of two independent experiments.

HCl (pH 7.5), and the retained radioactivity was quantitated by liquid scintillation counting in 5 ml of Hydrofluor liquid scintillation cocktail (National Diagnostics; Palmetto, FL), at an efficiency of 47%. All assays were performed in triplicate. At 0.5 nM [$^3$H]SCH-23390, typical binding values were 500 total cpm bound and 150 cpm nonspecifically bound.

**Protein determination.** Protein concentrations were determined using the bicinchoninic acid protein reagent (Pierce; Rockford, IL), as described (19).

**Data analysis.** Radioligand binding data were analyzed with the program LIGAND (20), which performs weighted nonlinear least squares curve fitting to the general model of Feldman (21) involving the interaction of several ligands with several independent classes of sites according to the law of mass action. Deviations of the observed points from the predicted values were weighted according to the reciprocal of the predicted variance. Competition curves were analyzed using models for competition of radioligand and competitor to one or two independent sites. Results from fits using a two-site model were retained only when the two-site model fit the data significantly better than a one-site model, as determined by the partial $F$ test at a significance level of $p < 0.05$. Experiments for which representative data are shown were replicated two or three times, with the results varying by <15% (SE).

## Results

We initially determined that the $D_1$ receptors on the NS20Y cells, which we had previously identified using radioligand binding methods, were functionally coupled to the stimulation of adenylyl cyclase activity. Fig. 1 and Table 1 show the effect of various dopaminergic and nondopaminergic agonists on adenylyl cyclase activity in NS20Y cell membranes. Dopamine and other agonists with efficacy at $D_1$ receptors stimulate NS20Y cell adenylyl cyclase in a dose-dependent fashion (Fig.

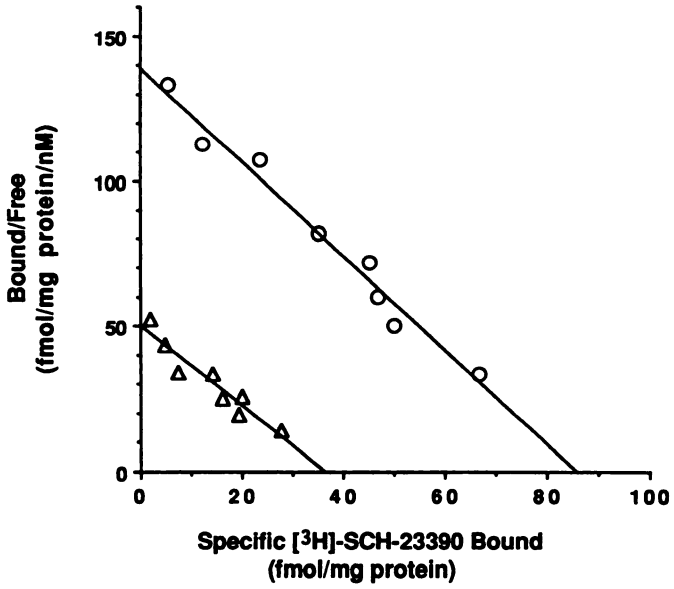


**Fig. 7.** Scatchard analysis of [$^3$H]SCH-23390 saturation binding isotherms in control and desensitized NS20Y cell membranes. Control (○) NS20Y cells were pretreated with Earle's balanced salt solution containing 200 μM sodium metabisulfite, whereas desensitized (△) NS20Y cells were pretreated with 100 μM dopamine in 200 μM sodium metabisulfite/Earle's balanced salt solution vehicle, for 1 hr at 37°. NS20Y cell membranes were prepared and $D_1$ receptor binding activity was assayed with 0.05 to 2 nM [$^3$H]SCH-23390, as described in Experimental Procedures. Scatchard analysis of the specific binding data reveals $K_D$ = 0.61 ± 0.01 nM and $B_{max}$ = 80.9 ± 6.9 fmol/mg of protein in control cell membranes, whereas $K_D$ = 0.70 ± 0.04 nM and $B_{max}$ = 36.5 ± 0.07 fmol/mg of protein for desensitized membranes.

1). The most potent agonists are the $D_1$-selective ligands (+)-SKF-38393 and (±)-SKF-82526 (Table 1), although neither of these drugs is as efficacious as dopamine in stimulating enzyme activity. (−)-NPA and (−)-apomorphine are also relatively potent (Table 1), although these drugs also exhibit partial agonist activity (Fig. 1). In contrast, dopamine and epinine (Fig. 1), as well as 6,7-ADTN, are full agonists at the $D_1$ receptor-stimulated adenylyl cyclase. Neither the $D_2$-selective agonist quinpirole, and $\beta$-adrenergic agonist isoproterenol, nor serotonin had any effect on NS20Y cell adenylyl cyclase, as indicated in Table 1.

Fig. 2 illustrates the dose-dependent inhibition of dopamine-stimulated adenylyl cyclase activity by the stereoisomers of SCH-23390 and butaclamol, $D_1$-selective and nonselective dopaminergic antagonists, respectively. As expected for a $D_1$ receptor, the (+)-isomers of both antagonists potently inhibit the dopamine-stimulated enzyme activity, whereas the (−)-isomers are relatively impotent. Table 1 indicates that (+)-SCH-23390 exhibits an approximate 370-fold stereoselectivity, whereas (+)-butaclamol is more than 10,000-fold more potent than its (−)-isomer. Overall, our data on the agonist potencies and efficacies, as well as the antagonist affinities, are in excellent agreement with previous pharmacological characterizations of $D_1$ receptor-coupled adenylyl cyclase in other tissues (22, 23). This observation, along with our previous radioligand binding data (16), provides convincing evidence for the presence of functional $D_1$ receptors on the NS20Y cells.

Because the initial goal was to evaluate the NS20Y cell line as a model system for investigating regulatory mechanisms associated with the $D_1$ receptor, the effect of dopamine preincubation on adenylyl cyclase activity was examined. Fig. 3 shows the results of such an experiment, where the cells were pretreated for 1 hr either with dopamine or with $PGE_2$ or adenosine, hormone agonists that activate adenylyl cyclase via their own unique receptor systems in these cells. None of the agonist pretreatments had any effect on basal adenylyl cyclase activity (data not shown) or that stimulated by forskolin (Fig. 3), a nonhormonal activator of adenylyl cyclase that directly activates the catalytic unit of the enzyme (24). In contrast, the dopamine-stimulated adenylyl cyclase activity was decreased by about 80% in response to dopamine pretreatment but was not affected by pretreatment with either $PGE_2$ or adenosine (Fig. 3). Similarly, the $PGE_2$- and adenosine-stimulated enzyme activities were each diminished by about 50% after $PGE_2$ and adenosine pretreatment, respectively, but were not affected by treatment with other agonists (Fig. 3). The adenylyl cyclase desensitization observed in the NS20Y cells, thus, appears to be "homologous" in nature, in that only the receptor system that was activated during the pretreatment shows a diminished response when subsequently examined.

In order to investigate the desensitization of dopamine-stimulated adenylyl cyclase further, the effect of pretreatment of the NS20Y cells with a number of additional agonists was examined. Fig. 4 shows that pretreatment with 6,7-ADTN, epinine, or (±)-SKF-82526 largely mimics that with dopamine, in terms of inducing desensitization of dopamine-stimulated adenylyl cyclase activity. Pretreatment with (−)-NPA, (+)-SKF-38393, or (−)-apomorphine, however, results in only a partial desensitization, relative to that observed with dopamine (Fig. 4). In contrast, preincubation with quinpirole, isoproterenol, or serotonin results in little to no desensitization of dopamine-stimulated enzyme activity (Fig. 4). This pharmacological profile is consistent with a $D_1$ receptor-mediated response. Moreover, the extent of desensitization observed appears to correlate with agonist efficacy for activating $D_1$ receptor-stimulated adenylyl cyclase activity (Fig. 1 and Table 1).

Fig. 5 shows that the ability of dopamine to induce desensitization during pretreatment of the NS20Y cells is dose dependent. The dopamine $EC_{50}$ for inducing desensitization is about 0.2–0.3 $\mu$M, which, interestingly, is about 20-fold higher affinity than that for dopamine stimulation of adenylyl cyclase activity in control NS20Y cell membranes (Table 1). Both the potency and efficacy for dopamine stimulation of adenylyl cyclase activity after desensitization were also examined. Fig. 6 shows dopamine dose-response curves for stimulation of adenylyl cyclase activity in control NS20Y cell membranes, as well as in membranes from cells that were desensitized for 5 or 20 min with dopamine. In each case, the desensitization appears to primarily involve a decrease in the maximum adenylyl cyclase response to dopamine, rather than a change in the potency of dopamine (Fig. 6).

In order to directly evaluate the status of the $D_1$ receptor following dopamine-induced desensitization, we examined the receptor binding activity of the radiolabeled $D_1$-selective antagonist [$^3$H]SCH-23390. Fig. 7 shows a Scatchard plot of [$^3$H]SCH-23390 saturation binding isotherms obtained in membranes prepared from control and dopamine-desensitized cells. A marked decrease in the receptor binding activity of [$^3$H]SCH-

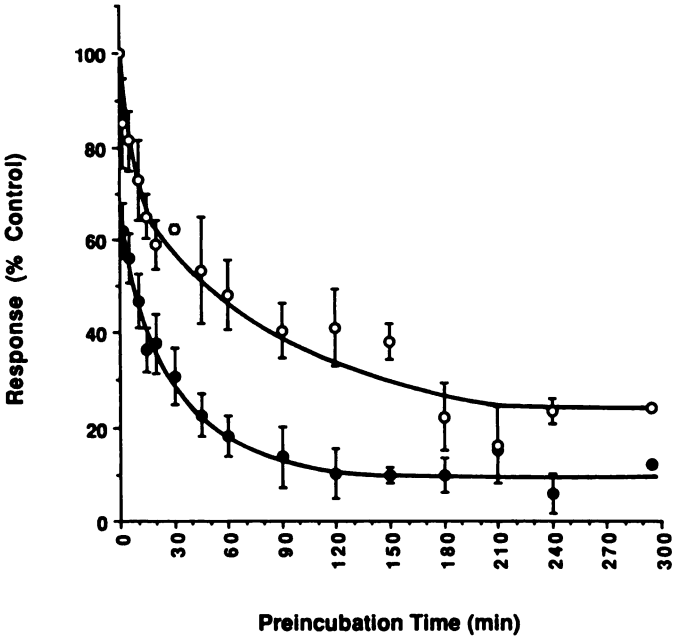


**Fig. 8.** Time-course for the effect of dopamine preincubation on $D_1$ receptor-coupled adenylyl cyclase activity and [$^3$H]SCH-23390 binding. NS20Y cells were preincubated with Earle's balanced salt solution containing 200 $\mu$M sodium metabisulfite (control) or with 100 $\mu$M dopamine in a 200 $\mu$M sodium metabisulfite/Earle's balanced salt solution vehicle for the indicated times. NS20Y cell membranes were then prepared and assayed for adenylyl cyclase activity in response to 100 $\mu$M dopamine (●), whereas specific $D_1$ receptor binding (○) was assayed using 0.5 nM [$^3$H]SCH-23390, as described in Experimental Procedures. The data are presented as a percentage of the control dopamine-stimulated cAMP response (10.9 ± 1.7 pmol/mg of protein/min) and control [$^3$H]SCH-23390 binding (40.6 ± 6.8 fmol/mg of protein) in the absence of dopamine preincubation. Values represent the means of standard errors from three independent experiments.

23390 appears to be associated with desensitization of the dopamine-stimulated adenylyl cyclase. Moreover, the decrease in [$^3$H]SCH-23390 binding is due to a reduction in the maximum receptor binding capacity ($B_{max}$) rather than a change in the receptor affinity ($K_D$) (Fig. 7). In order to further explore the relationship between the loss of receptor binding and desensitization of enzyme activity, we examined the time-course of each event in response to dopamine preincubation (Fig. 8). As early as 5 min after preincubation with dopamine, cAMP production is decreased by 45–50%, with maximal desensitization occurring by 90 min. The decrease in [$^3$H]SCH-23390 receptor binding occurs more slowly than the loss of enzyme activity, however, not achieving maximal levels until after 3 hr. Moreover, the total extent of receptor loss (65–70%) does not coincide with the maximal loss of dopamine-stimulated enzyme activity (85–90%) (Fig. 8).

Because the maximal and temporal decrease in $D_1$ receptor number does not correlate with the decrease in dopamine-stimulated enzyme activity, the desensitization may involve a functional uncoupling of the $D_1$ receptor, in addition to receptor down-regulation. This is further suggested by a loss of high affinity agonist binding observed in dopamine/[$^3$H]SCH-23390 competition experiments after desensitization. Fig. 9 shows dopamine/[$^3$H]SCH-23390 competition curves, in the absence and presence of Gpp(NH)p (a nonhydrolyzable analog of GTP), in membranes prepared from control (Fig. 9A) and desensitized (Fig. 9B) NS20Y cells. In the control membranes, the dopamine competition curve is heterogeneous (Hill coefficient <1) and best explained by assuming two agonist binding states of high and low affinity in the absence of Gpp(NH)p (Fig. 9A). In the presence of guanine nucleotides, however, the high affinity state is converted to low affinity and the dopamine competition curve is concomitantly shifted to the right and steepened (Hill coefficient = 1) (Fig. 9A). These data are identical to those previously demonstrated for $D_1$ receptors in brain (25, 26) and parathyroid gland (27, 28) membranes, as well as for many other catecholamine receptor systems (29), where it has been shown that the high affinity agonist binding state reflects functional receptor-G protein coupling. Interestingly, in membranes from cells that have been desensitized with dopamine, the competition curve for dopamine is of uniform low affinity and best described by a single low affinity receptor binding state (Fig. 9B). Moreover, the dopamine competition curve is minimally affected by guanine nucleotides in these membranes (Fig. 9B), indicating a relative lack of functional receptor-G protein coupling upon desensitization.

We were next interested in trying to determine the fate of the $D_1$ receptors that are apparently "lost" from the NS20Y plasma membrane fraction upon dopamine-induced desensitization. In initial experiments, both the microsomal membrane and cytoplasmic fractions obtained from high speed centrifugation (180,000 × $g$) of the supernatants derived from our crude plasma membrane preparations were assayed. Neither fraction, however, from control or desensitized cells exhibited any specific [$^3$H]SCH-23390 binding activity (data not shown). This observation suggests that the lost receptors may simply have been degraded or perhaps are still associated with the plasma membrane fraction in some "cryptic" non-ligand-binding state. In order to investigate these possibilities, resensitization experiments were performed both in the absence and in the presence of the protein synthesis inhibitor cycloheximide. Fig. 10A shows

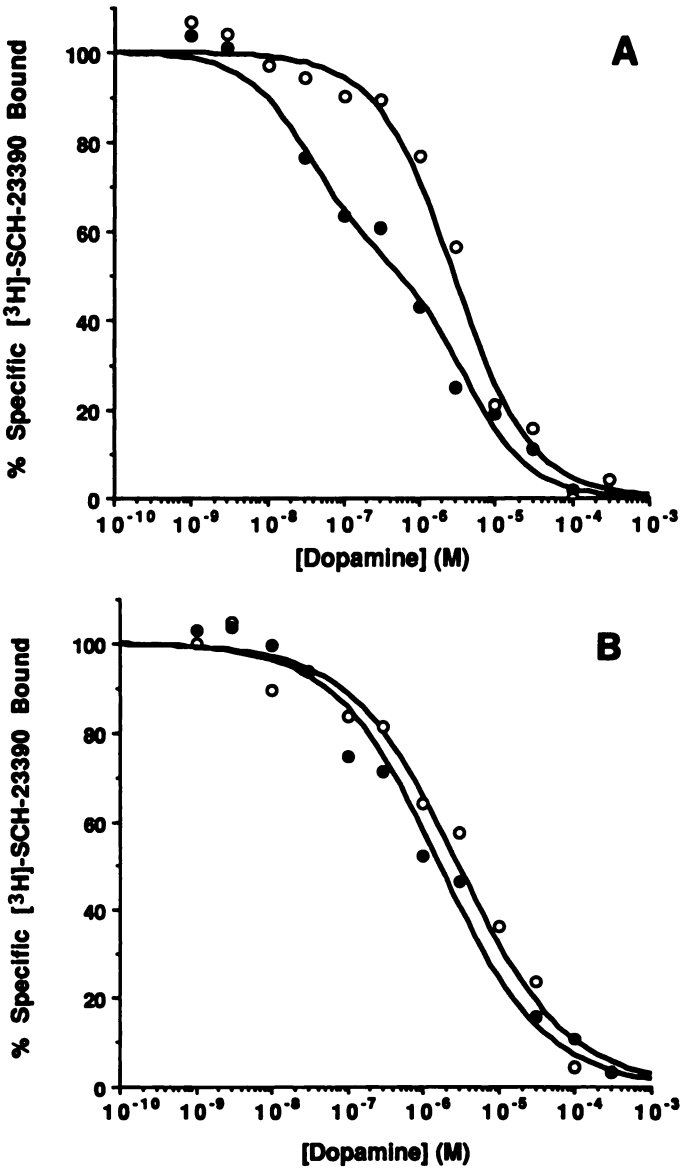


**Fig. 9.** Effect of Gpp(NH)p on dopamine competition for [$^3$H]SCH-23390 binding in membranes prepared from either control (A) or desensitized (B) NS20Y cells. NS20Y cells were preincubated with 200 μM sodium metabisulfite in Earle's balanced salt solution (control) or with 100 μM dopamine in a 200 μM sodium metabisulfite/Earle's balanced salt solution vehicle for 1 hr at 37°C. Membranes prepared from these cells were incubated with 0.5 nM [$^3$H]SCH-23390 and increasing concentrations of dopamine, in the absence (●) or presence (○) of 200 μM Gpp(NH)p, for 90 min at 22°, as described in Experimental Procedures. The data were computer analyzed using the curve-fitting program LIGAND, as described in Experimental Procedures. In the experiment shown (which is representative of two) the data points were experimentally determined; the *drawn lines* represent the computer-modeled best fit to the data. Only in control membranes, in the absence of Gpp(NH)p, did a two-site binding model fit the data significantly better than a one-site model. Average values from two control experiments revealed high and low affinity agonist dissociation constants ($K_H$ and $K_L$, respectively) and the proportion of high affinity sites (percentage of $R_H$) as follows (mean ± SD): $K_H$ = 3.05 ± 0.92 nM, $K_L$ = 2.36 ± 0.64 μM; and $R_H$ = 49.5 ± 0.7% in the absence of Gpp(NH)p, whereas in the presence of guanine nucleotides only the low affinity site was observed: $K_L$ = 1.51 ± 0.15 μM. In parallel experiments, desensitized NS20Y cell membranes demonstrated only a single low affinity agonist binding site in both the absence and the presence of Gpp(NH)p, with $K_L$ = 2.13 ± 0.70 μM and 2.65 ± 0.96 μM, respectively.

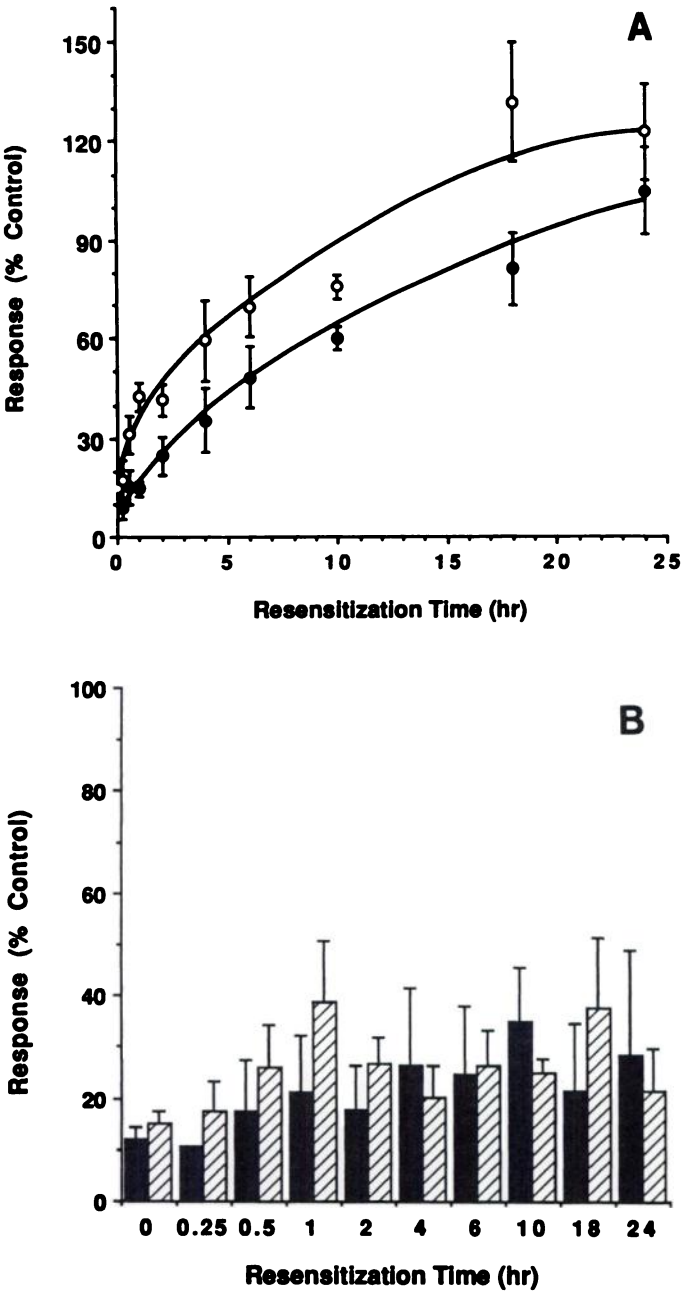


**Fig. 10.** Time course for resensitization of $D_1$ receptor-coupled adenylyl cyclase activity and [$^3$H]SCH-23390 binding after maximal desensitization/down-regulation. NS20Y cells were preincubated with 200 $\mu$M sodium metabisulfite in Earle's balanced salt solution (control) or 100 $\mu$M dopamine in 200 $\mu$M sodium metabisulfite/Earle's balanced salt solution at 37° for 3 hr. A, Following extensive washing with Earle's balanced salt solution, the treated cells were returned to Dulbecco's modified essential medium plus 10% fetal bovine serum for examination of recovery of adenylyl cyclase activity (●) and [$^3$H]SCH-23390 binding (○) at the indicated times. Zero time represents cells harvested immediately after the 3-hr preincubation. At each time point, NS20Y cell membranes were assayed for adenylyl cyclase activity in the presence of 100 $\mu$M dopamine and $D_1$ receptor binding using 0.5 nM [$^3$H]SCH-23390, as described in Experimental Procedures. B, The cells were treated as in A, except that after washing the dopamine-pretreated cells were returned to Dulbecco's modified essential medium plus 10% fetal bovine serum plus 5 $\mu$g/ml cycloheximide for examination of recovery of adenylyl cyclase activity (■) and [$^3$H]SCH-23390 binding (▨) at the indicated times. Under these conditions, cycloheximide did not affect cell viability or control receptor density during the experimental time period. The data are presented as a percentage of the control adenylyl cyclase and [$^3$H]SCH-23390 binding values, as described in Fig. 8. Values represent the means ± standard errors from three independent experiments.

that after a 3-hr pretreatment with 100 $\mu$M dopamine, which induces a maximal desensitization/down-regulation (Fig. 8), the $D_1$ receptor binding and adenylyl cyclase activities are both fully recovered by 24 hr. Interestingly, the receptor binding activity exhibits a more rapid rate of recovery, returning to control levels by about 18 hr (Fig. 10A). In contrast, Fig. 10B shows that inhibition of cellular protein synthesis with cycloheximide largely attenuates the $D_1$ receptor resensitization events after maximal desensitization/down-regulation. In this case, the $D_1$ receptor binding and adenylyl cyclase activities are still reduced to less than 40% of control values even after a 24-hr recovery period (Fig. 10B).

It was of interest to determine whether similar recovery phenomena would be observed if the cells were pretreated with dopamine for only 30 min (Fig. 8), resulting in partial desensitization/down-regulation. Fig. 11A shows that in this case the recovery rates are more rapid than those observed after maximal desensitization (Fig. 10A), with both the $D_1$ receptor binding and adenylyl cyclase activities returning to control levels by 2 hr. More importantly, both the rate and extent of recovery of the $D_1$ receptor binding and adenylyl cyclase activities now appear to be completely independent of new cellular protein synthesis, as determined in the presence of cycloheximide (Fig. 11B).

## Discussion

The present investigation illustrates the utility of the NS20Y cell line as a model system for investigating the mechanisms of agonist-induced stimulation and desensitization of $D_1$ receptor-coupled adenylyl cyclase activity. The $D_1$ receptor agonist potencies and efficacies for adenylyl cyclase stimulation and antagonist affinities for inhibition are in excellent agreement with those seen in other tissue systems (22, 23). A major advantage of the NS20Y cells, however, is their homogeneity and ease of manipulation for biochemical experimentation. The lack of significant $D_2$ receptor expression is another important feature of these cells (16). In most areas of the nervous system that express $D_1$ receptors, $D_2$ receptors are also present and act to inhibit $D_1$ receptor stimulation of adenylyl cyclase activity, thus complicating the analysis of $D_1$ receptor function and regulation. In addition, recent evidence has suggested the existence of a novel $D_1$ receptor subtype that is linked to phospholipase C activation, resulting in phosphatidylinositol turnover and $Ca^{2+}$ mobilization (5, 6). We have determined, however, that dopamine has no effect on phosphatidylinositol turnover or $Ca^{2+}$ mobilization in the NS20Y cells (data not shown), thus indicating expression of a single homogeneous $D_1$ receptor subtype.

The most significant finding of the present study is the demonstration that acute exposure of the NS20Y cells to dopamine results in a profound desensitization of the $D_1$ receptor-coupled adenylyl cyclase response. To our knowledge, this is the first characterization of dopamine receptor desensitization in any cultured cell system. The desensitization was found to be due to a reduction in the maximum responsiveness of the enzyme to dopamine, rather than a decrease in the potency of dopamine. Agonist occupancy and activation of the $D_1$ receptor were also shown to be directly linked to the desensitization

process, as demonstrated through several lines of evidence. For example, the agonist pharmacology for inducing desensitization (Fig. 4) was shown to be consistent with a $D_1$ receptor-mediated response. Also, the efficacies of various agonists for inducing desensitization were found to correlate with their efficacies for activating adenylyl cyclase activity (Fig. 4). This important observation suggests that receptor activation is directly coupled to desensitization. Finally, dopamine was shown to induce desensitization in a dose-dependent fashion (Fig. 5). It was of interest, however, that the potency of dopamine for inducing desensitization was about 20-fold higher than that for stimulating adenylyl cyclase activity. One possible explanation for this phenomenon is that the desensitization was performed in intact cells, whereas the adenylyl cyclase activity was measured in membrane preparations. This hypothesis seems unlikely, however, given that the dopamine $EC_{50}$ for stimulating cAMP accumulation in intact cells was determined to be identical to the $EC_{50}$ exhibited in adenylyl cyclase assays (data not shown). A similar phenomenon has been reported for agonist-induced desensitization of $\beta$-adrenergic receptor-coupled adenylyl cyclase in cultured cells (30). In this study, the potency of a $\beta$-adrenergic agonist for inducing desensitization was found to increase with prolonged times of preincubation. Whether a similar time dependency of agonist potency is observed for the $D_1$ receptor system will be the subject of future experimentation.

The agonist-induced desensitization of the $D_1$ receptor-coupled adenylyl cyclase system in NS20Y cells was observed to be homologous in nature. Other receptor systems that are linked to the stimulation of adenylyl cyclase have demonstrated both "homologous" and "heterologous" patterns of desensitization (31). The term homologous is used when the diminished response is observed only with the same receptor system that was activated during the agonist preincubation. Conversely, heterologous desensitization indicates that incubation with one agonist attenuates the response to multiple agonists operating through distinct receptors. In some instances, the pattern of unresponsiveness of adenylyl cyclase in heterologous desensitization may be so broad as to include decreased sensitivity to nonhormonal enzyme activators that bypass the receptors. In the case of the NS20Y cells, preincubation with dopamine resulted in attenuation of only the $D_1$ receptor-stimulated adenylyl cyclase response, without affecting the prostaglandin or adenosine receptor systems (Fig. 3). In addition, there was no effect on the nonhormonal activation of the enzyme by forskolin (Fig. 3) or fluoride ion (data not shown), further suggesting a homologous form of desensitization. These data also suggest that the "defect" associated with the desensitization is localized to the receptor protein and not to other distal components of the adenylyl cyclase system. Homologous but not heterologous desensitization of adenylyl cyclase is also usually associated with a translocation of the receptor from its normal position in the plasma membrane and a loss in its ligand-binding activity (31). This process was readily apparent in the NS20Y cells, as evidenced by a loss in $D_1$ receptor binding activity determined with the radiolabeled antagonist [$^3$H]SCH-23390. The data indicated that the loss was due to a decrease in the total number of assayable $D_1$ receptors, without a change in their affinity (Fig. 7). This loss of available receptors provides at least one mechanism for the diminished enzyme responsiveness to dopamine.

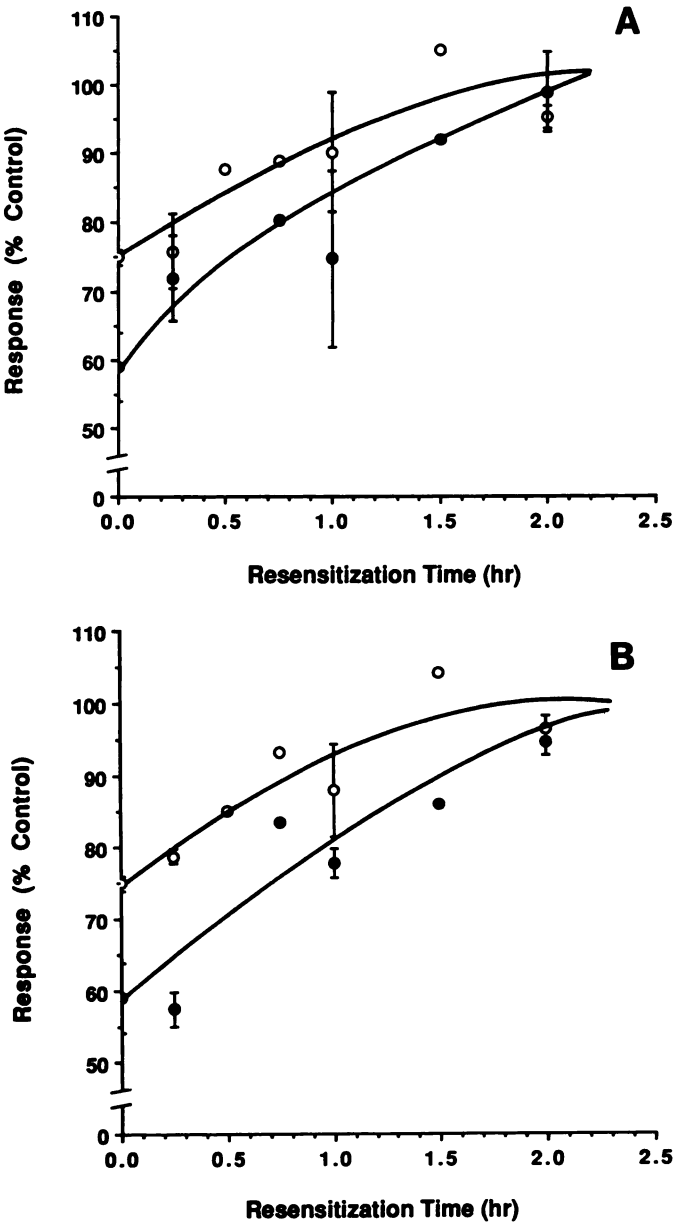


**Fig. 11.** Effect of protein synthesis inhibition on resensitization of $D_1$ receptor-coupled adenylyl cyclase activity and [$^3$H]SCH-23390 binding after partial desensitization/down-regulation. NS20Y cells were preincubated with 200 $\mu$M sodium metabisulfite in Earle's balanced salt solution (control) or 100 $\mu$M dopamine in 200 $\mu$M sodium metabisulfite/Earle's balanced salt solution at 37° for 30 min. A, Following extensive washing with Earle's balanced salt solution, the treated cells were returned to Dulbecco's modified essential medium plus 10% fetal bovine serum for examination of recovery of adenylyl cyclase activity (●) and [$^3$H]SCH-23390 binding (○) at the indicated times. Zero time represents cells harvested immediately after the 30-min preincubation. At each time point, NS20Y cell membranes were assayed for adenylyl cyclase activity in the presence of 100 $\mu$M dopamine and $D_1$ receptor binding using 0.5 nM [$^3$H] SCH-23390, as described in Experimental Procedures. B, The cells were treated as in A except that after washing the dopamine-pretreated cells were returned to Dulbecco's modified essential medium plus 10% fetal bovine serum plus 5 $\mu$g/ml cycloheximide for examination of recovery of adenylyl cyclase activity (●) and [$^3$H]SCH-23390 binding (○) at the indicated times. The data are presented as a percentage of the control adenylyl cyclase and [$^3$H]SCH-23390 binding values, as described in Fig. 8. Values represent the means ± standard errors from two independent experiments.

The loss of receptor binding activity, however, cannot completely explain the desensitization of the $D_1$ receptor-coupled adenylyl cyclase. A functional alteration or uncoupling of the receptor from the adenylyl cyclase system also seems to be involved, because the extent of enzyme desensitization was significantly greater than the observed decrease in receptor binding activity. Also, the desensitization did not appear to temporally correlate with the receptor down-regulation, as demonstrated in the time-courses for both onset of desensitization (Fig. 8) and recovery (Figs. 10 and 11). Finally, and perhaps most importantly, a loss of high affinity agonist binding was observed in dopamine/[$^3$H]SCH-23390 competition experiments after desensitization. In this experiment, the receptors that retained their ligand binding activity after desensitization were assayed for their ability to form an agonist-induced receptor-G protein complex, which serves as a functional intermediate in adenylyl cyclase activation (29). This complex is manifested as a high affinity, guanine nucleotide-sensitive agonist binding state in membrane preparations. Interestingly, a lack of detectable high affinity agonist binding was observed in the $D_1$ receptors remaining in the NS20Y cell membranes following dopamine-induced desensitization. All of these observations suggest that desensitization involves a functional alteration in the $D_1$ receptor protein that occurs independently of the regulation of its ligand binding activity.

The reduction in $D_1$ receptor binding activity that is associated with the desensitization response appears to occur through two different mechanisms. The first is readily reversible loss of ligand binding activity that is induced by a relatively short exposure to agonist. The second takes place upon longer agonist exposure and is a true down-regulation of the receptors, involving their degradation. Although not evident as an obvious biphasic down-regulation time-course, these two events can nonetheless be inferred from both the rate of the recovery process and its dependency on protein synthesis. Thus, after a short agonist exposure, which results in a partial loss of [$^3$H] SCH-23390 binding activity, the receptor recovery is rapid (within 2 hr) and not dependent on protein synthesis (Fig. 11). Conversely, after maximal down-regulation, the receptor binding activity does not fully recover until after 18 hr and requires protein synthesis (Fig. 10). The simplest interpretation of the latter event is that the receptors have been degraded and must be resynthesized for full recovery to occur. The former event, however, apparently involving a cryptic non-ligand-binding state of the receptor, remains more ill defined. One possibility is that the receptors have been translocated, sequestered, or oriented into a membrane environment such that their ligand-binding region is not readily exposed. Given that [$^3$H]SCH-23390 is a relatively hydrophilic antagonist ligand, there may, in this case, exist a permeability barrier that prevents normal ligand-receptor binding. A similar mechanism has been suggested for the sequestration process that is involved in $\beta$-adrenergic receptor desensitization (31–34). Future experiments will be directed at further investigating this desensitized state and cellular location of the $D_1$ receptor.

Based on our current data, we wish to propose the following scheme for agonist-induced desensitization of the $D_1$ receptor system in the NS20Y cells:

$$R_N \rightleftharpoons R_U \rightleftharpoons R_L \rightarrow R_D$$

In this scheme, the normal receptor ($R_N$) is rapidly converted upon agonist exposure to a functionally uncoupled form ($R_U$) before the loss in receptor-ligand binding activity ($R_L$). With further agonist exposure, the receptors undergo degradation ($R_D$) and require resynthesis for full recovery. We know from the desensitization and recovery time-course data that the formation of $R_U$ precedes that of $R_L$ and that both are readily reversible. The kinetics of the conversion of $R_L$ to $R_D$, however, remain poorly defined and require further characterization. At this point, the underlying biochemical mechanisms associated with the desensitization process can only be hypothesized. It is tempting to suggest that phosphorylation of the $D_1$ receptor protein may be involved, particularly with respect to the formation of the uncoupled state ($R_U$). Recent evidence has indicated that phosphorylation plays a role in the desensitization of many neurotransmitter and hormone receptor systems (35, 36). In particular, it has been shown that phosphorylation of the $\beta$-adrenergic receptor during homologous desensitization directly results in its functional uncoupling (37). This phosphorylation reaction is apparently mediated by a $\beta$-adrenergic receptor kinase (38). It will be of interest to determine whether a similar mechanism is operative for the $D_1$ receptor-coupled adenylyl cyclase system.

Finally, it should be noted that, in other receptor-linked adenylyl cyclase systems that have been examined, acute agonist exposure has usually demonstrated only a modest 40–60% desensitization of enzyme activity (31, 34, 39). The observation that dopaminergic agonists can induce a ≥90% desensitization of the $D_1$ receptor-coupled adenylyl cyclase system suggests that this process may be physiologically very significant. In fact, recent information concerning L-dihydroxyphenylalanine (L-DOPA) therapy in advanced Parkinson's disease has suggested that periodic dopaminergic agonist treatment can result in large oscillations in dopamine receptor responsiveness (12). Increased knowledge of the biochemical mechanisms of regulation for both $D_1$ and $D_2$ dopamine receptors will, hopefully, provide new insight into the role of dopaminergic function in a variety of neurological disorders.

**Acknowledgments**

We would like to thank Dr. Frederick J. Monsma, Jr., for helpful discussions and Dr. Marshall Nirenberg for generously providing the NS20Y cell line.

**References**

1. Creese, I., and C. M. Fraser, eds. *Receptor Biochemistry and Methodology: Dopamine Receptors*, Vol. 8. Alan R. Liss, Inc., New York (1987).
2. Freissmuth, M., P. J. Casey, and A. G. Gilman. G proteins control diverse pathways of transmembrane signaling. *FASEB J.* **3**:2125–2132 (1989).
3. Kebabian, J. W., and D. B. Caine. Multiple receptors for dopamine. *Nature (Lond.)* **277**:93–96 (1979).
4. Kebabian, J. W., T. Agui, J. C. van Oene, K. Shigematsu, and J. M. Saavedra. The $D_1$ dopamine receptor: new perspectives. *Trends Pharmacol. Sci.* **7**:96–99 (1986).
5. Felder, R. A., C. C. Felder, G. M. Eisner, and P. A. Jose. The dopamine receptor in adult and maturing kidney. *Am. J. Physiol.* **275**:F315–F327 (1989).
6. Mahan, L. C., R. M. Burch, F. J. Monsma, Jr., and D. R. Sibley. Expression of striatal $D_1$ dopamine receptors coupled to inositolphosphate production and $Ca^{2+}$ mobilization in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **87**:2196–2200 (1990).
7. Creese, I., D. R. Sibley, M. W. Hamblin, and S. E. Leff. The classification of dopamine receptors: relationship to radioligand binding. *Annu. Rev. Neurosci.* **6**:43–71 (1983).
8. Vallar, L., and J. Meldolesi. Mechanisms of signal transduction at the dopamine $D_2$ receptor. *Trends Pharmacol.* **10**:74–77 (1989).
9. Lacey, M. G., N. B. Mercuri, and R. A. North. Dopamine acts on $D_2$ receptors to increase potassium conductance in neurones of the rat substantia nigra zona compacta. *J. Physiol. (Lond.)* **392**:397–416 (1987).
10. Bigornia, L., M. Suozzo, K. A. Ryan, D. Napp, and A. S. Schneider. $D_2$ dopamine receptors modulate calcium channel currents and catecholamine

secretion in bovine adrenal chromaffin cells. *J. Neurochem.* **51**:999–1006 (1988).
11. Seeman, P. Dopamine receptors in human brain diseases, in *Receptor Biochemistry and Methodology: Dopamine Receptors* (I. Creese and C. M. Fraser, eds.), Vol. 8. Alan R. Liss, Inc., New York, 233–245 (1987).
12. Mouradian, M. M., J. L. Juncos, G. Fabbrini, J. Schlegel, J. J. Bartko, and T. N. Chase. Motor fluctuations in Parkinson's disease: central pathophysiological mechanisms. *Ann. Neurol.* **24**:372–378 (1988).
13. Creese, I., and D. R. Sibley. Receptor adaptations to centrally acting drugs. *Annu. Rev. Pharmacol. Toxicol.* **21**:357–391 (1981).
14. Memo, M., W. Lovenberg, and I. Hanbauer. Agonist-induced subsensitivity of adenylate cyclase coupled with a dopamine receptor in slices from rat striatum. *Proc. Natl. Acad. Sci. USA* **79**:4456–4460 (1982).
15. Roseboom, P., and M. E. Gnegy. Acute *in vivo* amphetamine produces a homologous desensitization of dopamine receptor-coupled adenylate cyclase activities and decreases agonist binding to the D1 site. *Mol. Pharmacol.* **35**:139–147 (1989).
16. Monsma, F. J., Jr., D. L. Brassard, and D. R. Sibley. Identification and characterization of $D_1$ and $D_2$ dopamine receptors in cultured neuroblastoma and retinoblastoma clonal cell lines. *Brain Res.* **492**:314–324 (1989).
17. Brown, B. L., J. D. M. Albano, R. P. Ekins, and A. M. Sghenzi. A simple and sensitive saturation assay method for the measurement of adenosine 3′:5′-cyclic monophosphate. *Biochem. J.* **121**:561–567 (1971).
18. Munemura, M., T. Agui, and D. R. Sibley. Chronic estrogen treatment promotes a functional uncoupling of the $D_1$ dopamine receptor in rat anterior pituitary gland. *Endocrinology* **124**:346–355 (1989).
19. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76–85 (1985).
20. Munson, P., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.* **107**:220–239 (1980).
21. Feldman, H. A. Mathematical theory of complex ligand-binding systems at equilibrium. *Anal. Biochem.* **48**:317–338 (1972).
22. Breese, G. R., and I. Creese, eds. *Advances in Experimental Medicine and Biology: Neurobiology of $D_1$-Dopamine Receptors*, Vol. 204. Plenum Publishing Corp., New York (1986).
23. Goldstein, M., K. Fuxe, and I. Tabachnick, eds. *Advances in Experimental Medicine and Biology: Central $D_1$ Dopamine Receptors*, Vol. 235. Plenum Publishing Corp., New York (1988).
24. Laurenza, A., E. Sutkowski, and K. B. Seamon. Forskolin: a specific stimulator of adenylyl cyclase or a diterpene with multiple sites of action. *Trends Pharmacol. Sci.* **10**:442–447 (1989).
25. Leff, S. E., M. W. Hamblin, and I. Creese. Interactions of dopamine agonists with brain $D_1$ receptors labeled by $^3$H-antagonists: evidence for the presence of high and low affinity agonist-binding states. *Mol. Pharmacol.* **27**:171–183 (1985).
26. Hess, E., G. Battaglia, A. B. Norman, L. Iorio, and I. Creese. Guanine nucleotide regulation of agonist interactions at [$^3$H]SCH-23390 labeled $D_1$ dopamine receptors in rat striatum. *Eur. J. Pharmacol.* **121**:31–38 (1986).
27. Monsma, F. J., Jr., and D. R. Sibley. Direct demonstration of $D_1$ dopamine receptors in bovine parathyroid gland membranes using the $D_1$ selective antagonist [$^{125}$I]-SCH 23982. *Synapse* **3**:193–199 (1989).
28. Niznik, H. B., E. L. Fogel, C. J. Chen, D. Congo, E. M. Brown, and P. Seeman. Dopamine $D_1$ receptors of the calf parathyroid gland: identification and characterization. *Mol. Pharmacol.* **34**:29–36 (1988).
29. Stadel, J. M., A. De Lean, and R. J. Lefkowitz. Molecular mechanisms of coupling in hormone receptor-adenylate cyclase systems. *Adv. Enzymol.* **53**:1–43 (1982).
30. Su, Y.-F., T. K. Harden, and J. P. Perkins. Catecholamine-specific desensitization of adenylate cyclase: evidence for a multistep process. *J. Biol. Chem.* **255**:7410–7419 (1980).
31. Sibley, D. R., and R. J. Lefkowitz. $\beta$-Adrenergic receptor-coupled adenylate cyclase: biochemical mechanisms of regulation. *Mol. Neurobiol.* **1**:121–154 (1987).
32. Strader, C. D., D. R. Sibley, and R. J. Lefkowitz. Association of sequestered *beta*-adrenergic receptors with the plasma membrane: a novel mechanism for receptor down-regulation. *Life Sci.* **35**:1601–1610 (1984).
33. Mahan, L. C., H. J. Motolsky, and P. A. Insel. Do agonists promote rapid internalization of $\beta$-adrenergic receptors? *Proc. Natl. Acad. Sci. USA* **82**:6566–6570 (1985).
34. Hertel, C., and J. P. Perkins. Receptor-specific mechanisms of desensitization of $\beta$-adrenergic receptor function. *Mol. Cell. Endocrinol.* **37**:245–256 (1984).
35. Sibley, D. R., J. L. Benovic, M. G. Caron, and R. J. Lefkowitz. Regulation of transmembrane signalling by receptor phosphorylation. *Cell* **48**:913–922 (1987).
36. Sibley, D. R., J. L. Benovic, M. G. Caron, and R. J. Lefkowitz. Phosphorylation of cell surface receptors: a mechanism for regulating signal transduction pathways. *Endocrine Rev.* **9**:121–157 (1988).
37. Sibley, D. R., R. H. Strasser, J. L. Benovic, K. Daniel, and R. J. Lefkowitz. Phosphorylation/dephosphorylation of the $\beta$-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution. *Proc. Natl. Acad. Sci. USA* **83**:9408–9412 (1986).
38. Benovic, J. L., A. DeBlasi, W. C. Stone, M. G. Caron, and R. J. Lefkowitz. $\beta$-Adrenergic receptor kinase: primary structure delineates a multigene family. *Science (Washington D. C.)* **246**:235–240 (1989).
39. Harden, T. K. Agonist-induced desensitization of the $\beta$-adrenergic receptor-linked adenylate cyclase. *Pharmacol. Rev.* **35**:5–32 (1983).
40. Cheng, Y.-C., and W. H. Prusoff. Relationship between the inhibition constant ($K_i$) and the concentration of inhibitor which causes 50 per cent inhibition ($I_{50}$) of an enzyme reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).

**Send reprint requests to:** Dr. David R. Sibley, Experimental Therapeutics Branch, Building 10, Room 5C-108, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892.