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Agonist-Induced Desensitization of D_2 Dopamine Receptors in Human Y-79 Retinoblastoma Cells

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SUMMARY

Y-79 human retinoblastoma cells can be induced to express significant quantities of functional D_2 dopamine receptors after attachment and differentiation with sodium butyrate. In membranes prepared from differentiated Y-79 cells, the D_2 dopaminergic antagonist [^{3}H]methylspiperone exhibits a K_D of 77 pM and a B_{max} of 60 fmol/mg of protein, whereas the antagonist [^{125}I]iodosulpride reveals a K_D of 0.77 nM and a B_{max} of 40 fmol/mg of protein. Dopamine also induces a pharmacologically specific, pertussis toxin-sensitive, dose-dependent inhibition of forskolin-stimulated adenylyl cyclase activity, with an EC_{50} of 2 μM and a maximal response at 100 μM (~50% enzyme inhibition). Pretreatment of the cells with dopamine results in a diminution in the subsequent ability of dopamine to inhibit adenylyl cyclase activity. This effect is time dependent, reaching maximal desensitization after ~24 hr. The dopamine dose-response curve for inducing desensitization exhibits an EC_{50} of ~2–3 μM and a maximal response at ~0.1–1 mM, similar to that for inhibiting adenylyl cyclase activity. After maximal desensitization, the EC_{50} for dopamine-induced inhibition of adenylyl cyclase activity is increased >20 fold (lower affinity) and the maximum inhibition is decreased to ~15%, representing an ~70% desensitization. The agonist-induced desensitization is pharmacologically specific, inasmuch as preincubation of the cells with the dopaminergic agonists epinine and (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene or the D_2-selective agonist N-0434 also results in desensitization of dopamine-induced inhibition of enzyme activity, whereas preincubation with the D_1-selective agonist SKF-38393 or with the nondopaminergic agonists isoproterenol and serotonin results in little or no desensitization. Preincubation of the cells with dopamine also promotes a time-dependent increase (~3-fold) in the K_D for [^{3}H]methylspiperone, with no change in its B_{max}. In contrast, after dopamine preincubation, the K_D for [^{125}I]iodosulpride is unchanged, whereas its B_{max} is reduced by ~50% upon maximum desensitization. In addition, agonist pretreatment promotes a functional uncoupling of the D_2 receptor, as suggested by a loss of high affinity agonist binding observed in radioligand competition binding assays after desensitization. Upon removal of agonist, the cellular D_2 receptor binding activity and functional response recover to control levels within a 24-hr period. These results suggest that prolonged exposure of cells to dopaminergic agonists initiates a desensitization process involving a functional uncoupling of the D_2 dopamine receptor as well as a loss of its ligand binding activity.

Dopamine receptors belong to a large superfamily of neurotransmitter receptors that are coupled to their specific effector functions via G proteins. Multiple criteria have been used to define two major subfamilies of dopamine receptors, which are referred to as D_1 and D_2 (1). D_1 receptors have been described as being coupled to the stimulation of adenylyl cyclase activity (2), although recent evidence has suggested that some D_1 receptors may activate phospholipase C (3, 4). In contrast, D_2 receptors have been suggested to be linked to various cellular responses (5), including inhibition of adenylyl cyclase activity, inhibition of phosphatidylinositol turnover, increase of K^+ channel activity, and inhibition of Ca^{2+} mobilization. Both dopamine receptor subtypes are predominantly found in the central nervous system, where they are critical for the regulation of cognitive function and motor control.

Defects in dopamine receptor function have been implicated in various neurological and endocrine disorders, including schizophrenia, Parkinson's disease, Tourette's syndrome, tardive dyskinesia, Huntington's chorea, and hyperprolactinemia (6). In many cases, these disorders and/or their therapy have been suggested to involve altered regulatory mechanisms of the dopaminergic receptor systems (7). Numerous *in vivo* investigations have, in fact, documented that dopamine receptors are subject to dynamic regulation in both a positive and a negative fashion (8). These regulatory phenomena have not been investigated in detail, however, and the underlying biochemical mechanisms are only now beginning to be addressed. This is primarily due to the inherent limitations of *in vivo* experimen-

ABBREVIATIONS: G protein, guanine nucleotide-binding regulatory protein; FBS, fetal bovine serum; MEM, Eagle's minimum essential medium; dY-79, differentiated Y-79; 6,7-ADTN, (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; Gpp(NH)p, guanylyl-5′-imidodiphosphate; EBSS, Earle's balanced salt solution.

tation and lack of cellular model systems for *in vitro* investigations of dopamine receptor function and regulation.

Recently, we reported that Y-79 retinoblastoma cells express D_2 dopamine receptor binding sites, as assessed using radioligand binding techniques, when they have been induced to differentiate by using dibutyryl-cAMP (9). 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_2 dopamine receptor. In addition, the presence of high affinity, guanine nucleotide-sensitive, agonist binding suggested that these receptors were capable of functional G protein coupling. Preliminary experiments also suggested that the Y-79 cell D_2 receptors were linked to the inhibition of adenylyl cyclase activity. In the present investigation, we demonstrate that the D_2 receptors expressed by Y-79 cells are indeed functionally coupled to the inhibition of adenylyl cyclase activity, with the appropriate pharmacology. More importantly, we find that treatment of the cells with dopamine or other dopaminergic agonists results in a profound desensitization of dopaminergic inhibition of adenylyl cyclase, as well as a down-regulation of the D_2 receptor ligand binding activity. Our data suggest that multiple biochemical mechanisms, including functional uncoupling and receptor sequestration, are involved in promoting these regulatory events.

Experimental Procedures

Materials. [^{3}H]Methylspiperone (60–80 Ci/mmol) was purchased from NEN/Dupont (Boston, MA). [^{3}H]cAMP (45 Ci/mmol) and [^{125}I] iodosulpride (2200 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). (+)-SKF-38393, (+)-butaclamol, (−)-butaclamol, (+)-apomorphine, (−)-apomorphine, ketanserin, phentolamine, spiperone, N-0434 [2-(*N*-phenethyl-*N*-propyl)amino-5-hydroxytetralin], 6,7-ADTN were purchased from Research Biochemicals, Inc. (Natick, MA). Dopamine and forskolin were purchased from Calbiochem (La Jolla, CA). Epinine, serotonin, (−)-isoproterenol, sodium butyrate, poly-D-lysine, fibronectin, 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). Pertussis toxin was purchased from List Biological Laboratories, Inc. (Campbell, CA). YM-09121-2 was a gift from Yamanouchi Pharmaceuticals (Japan). Cell culture media, reagents, and FBS were obtained from GIBCO (Grand Island, NY). All other chemicals were purchased from commercial suppliers and were of the highest quality available.

Cell culture. Y-79 cells were grown in suspension in MEM supplemented with 10% FBS, as previously described (9). Differentiation of Y-79 cells was carried out using a procedure similar to that described by us previously but modified as follows. Y-79 cells grown in MEM plus 10% FBS were collected by centrifugation, quantitated, triturated to produce a single-cell suspension, and plated at 5650 cells/cm^2 on 150- × 20-mm culture plates treated as follows for attachment: plates were incubated with 4 ml of poly-D-lysine (100 μg/ml) for 6 min at 37°, rinsed with 10 ml of serum-free MEM, and then treated with 4 ml of fibronectin (5 μg/ml) for 20 min at 37°. After removal of the fibronectin solution, the single-cell suspension was plated in MEM plus 10% FBS. The attached cells were allowed to grow for 8 days, with medium changes every third day, and then further differentiated by the addition of 1 mM sodium butyrate to the culture medium. Sodium butyrate treatment was routinely carried out for 8–12 days before each experiment, with medium changes on every third day.

Membrane preparation. dY-79 cells were harvested on day 16–20 after plating by treatment with 1 mM EDTA in Ca^{2+}/Mg^{2+}-free EBSS, followed by agitation and collection by centrifugation at 300 × *g* for 10 min. The cells were washed twice with cold EBSS (complete) and once with appropriate buffer (either binding buffer or adenylyl cyclase buffer; see below) and were quantitated using trypan blue. Cells were resuspended in buffer, transferred to a Dounce homogenizer, and homogenized using 15 strokes with an A pestle. The resulting membranes were pelleted by centrifugation at 34,500 × *g* for 10 min and resuspended in the appropriate assay volume using a motor-driven Teflon pestle. Before homogenization, in some experiments, the suspension of washed cells was split into two fractions. Thus, the same plate of dY-79 cells was used to generate cell membranes for radioligand binding analysis and cell lysates for adenylyl cyclase activity determinations.

Radioligand binding assays. One hundred microliters of dY-79 cell membranes, suspended in binding buffer (50 mM Tris·HCl, pH 7.4 at 22°, 1 mM EDTA, 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, 120 mM NaCl) at 1.5 mg of protein/ml (~0.5–1 × 10^6 original cells/ml), were added to a final volume of 1 ml of binding buffer containing 0.05–2 nM [^{3}H]methylspiperone for saturation analysis or 0.5 nM [^{3}H]methylspiperone for competition assays or single-point binding determinations. Alternatively, 25 μl of dY-79 cell membranes were added to a final volume of 100 μl of binding buffer containing 0.05–2 nM [^{125}I]iodosulpride for saturation analysis or 1 nM [^{125}I]iodosulpride for competition assays or single-point binding determinations. 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. Nonspecific binding was determined in the presence of 1 μM (+)-butaclamol. Incubations were carried out at room temperature for 1 hr for antagonist binding or 1.5 hr for agonist binding and were terminated by rapid filtration, under vacuum, through Whatman GF/C filters that were pretreated with 0.3% polyethyleneimine. The filters were washed with 5 × 4 ml of ice-cold 50 mM Tris·HCl (pH 7.4), and the retained ^{3}H radioactivity was quantitated by liquid scintillation counting in 5 ml of Aquasol (National Diagnostics) at a counting efficiency of 47%, whereas ^{125}I was quantitated in a γ counter at 75% efficiency.

Determination of cAMP production. Y-79 cells were washed as described above and then lysed by suspension and vortexing in 5 mM Tris·HCl (pH 7.4 at 4°), 5 mM $MgCl_2$, for 20 min at 4°. Fifty microliters of dY-79 cell lysate (50 μg of protein), suspended in adenylyl cyclase buffer (250 mM sucrose, 75 mM Tris·HCl, 12.5 mM $MgCl_2$, 1.5 mM EDTA, 1 mM dithiothreitol, 200 μM sodium metabisulfite) and supplemented with 2.75 mM phosphoenolpyruvate, 53 μM GTP, 0.12 mM ATP, 1.0 unit of myokinase, 0.2 unit of pyruvate kinase, and 100 μM RO-20-1724 (a phosphodiesterase inhibitor), were added to tubes containing either 50 μM forskolin or other appropriate test compounds, in a final volume of 0.06 ml on ice. The lysates were incubated for 5 min at 37° to generate cAMP, and the reaction was stopped by a 3-min incubation in boiling H_2O. The cAMP generated was assayed by the method of Brown *et al.* (10), 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 (11). After incubation with the cAMP-binding protein, free [^{3}H]cAMP was removed by treatment with charcoal/bovine serum albumin solution, and the bound [^{3}H]cAMP remaining in the supernatant was quantitated by liquid scintillation counting. The cAMP concentrations produced in the assay were determined by comparison with a standard curve, which was linear in the range of 1–30 pmol of cAMP/assay tube. Protein concentrations were determined using the bicinchoninic acid protein reagent (Pierce, Rockford, IL), as described (12). Average basal and forskolin-stimulated adenylyl cyclase activities were 119 ± 5.7 and 302 ± 19 pmol of cAMP/min/mg, respectively.

Data analysis. Radioligand binding data were analyzed with the program LIGAND (13), which performs weighted nonlinear least squares curve-fitting to the general model of Feldman (14) 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 recip-

rocal of the predicted variance. Competition curves were analyzed using models for competition of radioligand and competitor at 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 <20% (standard error).

Results

We have previously shown that differentiation of Y-79 cells by attachment and treatment with dibutyryl-cAMP in serum-free media induces the expression of D_2 receptors by about 10-fold (9). One problem associated with this differentiation protocol is the rather limited time that these cells can be maintained in culture (9). We have, thus, investigated another differentiation procedure, which involves attaching the cells and treating them with sodium butyrate in serum-containing media (15). In preliminary experiments, we determined that, in membranes prepared from Y-79 cells differentiated in this fashion, the D_2 dopaminergic antagonist [^{3}H]methylspiperone exhibits a dissociation constant (K_D) of 77 pM and a maximum binding capacity (B_{max}) of 60 fmol/mg of protein, whereas the radioiodinated antagonist [^{125}I]iodosulpride reveals a K_D of 0.77 nM and a B_{max} of 40 fmol/mg of protein (data not shown). This level of receptor expression is comparable to that observed previously (9); however, using this protocol, the Y-79 cells can be maintained in culture for ≥3 weeks after plating.

In order to determine whether the D_2 receptors expressed in the Y-79 cells after this new differentiation protocol are indeed functional, we examined their ability to mediate inhibition of adenylyl cyclase activity. Fig. 1 and Table 1 show the effect of various dopaminergic and nondopaminergic agonists on the inhibition of forskolin-stimulated adenylyl cyclase activity in dY-79 cell lysates. Dopamine and other agonists with efficacy at D_2 receptors inhibit the forskolin-stimulated enzyme activity in a dose-dependent fashion (Fig. 1). The most potent agonists include (−)-apomorphine, N-0434, 6,7-ADTN, and epinine (Table 1). The efficacy of the D_2-selective agonist N-0434, however, is consistently observed to be about 20% lower than that of the other D_2 agonists (Fig. 1). (+)-Apomorphine was also found to weakly inhibit adenylyl cyclase activity, although its potency is about 500-fold less than that observed with the more active (−)-isomer (Table 1). In contrast, serotonin, the β-adrenergic agonist isoproterenol, and the D_1-selective agonist SKF-38393 are all relatively ineffective in terms of inhibiting adenylyl cyclase activity (Table 1).

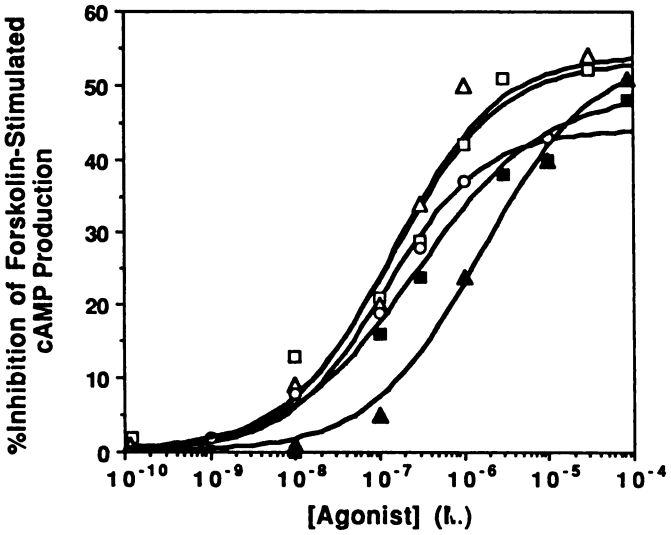

Fig. 1. Dose-response curves for dopaminergic agonist-induced inhibition of forskolin-stimulated cAMP production in dY-79 cells. Lysates were prepared from dY-79 cells and incubated with 50 μM forskolin and increasing concentrations of dopamine (▲), epinine (□), 6,7-ADTN (△), (−)-apomorphine (■), or N-0434 (○) for 5 min at 37°, as described in Experimental Procedures. The data are expressed as percentage of inhibition of the maximal forskolin-stimulated cAMP production observed in the absence of agonist. The curves shown are representative of two or three independent experiments for each agonist. Average EC_{50} values are presented in Table 1.

TABLE 1

Pharmacology of D_2 receptor-mediated inhibition of adenylyl cyclase activity in dY-79 cell lysates

Data presented are EC_{50} values for agonist-induced inhibition of forskolin-stimulated adenylyl cyclase activity and K_i values for antagonist blockade in dY-79 cell lysates. Experiments were performed as described in Figs. 1 and 2. EC_{50} values for agonists were graphically determined, whereas antagonist inhibition constants (K_i) were determined by Cheng and Prusoff (20) 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} = 1.95 μM. Values represent the means from two or three independent experiments (standard error values were ≤20% of the means).

Agonist	EC_{50}	Antagonist	K_i
	μM		nM
(−)-Apomorphine	0.11	YM-09121-2	0.06
N-0434	0.18	Spiperone	0.07
6,7-ADTN	0.20	(+)-Butaclamol	0.67
Epinine	0.22	(−)-Butaclamol	>10,000
Dopamine	1.95	Ketanserin	>100,000
(+)-Apomorphine	56.6	Phentolamine	>100,000
Serotonin	94.9		
Isoproterenol	>1,000		
(+)-SKF-38393	>1,000		

Fig. 2 and Table 1 demonstrate the dose-dependent blockade by antagonists of the dopamine-induced inhibition of adenylyl cyclase activity in the dY-79 cells. The most potent agents are the D_2-selective antagonists YM-09121-2 and spiperone. (+)-Butaclamol is also relatively potent and is over 10,000-fold more potent than its (−)-isomer, as expected for a D_2 dopamine receptor (Table 1). In contrast, the serotonin antagonist ketanserin and the α-adrenergic antagonist phentolamine are relatively ineffective (Table 1). These data thus indicate that the D_2 receptors expressed in the dY-79 cells are functionally coupled to the inhibition of adenylyl cyclase activity, in a pharmacologically specific fashion.

We were also interested in examining whether the dopaminergic inhibition of adenylyl cyclase is sensitive to pertussis toxin treatment. Fig. 3 shows that pretreatment of the dY-79 cells with pertussis toxin results in a dose-dependent inhibition of the subsequently tested dopamine response. Treatment of cells with 200 ng/ml pertussis toxin for 24 hr was found to completely abolish the ability of dopamine to inhibit adenylyl cyclase activity (Fig. 3). This observation suggests that the D_2 receptor-induced inhibition of adenylyl cyclase is mediated by a pertussis toxin-sensitive G protein, presumably one of the G_i proteins (16). Overall, the data in Figs. 1–3 and Table 1 provide convincing evidence for the expression of functional, G protein-linked, D_2 dopamine receptors on the dY-79 cells and, most importantly, document the efficacy of our new cellular differentiation procedure.

Because our initial interest in this study was to evaluate the

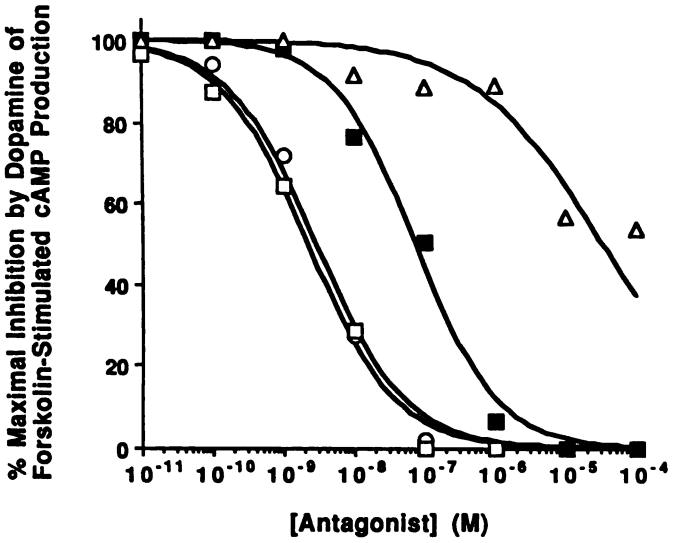

Fig. 2. Pharmacology of antagonist blockade of dopamine-induced inhibition of cAMP production in dY-79 cells. Lysates prepared from dY-79 cells were incubated with 50 μM forskolin, with or without 100 μM dopamine, and increasing concentrations of YM-09151-2 (□), spiperone (○), (+)-butaclamol (■), or (−)-butaclamol (△) for 5 min at 37°, as described in Fig. 1. The data are expressed as a percentage of the maximal enzyme inhibition by dopamine observed in the absence of antagonist. The curves shown are representative of two or three independent experiments for each antagonist. Average K_i values are presented in Table 1.

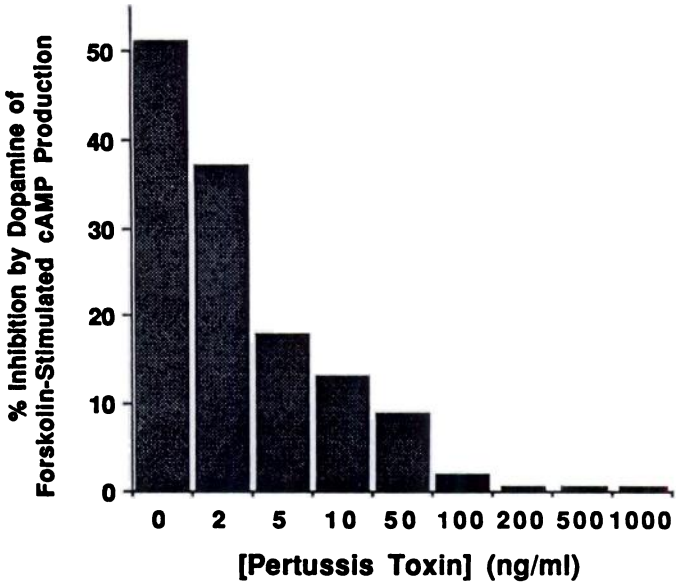

Fig. 3. Prevention of dopaminergic inhibition of adenylyl cyclase activity in dY-79 cells by pertussis toxin treatment. Y-79 cells were cultured in the presence of the indicated concentrations of pertussis toxin for 24 hr. After this exposure, the cells were harvested and washed extensively, and dopaminergic inhibition of adenylyl cyclase activity was examined using 100 μM dopamine. The data are expressed as a percentage of inhibition of the forskolin-stimulated enzyme activity. Results shown are those from a single experiment, which was performed twice with similar results.

dY-79 cells as a model system for investigating regulatory mechanisms associated with the D_2 receptor, the effect of dopamine preincubation on the dY-79 cells was examined. Fig. 4A demonstrates that pretreatment of the cells with dopamine results in a diminution in the subsequent ability of dopamine

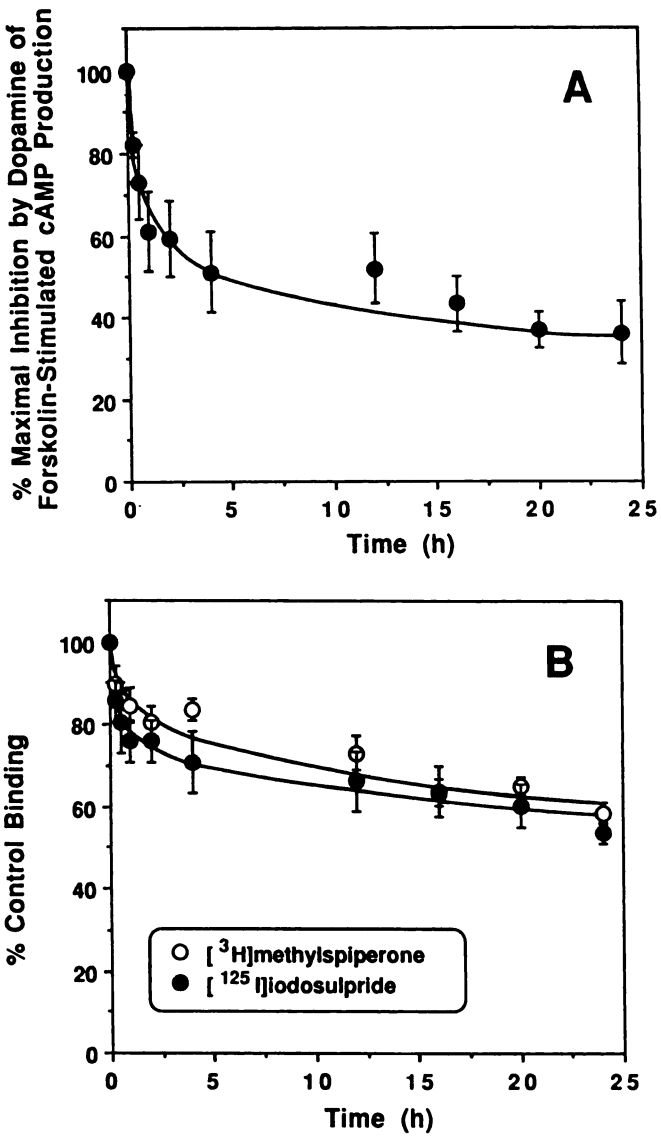

Fig. 4. Time course for the desensitizing effect of dopamine preincubation on D_2 receptor-mediated inhibition of cAMP production and [^{3}H]methylspiperone and [^{125}I]iodosulpride binding. Y-79 cells were cultured in the presence of 200 μM sodium metabisulfite (control) or 500 μM dopamine plus 200 μM sodium metabisulfite, for the indicated times. After extensive washing of the cells with EBSS, both cell lysates and membranes were prepared from each pretreatment group and assayed as described in Experimental Procedures. A, dY-79 cell lysates were incubated with 50 μM forskolin, in the presence or absence of 100 μM dopamine, for 5 min at 37° to generate cAMP. In control cells, 100 μM dopamine produced a maximal 45–55% inhibition of cAMP production, and the data are presented as a percentage of this inhibition. B, dY-79 cell membranes were assayed for D_2 receptor radioligand binding activity using 0.5 nM [^{3}H]methylspiperone or 1.0 nM [^{125}I]iodosulpride. The data are presented as a percentage of the control [^{3}H]methylspiperone (31.7 ± 5.4 fmol/mg of protein) or [^{125}I]iodosulpride (17.8 ± 1.9 fmol/mg of protein) receptor binding activities. Values shown are means ± standard errors of three independent experiments.

to inhibit adenylyl cyclase activity. This effect is time dependent, reaching maximal desensitization after about 20 hr, with a $t_{1/2}$ of about 1 hr. Fig. 4B shows that dopamine pretreatment also results in a time-dependent loss of the ligand binding activity of the D_2 receptor, as detected with the radiolabeled antagonists [^{3}H]methylspiperone and [^{125}I]iodosulpride. This decrease in radioligand binding exhibits a $t_{1/2}$ of about 4 hr,

with a maximal loss occurring after ~24 hr of dopamine treatment (Fig. 4B). The dopamine-induced decline in functional response (Fig. 4A) thus appears to occur somewhat more rapidly than the loss of receptor binding activity (Fig. 4B).

Fig. 5 shows that the ability of dopamine to induce desensitization (reduction in dopamine inhibition of adenylyl cyclase) and receptor down-regulation (loss of [^{125}I]iodosulpride binding) during pretreatment of the dY-79 cells is dose dependent. The dopamine EC_{50} for inducing both desensitization and down-regulation is about 2–3 μM, with a maximal response at 0.1–1 mM (Fig. 5). These dose-response parameters are remarkably similar to those for dopamine inhibition of adenylyl cyclase activity in control dY-79 cell lysates (Fig. 1 and Table 1).

Given the observation described above, it was also of interest to examine the dose-response relationship for dopamine inhibition of enzyme activity after the induction of desensitization. Fig. 6 shows dopamine dose-response curves for the inhibition of forskolin-stimulated adenylyl cyclase activity in control dY-79 cell lysates, as well as from cells that were desensitized for 4, 12, or 24 hr with dopamine. In each case, the desensitization appears to involve both a reduction in the maximum inhibition of the enzyme by dopamine and a decrease in the potency of dopamine. After maximal desensitization with dopamine for 24 hr, the EC_{50} for dopamine-induced inhibition of adenylyl cyclase activity is increased by 30-fold to lower potency and the maximum inhibition is decreased to about 15%, representing an ~70% desensitization of activity (Fig. 6). The reversal of inhibition seen at dopamine concentrations of >100 μM is presumably due to stimulation of the Y-79 cell β-adrenergic receptor, as we have previously characterized (9).

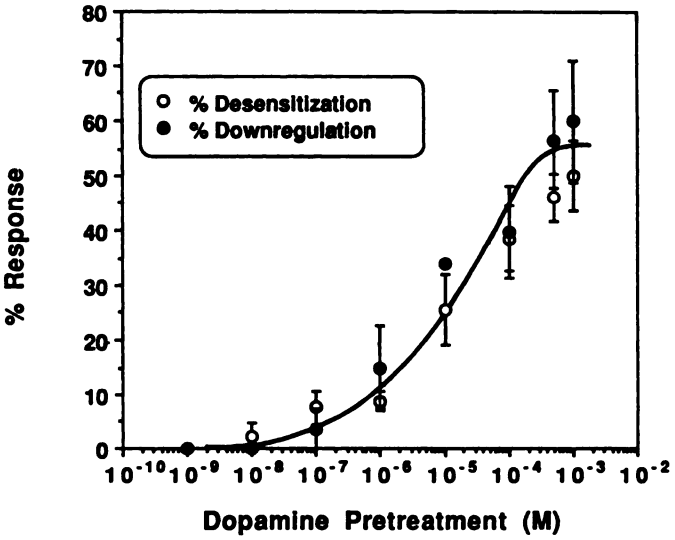

Fig. 5. Dose-response curve for dopamine-induced D_2 receptor desensitization and down-regulation in dY-79 cells. Y-79 cells were cultured in the presence of 200 μM sodium metabisulfite (control) or with 200 μM sodium metabisulfite plus increasing concentrations of dopamine, for 24 hr at 37°. After extensive washing of the cells with EBSS, both cell lysates and membranes were prepared from each pretreatment group and assayed as described in Experimental Procedures. D_2 receptor function (○) was evaluated by measuring cAMP production in response to 50 μM forskolin in the presence of 100 μM dopamine in lysates prepared from each treatment group. The data are expressed as the percentage of reduction in the maximal dopamine response observed relative to the control group (45–50% enzyme inhibition). D_2 receptor radioligand binding activity (●) in cell membranes prepared from the same pretreatment groups was evaluated using 1 nM [^{125}I]iodosulpride. The data are expressed as the percentage of reduction in receptor binding activity relative to control levels. Values shown are means ± standard errors of three independent experiments.

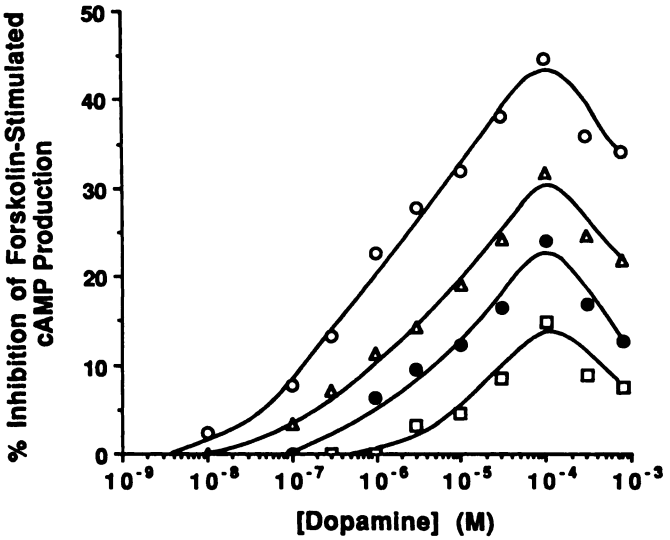

Fig. 6. Dopamine dose-response curves for adenylyl cyclase inhibition in control and desensitized dY-79 cell lysates. Y-79 cells were cultured in the presence of 200 μM sodium metabisulfite (control) (○) or with 200 μM sodium metabisulfite plus 500 μM dopamine for 4 (△), 12 (●), or 24 (□) hr, at 37°. After extensive washing of the cells with EBSS, cell lysates were prepared from each pretreatment group and assayed with increasing concentrations of dopamine, as described in Experimental Procedures. The data are presented as the average percentage of inhibition of the forskolin-stimulated cAMP production from three independent experiments. The EC_{50} values for the control and treatment groups are as follows: control, 1.25 ± 0.11 μM; 4 hr, 4.61 ± 0.74 μM; 12 hr, 7.48 ± 0.31 μM; and 24 hr, 39.1 ± 0.84 μM.

We next sought to examine in more detail the loss of radiolabeled antagonist binding observed upon dopamine-induced desensitization. Fig. 7 shows Scatchard plots of [^{3}H]methylspiperone and [^{125}I]iodosulpride saturation binding isotherms obtained in membranes prepared from control dY-79 cells, as well as from cells that had been pretreated for 4, 12, or 24 hr with dopamine. Fig. 7A shows that the predominant effect of dopamine treatment on [^{3}H]methylspiperone binding is a time-dependent reduction in the affinity (increase in K_D) for the receptor, without a change in the maximum receptor binding capacity (B_{max}). After maximal desensitization with dopamine for 24 hr, the [^{3}H]methylspiperone K_D is increased by about 5-fold. In contrast, Fig. 7B shows that the major effect of dopamine desensitization on [^{125}I]iodosulpride binding is a time-dependent reduction in the maximum binding capacity, without a significant change in receptor affinity. In this case, the B_{max} for [^{125}I]iodosulpride binding is reduced to about 40% of control after maximal dopamine-induced desensitization. The nature of the loss of antagonist binding upon desensitization thus appears to be ligand dependent and may be related to sequestration or internalization of the receptors (see Discussion).

To further characterize the agonist-induced desensitization of the D_2 receptor system, we investigated the pharmacology of this response. Fig. 8 shows the effect of pretreating the dY-79 cells with various dopaminergic and nondopaminergic agonists. The nonselective agonists epinine and 6,7-ADTN, as well as the D_2-selective agonist N-0434, were as effective as dopamine in desensitizing the D_2 receptor-mediated inhibition of adenylyl cyclase and promoting down-regulation of [^{125}I]iodosulpride binding. In contrast, preincubation with the D_1-selective ago-

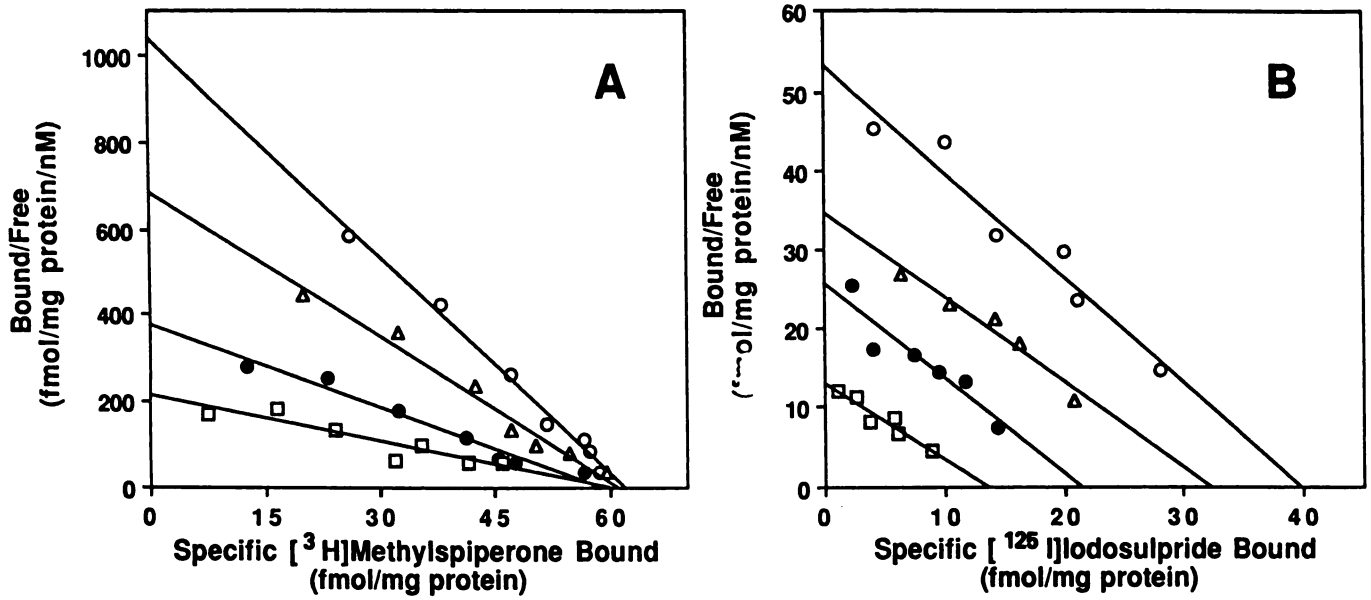

Fig. 7. Scatchard analysis of radiolabeled antagonist saturation binding isotherms in control and desensitized dY-79 cell membranes. Y-79 cells were cultured in the absence (○) or presence of 500 μM dopamine for 4 (△), 12 (●), or 24 (□) hr, at 37°, as described in Fig. 6. After extensive washing of the cells with EBSS, membranes were prepared from each pretreatment group and saturation radioligand binding assays were performed as described in Experimental Procedures. A, D_2 receptor binding activity was assayed with 0.05–2 nM [^{3}H]methylspiperone. The data shown are from a single representative experiment. Average values ± standard errors from three experiments indicated that the K_D increased from 76.7 ± 8.1 pM in control membranes to 106 ± 28.5, 182 ± 34.5, and 436 ± 72 pM after 4, 12, and 24 hr of dopamine preincubation, respectively, whereas the B_{max} (57 ± 9.3 fmol/mg of protein) was unchanged. B, The data from a single representative experiment using 0.05–2 nM [^{125}I]iodosulpride are shown. Average values ± standard errors from three experiments revealed no change in the K_D values, which were 0.77 ± 0.06, 0.87 ± 0.17, 0.84 ± 0.09, and 0.83 ± 0.04 nM for control and 4-, 12-, and 24-hr treatment groups, respectively. In contrast, the B_{max} was reduced from 43.2 ± 5 fmol/mg of protein in control to 30.3 ± 5.9, 22 ± 5.3, and 17.3 ± 5.5 fmol/mg after 4, 12, and 24 hr of dopamine preincubation, respectively.

nist SKF-38393, the β-adrenergic agonist isoproterenol, or serotonin resulted in little desensitization or down-regulation of the D_2 receptor system. This pharmacological profile is thus consistent with a D_2 receptor-mediated response.

It was also of interest to directly examine the effect of dopamine-induced desensitization on agonist binding to the D_2 receptor. Fig. 9 shows dopamine competition curves for [^{3}H]methylspiperone (Fig. 9A) and [^{125}I]iodosulpride (Fig. 9B) binding, in the absence and presence of Gpp(NH)p (a nonhydrolyzable analog of GTP), in membranes prepared from control and desensitized dY-79 cells. In the control membranes, the dopamine competition curves are 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. In the presence of guanine nucleotides, however, the high affinity state is converted to low affinity and the dopamine competition curves are concomitantly shifted to the right and steepened (Hill coefficient = 1). These results are identical for both radioligands (Fig. 9) and also to data previously reported for D_2 receptors in brain (17) and pituitary (18), as well as for other catecholamine receptor systems (19) where it has been shown that the high affinity agonist binding state reflects functional receptor-G protein coupling. Interestingly, in membranes from cells that were desensitized with dopamine, the competition curves for dopamine are shifted to the right and steepened, exhibiting lower affinity for receptor binding (Fig. 9). In the case of [^{3}H]methylspiperone binding, the desensitization appears to be associated with a reduction in affinity as well as quantity of the high affinity agonist binding state (Fig. 9A). In contrast, this high affinity state appears to be completely lost after desensitization, as detected using [^{125}I]iodosulpride binding (Fig. 9B). Moreover, the dopamine competition curves are only minimally affected by guanine nucleotides in membranes from the desensitized cells (Fig. 9), indicating a reduction of functional D_2 receptor-G protein coupling upon desensitization.

Finally, we characterized the ability of the D_2 receptor system to recover to control levels of activity after agonist-induced desensitization. Fig. 10 demonstrates the rate of resensitization of both the receptor binding activity and functional response subsequent to a maximal desensitization by dopamine. As can be seen, the [^{125}I]iodosulpride binding activity and the dopamine inhibition of adenylyl cyclase activity recover with similar rates, exhibiting a $t_{1/2}$ of about 4–8 hr (Fig. 10). Full recovery for both the [^{125}I]iodosulpride receptor binding activity and adenylyl cyclase response occurs within a 24-hr period (Fig. 10).

Discussion

The current investigation confirms and extends our previous results demonstrating the utility of the Y-79 cell as a model system for investigating the function and regulation of the human D_2 dopamine receptor system (9). We had previously shown that differentiation of Y-79 cells by attachment and treatment with dibutyryl-cAMP in serum-free media promotes the expression of functional D_2 receptors (9). One problem associated with this differentiation protocol, however, is the rather limited time that these cells can be maintained in culture. We have, thus, developed another differentiation procedure, which involves attaching the cells and treating them

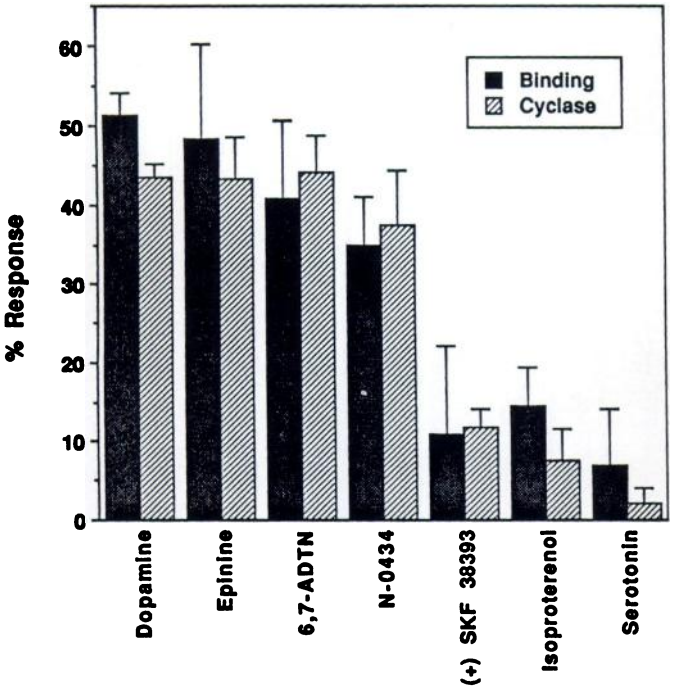

Fig. 8. Pharmacology of agonist-induced desensitization of the D_2 receptor in dY-79 cells. The cells were cultured in the absence (control) or presence of 100 μM concentrations of the indicated agonists (plus 200 μM sodium metabisulfite) for 24 hr at 37°. After extensive washing of the cells with EBSS, both cell lysates and membranes were prepared from each treatment group and assayed for maximal (100 μM) dopamine-induced inhibition of forskolin-stimulated cAMP production and [^{125}I] iodosulpride (1 nM) receptor binding activity. The cyclase data are expressed as the percentage of reduction in the maximal dopamine response observed relative to the control group (45–50% enzyme inhibition), whereas the binding data are expressed as the percentage of reduction in receptor binding activity relative to control levels. Values shown are means ± standard errors of three independent experiments.

with sodium butyrate in serum-containing media. This new protocol allows for a longer period of cell culture and was found to result in a comparable expression of D_2 receptors, as detected using radioligand binding techniques. In addition, we established that the D_2 receptor agonist potencies and efficacies for adenylyl cyclase inhibition (Fig. 1) and antagonist affinities for blocking the dopamine response (Fig. 2) are in excellent agreement with those seen in other tissues that express D_2 receptors (5, 21). Major advantages of the Y-79 cells, however, are their homogeneity, ability to be grown in large quantity, and ease of manipulation for biochemical experimentation.

The most significant finding of the present study, however, is the demonstration that exposure of the dY-79 cells to dopamine results in a profound desensitization of the D_2 receptor system linked to adenylyl cyclase inhibition. To our knowledge, this is the first characterization of D_2 receptor desensitization in any cultured cell system. This agonist-induced desensitization appears to be due to a reduction in both the maximum inhibition of adenylyl cyclase and the potency of dopamine for producing this response (Fig. 6). Several lines of evidence indicate that the induction of receptor desensitization is closely correlated with agonist occupancy. Firstly, dopamine was found to promote desensitization in a dose-dependent fashion (Fig. 5). Moreover, the EC_{50} for the dopamine-induced desensitization is similar to that for dopamine inhibition of adenylyl cyclase activity. Secondly, the agonist pharmacology for promoting desensitization corresponds to a D_2 dopaminergic response and closely matches that for inhibition of adenylyl cyclase activity (Fig. 8). Finally, recovery of the D_2 receptor system to control levels of activity is initiated immediately upon removal of agonist exposure (Fig. 10). All of these data suggest that desensitization of the D_2 receptor is tightly coupled to its activation by dopamine.

The loss of functional activity of the D_2 receptor appears to be associated with a reduction in ligand binding activity, as detected using radiolabeled antagonists. The desensitization of receptor response and the down-regulation of receptor binding exhibit the same dopamine dose dependency (Fig. 5), agonist pharmacology (Fig. 8), and time course for recovery (Fig. 10). The nature of the loss of antagonist binding upon desensitization, however, appears to be ligand dependent. Using [^{3}H] methylspiperone, the loss of binding activity is manifested as a reduction in the affinity of the receptor-ligand interaction, with no observable loss of receptor number (Fig. 7). In contrast, using [^{125}I]iodosulpride, there is a decrease in the number of receptors detected with the radioligand, with no change in receptor affinity (Fig. 7). The fact that there is no loss of the total receptor number detected using [^{3}H]methylspiperone argues against receptor degradation as an explanation for the observed reduction in receptor binding after desensitization. Instead, we would like to hypothesize that the D_2 receptors undergo an agonist-induced translocation or sequestration event that orients them into a membrane environment, such that their ligand binding region is not readily exposed. In this hypothesis, [^{125}I]iodosulpride, which is a member of the relatively hydrophilic benzamide class of dopamine antagonists (22–24) and is probably membrane impermeable, can only detect those receptors remaining fully exposed on the surface of the dY-79 cell membranes after desensitization. Conversely, [^{3}H]methylspiperone is a very hydrophobic, membrane-permeable, antagonist ligand and is able to detect the full complement of receptors associated with the membrane fraction. The small reduction in affinity of [^{3}H]methylspiperone binding after desensitization may be reflective of only a kinetic barrier for penetration of this ligand into the sequestered membrane environment. A similar mechanism has been suggested, based partially on data generated using hydrophilic and hydrophobic radiolabeled antagonist ligands, for the sequestration process that is involved in β-adrenergic receptor desensitization (25–29). This hypothesis might also explain the observation that a greater number of D_2 receptors are detected using [^{3}H]methylspiperone, compared with [^{125}I]iodosulpride, in membranes prepared from control dY-79 cells (Fig. 7). Thus, under basal conditions, a significant fraction of the dY-79 cell D_2 receptors may already exist in a sequestered binding state. Future experiments will obviously be directed at further investigating the ligand binding properties and membrane location of the desensitized/sequestered D_2 receptors.

The loss of receptor binding activity, however, cannot completely explain the desensitization of the D_2 receptor-mediated adenylyl cyclase inhibition. A functional alteration or uncoupling of the receptor from its signal-transducing G protein also seems to be involved. This was initially suggested by the observation that the desensitization appears to precede the loss of radioligand binding activity (Fig. 4), indicating that these processes can be temporally separated. Secondly, and more importantly, a loss of high affinity agonist binding was observed in

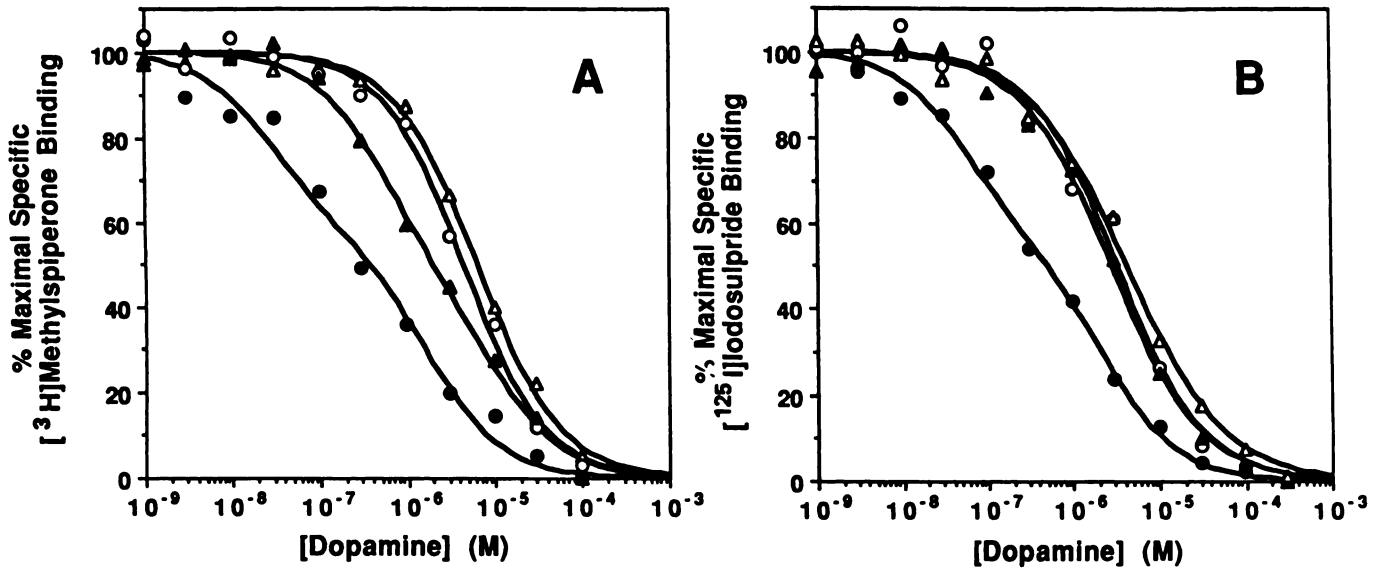

Fig. 9. Dopamine competition for radiolabeled antagonist binding in control and desensitized dY-79 cell membranes. Cells were pretreated with 200 μM sodium metabisulfite without (control) (●, ○) or with 500 μM dopamine (desensitized) (▲, △), for 24 hr at 37°. After extensive washing of the cells with EBSS, membranes were prepared from each pretreatment group and competition radioligand binding assays were performed, as described in Experimental Procedures, in the absence (●, ▲) or presence (○, △) of 200 μM Gpp(NH)p. The data were computer analyzed using the curve-fitting program LIGAND, as described in Experimental Procedures. In the experiment shown, which is representative of three, the *data points* were experimentally determined, whereas the *drawn lines* represent the computer-modeled best fit to the data. A, [^{3}H]Methylspiperone competition curves exhibited the following computer-derived binding parameters: control, K_H = 0.033 μM, K_L = 1.6 μM, R_H = 58%; control plus Gpp(NH)p, K_L = 1.7 μM; desensitized, K_H = 0.15 μM, K_L = 1.5 μM, R_H = 46%; desensitized plus Gpp(NH)p, K_L = 1.8 μM. B, [^{125}I]Iodosulpride competition curves exhibited the following computer-derived binding parameters: control, K_H = 0.031 μM, K_L = 1.3 μM, R_H = 51%; control plus Gpp(NH)p, K_L = 2.2 μM; desensitized, K_L = 2.1 μM; desensitized plus Gpp(NH)p, K_L = 2.8 μM.

dopamine competition for [^{3}H]methylspiperone and [^{125}I]iodosulpride binding after desensitization (Fig. 9). 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 is believed to serve as a functional intermediate in adenylyl cyclase inhibition (17, 18). This complex is manifested as a high affinity, guanine nucleotide-sensitive, agonist binding state in membrane preparations. Thus, a reduction of high affinity agonist binding was observed for the D_2 receptors remaining in the dY-79 cell membranes after dopamine-induced desensitization (Fig. 9). Interestingly, the loss of high affinity agonist binding appeared to be complete for the subset of receptors labeled with [^{125}I]iodosulpride, but only a partial loss was apparent when the total complement of receptors was examined using [^{3}H] methylspiperone. Although this observation requires additional investigation, this finding might suggest that cell surface receptors detected with [^{125}I]iodosulpride exhibit a greater degree of desensitization. In any case, these data suggest that the desensitization involves a functional alteration in the D_2 receptor protein, which occurs independently from the regulation of its ligand binding activity.

Based on the present data, we would like to propose the following two-step process for agonist-induced desensitization of the D_2 receptor system in the dY-79 cells:

$$R_N \leftrightharpoons R_U \leftrightharpoons R_L \qquad (2)$$

In this scheme, the normal receptor (R_N) is converted by agonist exposure to a functionally uncoupled form (R_U) before a loss in receptor binding activity (R_L), the latter of which is presumably due to a sequestration process. These events appear to be readily reversible. The observation that the rate of desensitization is faster than the loss of antagonist binding suggests that the formation of R_U precedes that of R_L. What is not known, however, is whether the formation of R_U is obligatory for the conversion to R_L. Future experiments, designed to test whether receptor-G protein coupling is required for the agonist-induced loss of antagonist binding, may shed some light on this issue.

It is interesting to compare our findings on D_2 receptor desensitization with those observed in other neurotransmitter receptor systems. Recently, we reported on agonist-induced desensitization of the D_1 dopamine receptor subtype in cultured neuroblastoma cells (30). We found that agonist exposure of D_1 receptors promoted their functional uncoupling, sequestration, and, in addition, degradation. In contrast, we have found no evidence for agonist-induced degradation of D_2 receptors, even after 2 days of dopamine exposure (data not shown). In addition, the rate of the agonist-induced desensitization is much faster for the D_1 receptors, achieving maximal levels within 3 hr of treatment. This is also true for other receptor systems linked to adenylyl cyclase stimulation, such as the β-adrenergic receptor (25, 28, 31), suggesting that fundamental differences may exist between the regulation of adenylyl cyclase-linked stimulatory and inhibitory receptors. At this point, the underlying biochemical mechanisms associated with the D_2 receptor desensitization can only be hypothesized. An emerging concept, however, is the critical role that protein phosphorylation plays in the regulation of a number of neurotransmitter and hormone receptor systems (32, 33). Of particular relevance is the observation that α_2-adrenergic and muscarinic cholinergic receptors, both of which inhibit adenylyl cyclase activity,

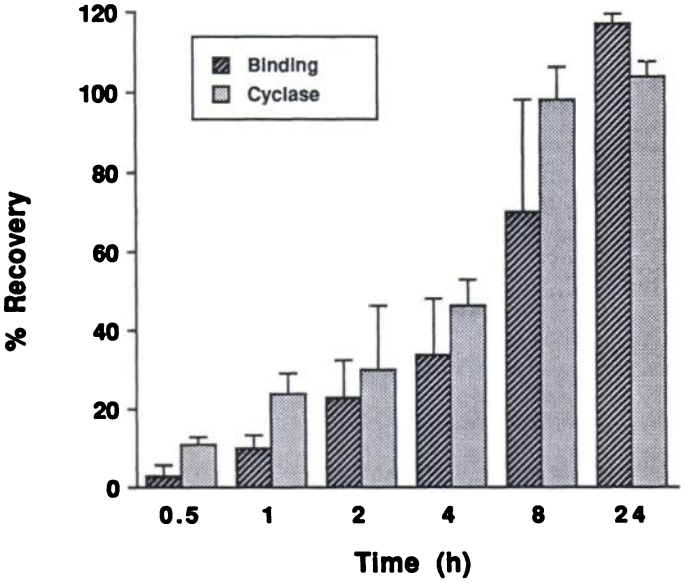

Fig. 10. Time course of recovery of D_2 receptor function and ligand binding activity after dopamine-induced desensitization and down-regulation. Cell cultures were incubated with 200 μM sodium metabisulfite (control) or 500 μM dopamine plus 200 μM sodium metabisulfite, for a period of 24 hr. At this time, some cells were harvested for the determination of maximal desensitization of dopamine inhibition of enzyme activity and loss of receptor binding, as measured with 1 nM [^{125}I] iodosulpride, as described in Fig. 8. The remaining cell groups were washed extensively and allowed to continue in culture in the presence of fresh medium for the indicated times. At each time point, the cells were harvested and adenylyl cyclase and binding activities were determined as described above. The data are expressed as percentage of recovery, where 0% represents the maximal desensitization/down-regulation observed after 24 hr of dopamine treatment and 100% represents the control values. Results shown are means ± standard errors of three independent experiments.

serve as substrates for a number of different protein kinases (34–37). It will be of interest to determine in future experiments whether this covalent modification is also involved in desensitization of the D_2 dopamine receptor system.

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