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The D_{2S} and D_{2L} Dopamine Receptor Isoforms Are Differentially Regulated in Chinese Hamster Ovary Cells

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SUMMARY

To investigate and compare the regulatory properties of the two isoforms of the D_2 dopamine receptor, we have stably expressed their cDNAs in Chinese hamster ovary (CHO) cells. Cell lines were selected that express similar levels of [^{3}H]methylspiperone-binding activity. Both isoforms mediate a dose-dependent and pharmacologically specific inhibition of adenylyl cyclase activity in both intact cell and membrane preparations. Pretreatment of both D_{2L} and D_{2S} receptor-expressing cells with 100 μM dopamine produces a ~5-fold shift (to lower affinity) in the EC_{50} for dopamine inhibition of cAMP accumulation, with a 25–30% decrease in the maximum response. Dopamine treatment also results in a ~25% decrease in the maximum receptor binding activity of the D_{2S} receptor-expressing cells. In contrast, the D_{2L} receptors are up-regulated by about 2-fold in response to dopamine exposure. This difference in response between the D_{2S} and D_{2L} receptors is not cell line specific, inasmuch as other CHO clones expressing these isoforms show identical responses. The dopamine-induced up-regulation of D_{2L} receptor binding is time dependent, reaching maximal levels after 10 hr ($t_{1/2}$ = 2 hr). Upon removal of dopamine, the receptor binding activity returns to control levels within 20 hr. The adenylyl cyclase desensitization response is also time dependent but exhibits a slower time course ($t_{1/2}$ = 5 hr) than the receptor up-regulation. Both regulatory responses are induced in a dose-dependent fashion by dopamine, albeit with different potencies (up-regulation EC_{50} = 100 nM, desensitization EC_{50} = 2 μM). These regulatory effects are pharmacologically specific, being mimicked by D_2-selective agonists but not by agonists of other receptor subtypes. The dopamine-induced receptor up-regulation is blocked by prior treatment of the cells with pertussis toxin and is not mimicked by cAMP analogs. Conversely, elevation of intracellular cAMP levels results in down-regulation of the D_{2L} receptor activity. To test whether protein synthesis is required for the D_{2L} receptor up-regulation, cycloheximide was used to block mRNA translation. This was found to completely inhibit the up-regulation of D_{2L} binding activity; however, there was no effect on the desensitization of the adenylyl cyclase response. RNA dot-blot analyses indicate that dopamine treatment is associated with a sustained 2-fold increase in the steady state levels of D_{2L} mRNA, whereas D_{2S} mRNA is transiently increased by only 50%. Our data suggest that dopamine can promote a number of regulatory events in the CHO cells, including desensitization of both D_2 receptor isoforms, down-regulation of the D_{2S} isoform, and up-regulation of the D_{2L} receptor.

The transduction of dopaminergic signals across cellular membranes is mediated by a diversity of receptor subtypes that belong to the gene superfamily of G protein-linked receptors (1–3). To date, cDNAs and/or genes encoding five different dopamine receptors have been cloned and their primary structures elucidated (1–3). These include the D_1 and D_5 receptor subtypes (also referred to as D_{1A} and D_{1B}), which exhibit a classical D_1 pharmacological profile, as well as the D_2, D_3, and D_4 receptors, all of which demonstrate a D_2-like pharmacology. The D_1-like dopamine receptors are functionally linked to increasing intracellular cAMP levels through activation of the enzyme adenylyl cyclase (4, 5). In contrast, D_2-like receptors have been suggested to activate a wide variety of signal transduction pathways (6). When expressed in various cells, the cloned D_2 receptor has been shown to inhibit adenylyl cyclase activity (7), potentiate arachidonic acid release (8), stimulate phosphatidylinositol turnover (9), and regulate K^+ and Ca^{2+} channel activity (9, 10). The D_4 receptor has also been suggested to inhibit adenylyl cyclase activity (11), whereas the transduction pathway for the D_3 receptor is at present unclear. All of these dopamine receptor subtypes are expressed in specific regions of the central nervous system, where they play important roles in the regulation of cognitive function and motor control.

Regulation of receptor responsiveness by neurotransmitters and hormones is a well recognized phenomenon that has been demonstrated for most receptor systems (12). Dopaminergic receptors are no exception in this regard, because numerous *in*

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ABBREVIATIONS: CHO, Chinese hamster ovary; Sp-cAMPS and Rp-cAMPS, Sp- and Rp-isomers of adenosine-3′,5′-cyclic monophosphorothioate; SSC, standard saline citrate; 5-HT, 5-hydroxytryptamine; EBSS, Earle's balanced salt solution.

vivo studies have shown that these receptors are subject to dynamic regulation in both positive and negative fashions (13). Studies carried out *in vitro* have further indicated that exposure of D_1 receptors to dopaminergic agonists results in a profound desensitization of the adenylyl cyclase response as well as a loss of receptor binding activity (14–22). In contrast, similar *in vitro* investigations of D_2 receptor regulation have produced conflicting results. Whereas some studies have shown agonist treatment to result in functional desensitization and receptor down-regulation (23), others have observed desensitization without changes in receptor levels (24–26) or no desensitization response at all (27, 28). In one study, agonist treatment resulted in up-regulation of receptor binding activity (28). To sort out these various regulatory events and investigate their potential mechanisms, we have initiated studies using CHO cells transfected with either the short (D_{2S}) or long (D_{2L}) isoform of the rat D_2 receptor and treated with dopaminergic agonists. Our present results indicate that, whereas agonist activation results in functional desensitization of both receptor isoforms, the receptor variants are differentially regulated with respect to their expression levels. Thus, receptor occupancy by agonists promotes a loss of D_{2S} receptor binding, whereas the expression of the D_{2L} receptor isoform is paradoxically increased. The later event appears to be due to increased synthesis of the D_{2L} receptors, because the corresponding mRNA level is increased in response to agonist treatment.

Experimental Procedures

Materials. [^{3}H]Methylspiperone (80–90 Ci/mmol), [^{3}H]cAMP (45 Ci/mmol), and GeneScreen Plus nitrocellulose membranes were purchased from NEN/DuPont (Boston, MA). SKF-38393, quinpirole, and (+)-butaclamol were purchased from Research Biochemicals, Inc. (Natick, MA). Dopamine, forskolin, serotonin, isoproterenol, cycloheximide, and protein kinase A 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). Sp-cAMPS and Rp-cAMPS were purchased from Biolog Life Science Institute (La Jolla, CA). Cell culture media, transfection reagents, and fetal bovine serum were obtained from GIBCO (Grand Island, NY). All other chemicals were purchased from commercial suppliers and were of the highest quality available.

CHO cell culture and transfection. CHO-K1 cells (American Type Culture Collection) were routinely cultured in Ham's F-12 medium (GIBCO) containing 1 mM sodium pyruvate, 10% fetal bovine serum, and 30 μg/ml gentamycin. The medium was usually replaced every other day. The D_{2L} receptor cDNA used in this study has been described previously (29). A cDNA encoding the full length D_{2S} receptor was isolated from a rat striatal library and sequenced in pBluescript. Both D_{2L} and D_{2S} receptor cDNAs were subcloned from pBluescript by excision with *Xho*I, which cuts approximately 80 base pairs upstream of the translational start site, and *Xba*I, which cuts in the pBluescript polylinker downstream of the 3′ end of the cDNAs. The resulting *Xho*I/*Xba*I fragments were ligated into the eukaryotic expression vector pCD-SRα (30), yielding pSR-D2L and pSR-D2S. These D_{2L} and D_{2S} expression constructs thus contain identical 5′ and 3′ untranslated sequences, including the native polyadenylation signal sites. CHO cells were transfected with 30 μg of either pSR-D2L or pSR-D2S and 3 μg of pMAM-Neo (Clontech) by the $CaPO_4$ precipitation method. Stable transfectants were selected in the presence of 500 μg/ml G418 (GIBCO) and were subcloned by limiting dilution. Positive receptor-expressing cell lines were identified via [^{3}H]methylspiperone binding. Some of the D_{2L} and D_{2S} receptor-expressing CHO cell lines used in this study have been described previously (8, 31). All transfected cell lines were maintained in the presence of 500 μg/ml G418.

Radioligand binding assays. CHO cells were harvested with 1 mM EDTA in Ca^{2+}/Mg^{2+}-free EBSS, using agitation and collection by centrifugation at 300 × *g* for 10 min. The cells were washed twice with EBSS (complete) and resuspended in 5 mM Tris, pH 7.4 at 4°, 5 mM $MgCl_2$. Disruption of the cells was accomplished using a Dounce homogenizer. Crude membranes were collected by centrifugation at 34,000 × *g* for 10 min and were resuspended 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 0.3 mg of protein/ml, using a motor-driven Teflon pestle. Membrane suspension (100 μl) was added to triplicate assay tubes containing 0.03–1 nM [^{3}H]methylspiperone, in a final volume of 1 ml. (+)-Butaclamol (1 μM) was used to define nonspecific binding. The assay tubes were incubated for 1 hr at room temperature, and the assay was terminated by rapid filtration through GF/C filters that had been pretreated with 0.3% polyethyleneimine. The filters were rapidly washed with 5 × 4 ml of 50 mM Tris·HCl, pH 7.4 at 4°. Radioactivity bound to the filters was quantitated by scintillation counting, using Hydroflour (National Diagnostics), at an counting efficiency of 47%. Protein concentrations were determined using the bicinchoninic acid protein reagent (Pierce, Rockville, IL).

Determination of cAMP production. With the exception of the experiment shown in Fig. 8, the production of cAMP was measured using intact cell assays. CHO cells were harvested and washed as described for radioligand binding assays but final resuspension was in AC buffer (250 mM sucrose, 75 mM Tris·HCl, pH 7.4 at 37°, 12.5 mM $MgCl_2$, 1.5 mM EDTA, 1 mM dithiothreitol, 0.2 mM sodium metabisulfite, with 100 μM RO-20–1724, a phosphodiesterase inhibitor). After a 30-min preincubation at room temperature, 50 μl of cell suspension containing 250,000 cells were added to triplicate assay tubes containing 10 μl of drugs or buffer, on ice, followed by a 5-min incubation at 37°. The reaction was terminated by placing the assay tubes in boiling water for 3 min. The cAMP generated was quantitated with a competitive binding assay described previously (32), except that protein kinase A isolated from bovine heart (Sigma) was used instead of adrenal cAMP-binding protein. The cAMP concentrations produced in this assay were determined by comparison with a standard curve, which was linear in the range of 0.5–25 pmol of cAMP/assay tube. For the experiment shown in Fig. 8, cAMP production was assessed using a broken cell/membrane assay, as follows. CHO cells were harvested and membranes were prepared as described for the radioligand binding assays. Final resuspension of the membranes was performed in AC buffer supplemented with 2.75 mM phosphoenolpyruvate, 53 μM GTP, 0.12 mM ATP, 1.0 unit of myokinase, and 0.2 unit of pyruvate kinase. cAMP generation and assessment were subsequently accomplished as described for intact cells.

RNA dot-blot analysis. CHO cells (50–100 × 10^3) were collected by vacuum on GeneScreen Plus (NEN) nitrocellulose filter sheets that had been pretreated with 10× SSC. RNA was cross-linked to the nitrocellulose filters by using UV radiation set at 1200 μJ. The GeneScreen Plus membranes were prehybridized in 50% formamide, 10% dextran sulfate, 1% sodium dodecyl sulfate, 1 M NaCl, for 1 hr at 42°. Hybridization probes consisted of 48-mer antisense oligodeoxynucleotides derived from sequences found in either rat β-actin or the third cytoplasmic loop of the rat D_2 receptor in a region that is common to both long and short isoforms. The sequence of the D_2 receptor mRNA probe was 5′-AGGACTGTCAGGGTTGCTATGTAGGCCGT GGTGGGATGGATCAGGGAG-3′, whereas that of the β-actin probe was 5′-CTCCTGCTTGCTGATCCACATCTGCTGGAAGGTGGACA GTGAGGCCAG-3′. The oligodeoxynucleotides were synthesized on an Applied Biosystems Inc. model 394 DNA synthesizer, using cyanoethyl phosphoramidites, and were deprotected at 55° for 8 hr. The 5′-dimethoxyltrityl moiety was not cleaved after synthesis, to allow separation of full length from partial length oligodeoxynucleotides on a reverse phase purification column (Research Genetics, Huntsville, AL). The oligodeoxynucleotides were ^{32}P-labeled by 3′-end tailing using 25

pmol of [γ-^{32}P]ATP (3000 Ci/mmol; NEN/DuPont) and 100 units of terminal deoxynucleotidyl transferase (Promega), in the presence of 2.5 mM $CoCl_2$, for 5 min at 37°. Unincorporated dATP was eliminated using Nuctrap purification columns (Stratagene). After prehybridization for 30 min, 2×10^6 dpm of probe and 0.1 mg/ml salmon testes DNA were added to the hybridization solution and the filters were incubated overnight at 42° in a hybridization oven. The filters subsequently underwent two 5-min washes in 2× SSC at room temperature, followed by two 30-min washes in 2× SSC/1% sodium dodecyl sulfate at 65°. The filters were exposed to Kodak XAR X-ray film for 24–48 hr. The RNA hybridization dots were quantitated by excision from the filters and counting of the ^{32}P incorporation via scintillation counting. Controls consisted of hybridizing the filters with the corresponding sense strand probes, which did not exhibit any measurable hybridization.

Results

Our initial experiments were directed at determining whether the different D_2 receptor isoforms are subject to agonist-induced regulatory phenomena when expressed in CHO cells. Fig. 1 shows the effects of culturing CHO cells, expressing either the short or long isoform, in the presence of dopamine for 24 hr. For the short isoform, dopamine treatment resulted in a small but consistent ~25% decline in the maximum receptor binding capacity, without a significant change in affinity (Fig. 1A). The functional activity of the D_{2S} receptor was similarly depressed after this treatment, as shown in Fig. 1C. The EC_{50} for dopamine inhibition of cAMP accumulation was shifted by about 5-fold to lower potency, whereas the maximum inhibition was reduced by ~30%. In contrast, the D_{2L} isoform exhibited somewhat different results. Surprisingly, in this case dopamine pretreatment resulted in an approximate doubling of the maximum receptor binding activity (Fig. 1B), without a change in affinity. The functional activity of the D_{2L} receptor, however, was diminished in a manner identical to that seen for the short isoform (Fig. 1D). In general, the shift in potency (EC_{50}) for dopamine was more consistent in magnitude than the decrease in maximum response for both the D_{2S} and D_{2L} receptors, after agonist pretreatment. Treatment of the cells with dopamine also frequently produced a 25–50% increase in basal and/or forskolin-stimulated cAMP levels. This effect was highly variable, however, and not always seen. The difference in the D_{2S} and D_{2L} receptor regulation was not unique to the cells examined, because other clonal CHO cell lines expressing either the D_{2S} (data not shown) or D_{2L} (see below) receptors exhibited identical responses, as shown in Fig. 1.

Because of the unexpected up-regulation of D_{2L} receptor expression, coupled with a functional desensitization response, we chose to examine these agonist-induced regulatory events in more detail. Fig. 2 shows a time course for the effects of dopamine pretreatment on D_{2L} receptor binding and functional activity. The increase in receptor binding activity occurred relatively quickly upon agonist exposure, exhibiting a $t_{1/2}$ of about 2 hr and a maximum increase at about 10 hr (Fig. 2A). In contrast, the functional desensitization, as measured by the shift in the EC_{50} for dopamine inhibition of cAMP accumulation, occurred more slowly, with a $t_{1/2}$ of about 5 hr and a maximal effect at about 24 hr (Fig. 2B). These data indicate that both the receptor up-regulation and desensitization responses are dependent on agonist exposure time and appear to be temporally separable processes.

We next sought to investigate the dose-response relationship for the dopamine-induced regulatory events. Fig. 3A shows that the increase in D_{2L} receptor binding was indeed dependent on the concentration of dopamine used in the pretreatment period. The EC_{50} for this response was about 100 nM (Fig. 3A, *inset*), which is similar to the potency for dopamine inhibition of cAMP accumulation in control cells (see Fig. 3B). In contrast, although the dopamine-induced shift in the EC_{50} for the inhibition curve was dose dependent, the potency for producing this effect was about 1 order of magnitude lower than that observed for receptor up-regulation (Fig. 3B). These two regulatory responses thus appear to exhibit different dose-response relationships with respect to their induction by dopamine.

The pharmacology of the dopaminergic regulatory responses was investigated in the experiment shown in Fig. 4. A number of compounds with agonist activity at D_1 and D_2 dopaminergic, adrenergic, and serotonergic receptors were incubated with the CHO cells. Only the D_2-selective agonist quinpirole could mimic the dopamine-induced increase in ligand-binding activity (Fig. 4A), as well as functional desensitization (Fig. 4B). In contrast, other receptor agonists, including the D_1-selective agonist SKF-38393, the β-adrenergic receptor agonist isoproterenol, and serotonin, had no effect on either the receptor binding activity or the functional response. These data suggest that both the dopaminergic receptor up-regulation and functional desensitization responses are pharmacologically specific and coupled to receptor occupancy by D_2-selective agonists.

We were next interested in determining whether the dopamine-induced regulatory responses, especially the increase in receptor binding, might be a function of the level of receptor expression. Fig. 5 shows results obtained using transfected CHO cells expressing either higher or lower D_{2L} receptor levels, compared with the cells used in Figs. 1–4. In this experiment, the different transfected CHO cell lines expressed approximately 4-fold lower or higher receptor levels than those in the cells used previously. As seen in Fig. 5, both the lower and higher level receptor-expressing cell lines exhibited identical responses in terms of receptor up-regulation and functional desensitization after dopamine pretreatment. Moreover, the regulatory responses with these cells were identical to those demonstrated in Fig. 1 using an intermediate level receptor-expressing cell line. These results suggest that the receptor up-regulation and desensitization responses are neither dependent on the level of receptor expression nor cell line specific.

To evaluate the reversibility of the receptor up-regulation response, we performed the experiment shown in Fig. 6. In this case, the CHO cells were exposed to dopamine for 24 hr, extensively washed, and then further cultured in the absence of dopamine. The dopamine-induced increase in receptor binding activity was found to be fully reversible, returning to baseline levels by about 20 hr (Fig. 6). The $t_{1/2}$ for recovery was estimated to be about 15 hr (Fig. 6). The receptor up-regulation response thus does not appear to be due to any irreversible change in the cellular expression of the D_{2L} receptor or in the CHO cells *per se*.

As an initial approach to investigating potential mechanisms for the dopamine-induced regulatory responses, we evaluated the role of receptor-G protein coupling by using pertussis toxin to inactivate G_i/G_o or similar G proteins with which the D_2 receptor is known to be functionally coupled (1–3). Fig. 7 shows the results of pretreatment of the CHO cells with 1 μg/ml pertussis toxin for 24 hr, followed by dopamine treatment. This

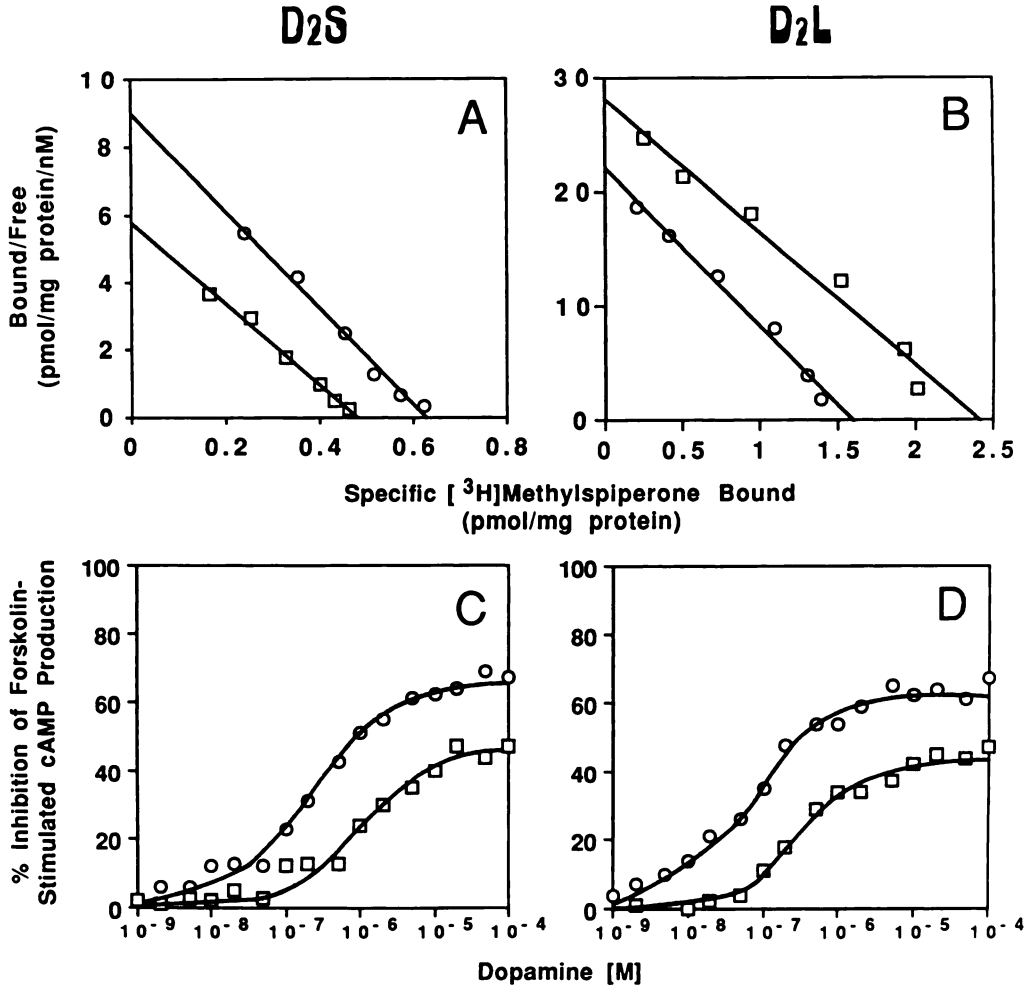

Fig. 1. Dopamine regulation of D_2 receptor isoform activity in CHO cells. CHO cells were cultured in the presence of 0.2 mM sodium metabisulfite (control) (○) or 0.2 mM sodium metabisulfite (to prevent oxidation of dopamine) plus 100 μM dopamine (□) for 24 hr. After extensive washing with EBSS, the cells were harvested and prepared for radioligand binding and cAMP analyses as described in Experimental Procedures. The data shown are from a single representative experiment, which was performed three times with similar results. A, Radioligand binding to CHO cell membranes expressing the D_{2S} receptor isoform. The saturation isotherm is presented in Scatchard coordinates. In this experiment the ligand binding parameters were as follows: control, K_d = 70 pM, B_{max} = 0.63 pmol/mg of protein; dopamine-treated, K_d = 83 pM, B_{max} = 0.48 pmol/mg of protein. B, Radioligand binding to CHO cells expressing the D_{2L} receptor isoform. In this experiment the ligand binding parameters were as follows: control, K_d = 72 pM, B_{max} = 1.6 pmol/mg of protein; dopamine-treated, K_d = 86 pM, B_{max} = 2.4 pmol/mg of protein. C, cAMP accumulation in CHO cells expressing the D_{2S} receptor isoform. After harvesting, the cells were incubated in the presence of either 0.2 mM sodium metabisulfite (basal activity), 0.2 mM sodium metabisulfite plus 10 μM forskolin, or 0.2 mM sodium metabisulfite, 10 μM forskolin, and the indicated concentrations of dopamine. The data shown were derived by first subtracting the basal cAMP levels and then calculating the percentage inhibition by dopamine of the forskolin-stimulated cAMP response. In this experiment the basal and forskolin-stimulated cAMP levels were as follows: control, 1.1 pmol/tube and 31 pmol/tube, respectively; dopamine-treated, 1.1 pmol/tube and 46 pmol/tube, respectively. The estimated EC_{50} parameters were as follows: control, 0.2 μM; dopamine-treated, 1 μM. D, cAMP accumulation in CHO cells expressing the D_{2L} isoform. The experiment was performed as described for C. The basal and forskolin-stimulated cAMP levels were as follows: control, 1.2 pmol/tube and 34 pmol/tube, respectively; dopamine-treated, 1.8 pmol/tube and 44 pmol/tube, respectively. The estimated EC_{50} parameters were as follows: control, 0.15 μM; dopamine-treated, 0.8 μM.

treatment with pertussis toxin was shown to completely abolish dopaminergic inhibition of adenylyl cyclase activity in the cells (data not shown), indicating functional uncoupling of the receptor from the G protein(s) mediating this response. Interestingly, the pertussis toxin pretreatment also blocked the ability of dopamine to subsequently promote up-regulation of the D_{2L} receptor binding activity (Fig. 7). This suggests that the receptor up-regulation response is dependent on the coupling of the D_{2L} receptor to G_i/G_o or related pertussis toxin-sensitive G proteins in the CHO cells. Unfortunately, due to the complete uncoupling of the receptors, the effect of pertussis toxin pretreatment on the ability of agonists to promote desensitization of the adenylyl cyclase response could not be evaluated.

Because functional receptor-G protein coupling appears necessary for the agonist-induced receptor up-regulation response, we evaluated the role of the second messenger cAMP in regulating D_{2L} receptor activity. Fig. 8 shows the effects of treating the CHO cells with the membrane-permeable cAMP analogs Sp-cAMPS and Rp-cAMPS, which either mimic or block the action of cAMP, respectively (33). In this experiment, the cells were also treated with the membrane-permeable phosphodiesterase inhibitor RO-20–1724, to preclude degradation of the cAMP analogs. As seen in Fig. 8A, treatment with the phosphodiesterase inhibitor, either alone or with the cAMP antagonist Rp-cAMPS, had little or no effect on the binding activity of the D_{2L} receptor. In contrast, treatment of the cells with the cAMP agonist Sp-cAMPS resulted in a dramatic decline in the level of receptor binding, without a change in receptor affinity.

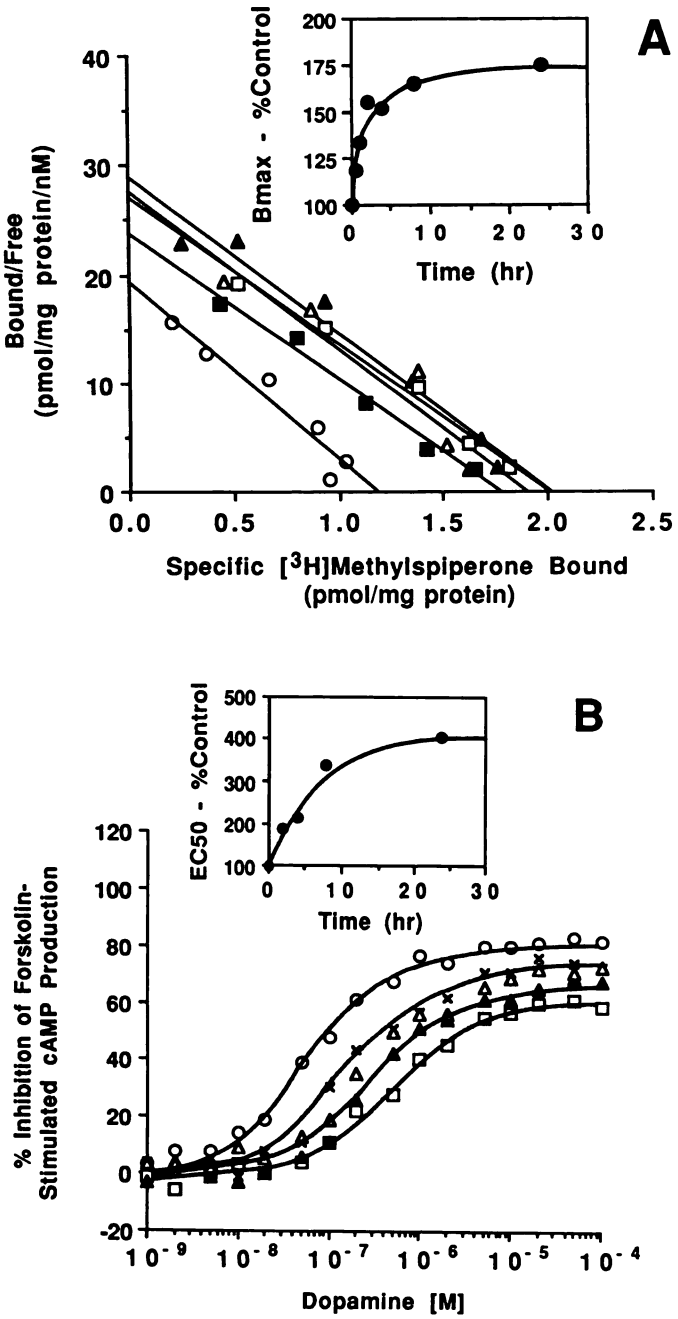

Fig. 2. Time course for dopamine regulation of the D_{2L} receptor isoform in CHO cells. CHO cells were cultured in the presence of 0.2 mM sodium metabisulfite (control) (○) or 0.2 mM sodium metabisulfite plus 100 μM dopamine for 1 hr (■), 2 hr (×), 4 hr (△), 8 hr (▲), or 24 hr (□). After extensive washing with EBSS, the cells were harvested and prepared for radioligand binding and cAMP analyses as described in Experimental Procedures. A, Saturation isotherms are presented from a single experiment that was replicated three times with similar results. In this experiment, the K_d values were as follows: control, 61 pM; 1 hr, 74 pM; 4 hr, 69 pM; 8 hr, 69 pM; 24 hr, 74 pM. The B_{max} values were as follows: control, 1.2 pmol/mg; 1 hr, 1.8 pmol/mg; 4 hr, 1.9 pmol/mg; 8 hr, 2.0 pmol/mg; 24 hr, 2.0 pmol/mg. *Inset*, B_{max} values, as a percentage of the control value, are plotted as a function of dopamine pretreatment time. The data points represent the mean values for three independent experiments. B, Dopamine dose-response curves for inhibition of forskolin-stimulated cAMP accumulation. The experiment was performed using the same cells as in A and the data were calculated as described for Fig. 1. In this experiment, the estimated EC_{50} values were as follows: control, 70 nM; 2 hr, 200 nM; 4 hr, 200 nM; 8 hr, 300 nM; 24 hr, 400 nM. *Inset*, the EC_{50} values, as a percentage of the control value, are plotted as a function of dopamine pretreatment time. The data points represent the mean values for three independent experiments.

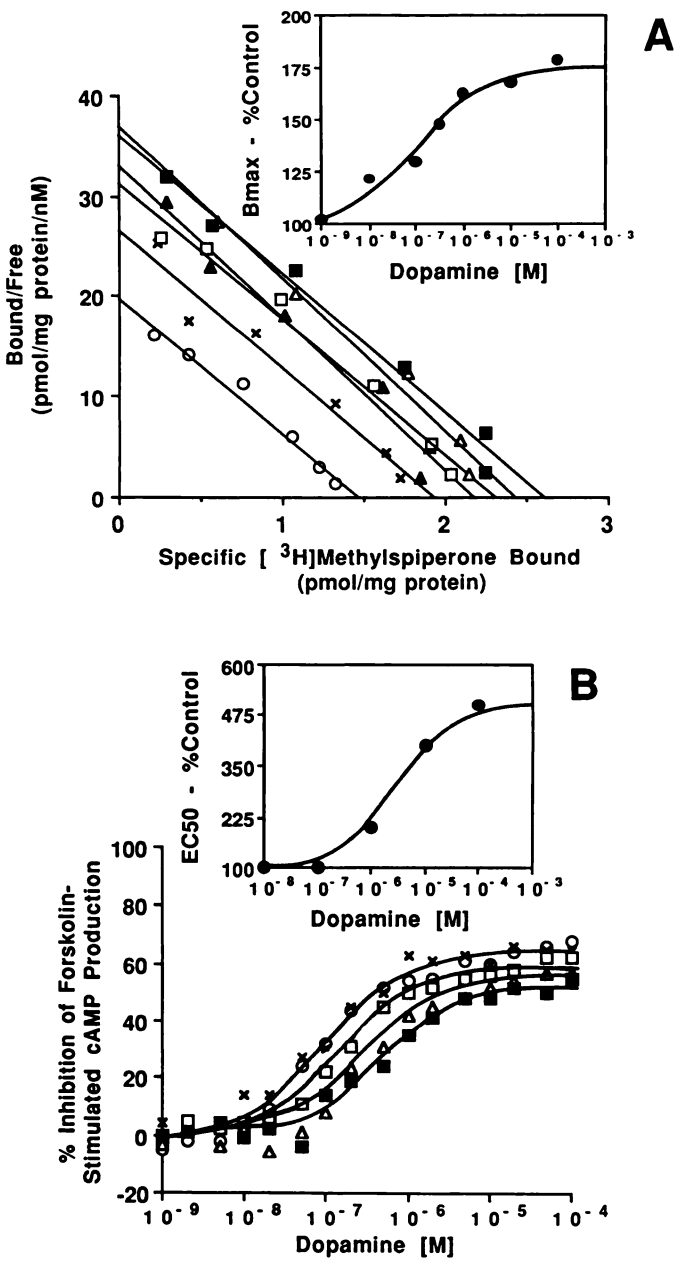

Fig. 3. Dose-response curves for dopamine regulation of the D_{2L} receptor isoform in CHO cells. CHO cells were cultured for 24 hr in the presence of 0.2 mM sodium metabisulfite (control) (○) or 0.2 mM sodium metabisulfite plus the following concentrations of dopamine: 0.1 μM (×), 0.3 μM (▲), 1 μM (□), 10 μM (△), or 100 μM (■). After extensive washing with EBSS, the cells were harvested and prepared for radioligand binding and cAMP analyses as described in Experimental Procedures. A, Saturation isotherms from a single experiment, which was performed three times with similar results. In this experiment, the K_d values were as follows: control, 74 pM; 0.1 μM dopamine, 73 pM; 0.3 μM dopamine, 66 pM; 1 μM dopamine, 74 pM; 10 μM dopamine, 66 pM; 100 μM dopamine, 72 pM. The B_{max} values were as follows: control, 1.5 pmol/mg; 0.1 μM dopamine, 1.9 pmol/mg; 0.3 μM dopamine, 2.2 pmol/mg; 1 μM dopamine, 2.3 pmol/mg; 10 μM dopamine, 2.4 pmol/mg; 100 μM dopamine, 2.6 pmol/mg. *Inset*, B_{max} values, as a percentage of the control value, are plotted as a function of the dopamine pretreatment dose. The data points represent the mean values for three independent experiments. B, Dopamine dose-response curves for inhibition of forskolin-stimulated cAMP accumulation. The experiment was performed using the same cells as in A, and the data were calculated as described for Fig. 1. In this experiment, the EC_{50} values were as follows: control, 100 nM; 0.1 μM dopamine, 100 nM; 1 μM dopamine, 200 nM; 10 μM dopamine, 400 nM; 100 μM dopamine, 500 nM. *Inset*, the EC_{50} values, as a percentage of the control value, are plotted as a function of the dopamine pretreatment dose. The data points represent the mean values for three independent experiments.

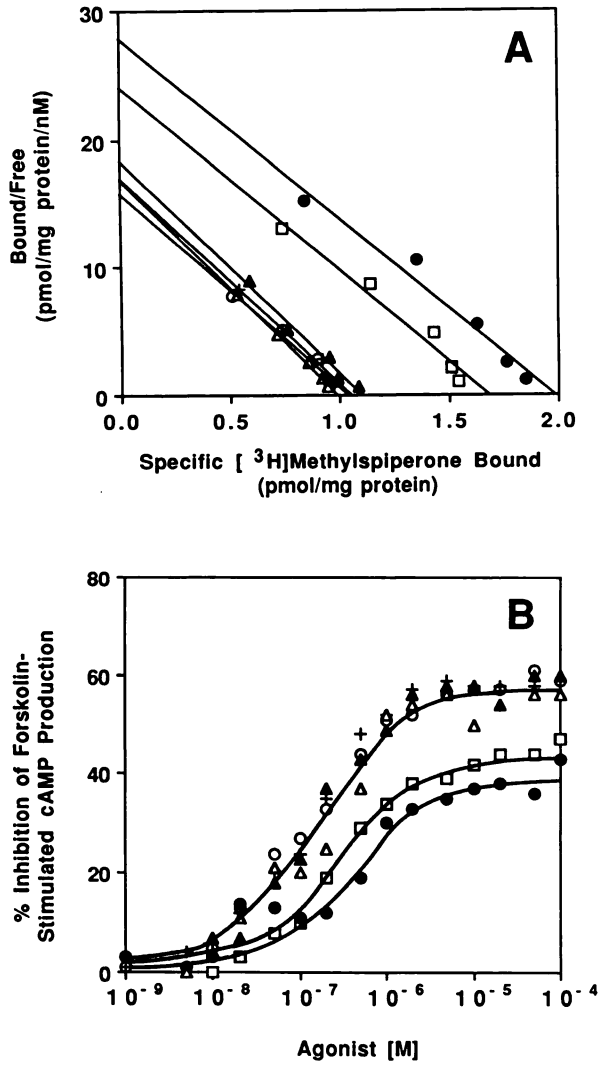

Fig. 4. Pharmacology of agonist-induced regulation of the D_{2L} receptor in CHO cells. CHO cells were cultured for 24 hr in the presence of 0.2 mM sodium metabisulfite (control) (○) or 0.2 mM sodium metabisulfite plus 100 μM concentrations of the following receptor agonists: dopamine (□), quinpirole (●), SKF-38393 (+), isoproterenol (▲), and serotonin (△). After extensive washing with EBSS, the cells were harvested and prepared for radioligand binding and cAMP analyses as described in Experimental Procedures. A, Saturation isotherms from a single experiment, which was performed three times with similar results, are shown. In this experiment, the K_d values were as follows: control, 65 pM; dopamine, 70 pM; quinpirole, 71 pM; SKF-38393, 59 pM; isoproterenol, 60 pM; serotonin, 62 pM. The B_{max} values were as follows: control, 1.0 pmol/mg; dopamine, 1.7 pmol/mg; quinpirole, 2.0 pmol/mg; SKF-38393, 1.0 pmol/mg; isoproterenol, 1.1 pmol/mg; serotonin, 1.1 pmol/mg. B, Dopamine dose-response curves for inhibition of forskolin-stimulated cAMP accumulation. The experiment was performed using the same cells as in A, and the data were calculated as described for Fig. 1. In this experiment, the estimated EC_{50} values were as follows: control, 150 nM; dopamine, 400 nM; quinpirole, 500 nM; SKF-38393, 150 nM; isoproterenol, 150 nM; serotonin, 150 nM.

Similar results were seen with respect to functional activity of the D_{2L} receptor (Fig. 8B). Treatment with Sp-cAMPS resulted in an approximately 5-fold shift in the potency (EC_{50}) for dopamine inhibition of cAMP adenylyl cyclase, as well as a small decline in the maximum response. In contrast, treatment with Rp-cAMPS had minimal effect. It thus appears that increasing intracellular cAMP levels mimic the action of dopamine agonists with respect to the functional desensitization of the D_{2L} receptor but produce an opposite response in terms of the receptor binding activity.

We next wished to further explore the mechanism by which dopamine treatment results in an increased level of receptor binding activity. Conceivably, this could be due to several events, including, but not restricted to, enhanced receptor synthesis, decreased receptor degradation, modification of receptor structure, and recruitment of intracellular receptors to the plasma membrane. In an initial test of these possibilities, we used the protein synthesis inhibitor cycloheximide, which disrupts mRNA translation. Fig. 9A shows that treatment of the CHO cells with cycloheximide alone did not significantly affect the D_{2L} receptor binding activity but when cycloheximide was combined with dopamine the treatment resulted in complete blockade of the receptor up-regulation. Conversely, cycloheximide treatment did not affect the ability of dopamine to produce functional desensitization of the D_{2L} receptor (Fig. 9B). It should be noted that, under the conditions of the experiment shown in Fig. 9, there was no effect of cycloheximide on cell viability (data not shown). These data suggest that, whereas the receptor up-regulation response is dependent on new protein synthesis, the desensitization response is not.

To determine whether the protein synthesis dependence of the receptor up-regulation response is indicative of increased receptor synthesis, we examined D_2 receptor mRNA levels after dopamine treatment. Fig. 10 shows cellular RNA dot-blot analyses for CHO cells, expressing either the D_{2L} or D_{2S} receptor, after treatment with dopamine for the indicated times. Fig. 10A shows hybridization using an antisense oligodeoxynucleotide probe for detecting either D_{2L} or D_{2S} receptor mRNA, whereas Fig. 10B shows duplicate blots that were hybridized with an antisense oligodeoxynucleotide probe for β-actin mRNA. It should be noted that the autoradiograms reflect detection of RNA and not DNA, because hybridization with oligodeoxynucleotide probes representing the sense strands of the corresponding probes failed to produce a signal. Quantitation and normalization, using the β-actin values (which were unchanged by the treatments) (Fig. 10B), of the data are shown in Fig. 10C. As can be seen, dopamine treatment resulted in more than doubling of the D_{2L} receptor mRNA. This occurred with a $t_{1/2}$ of about 30 min, was maximal within 2 hr of treatment, and was sustained for up to 24 hr. In contrast, dopamine treatment resulted in only a transient increase of about 50% in the level of D_{2S} receptor mRNA. It is notable that the increase in the steady state levels of D_{2L} receptor mRNA exhibits a time course preceding that for the increase in receptor binding (see Fig. 2A). Moreover, the approximately 2-fold magnitude of the mRNA increase corresponds closely to that seen for the increase in D_{2L} receptor expression levels.

Because of the small increase observed in D_{2S} receptor mRNA levels after a brief exposure to dopamine, we wished to re-evaluate the expression status of the D_{2S} receptor at these early time points. Exposure of the CHO cells to 100 μM dopamine for 30 min to 24 hr did not, however, result in increased D_{2S} receptor binding at any time point examined (data not shown). Rather, the binding activity only declined (after a lag of about 2 hr), as illustrated in Fig. 1A for a 24-hr treatment period.

To evaluate the specificity of the dopamine-induced increase in D_2 receptor mRNA levels, we attempted to block this response with an antagonist ligand. Fig. 11 shows that co-treatment with the D_2-selective antagonist (−)-sulpiride could com-

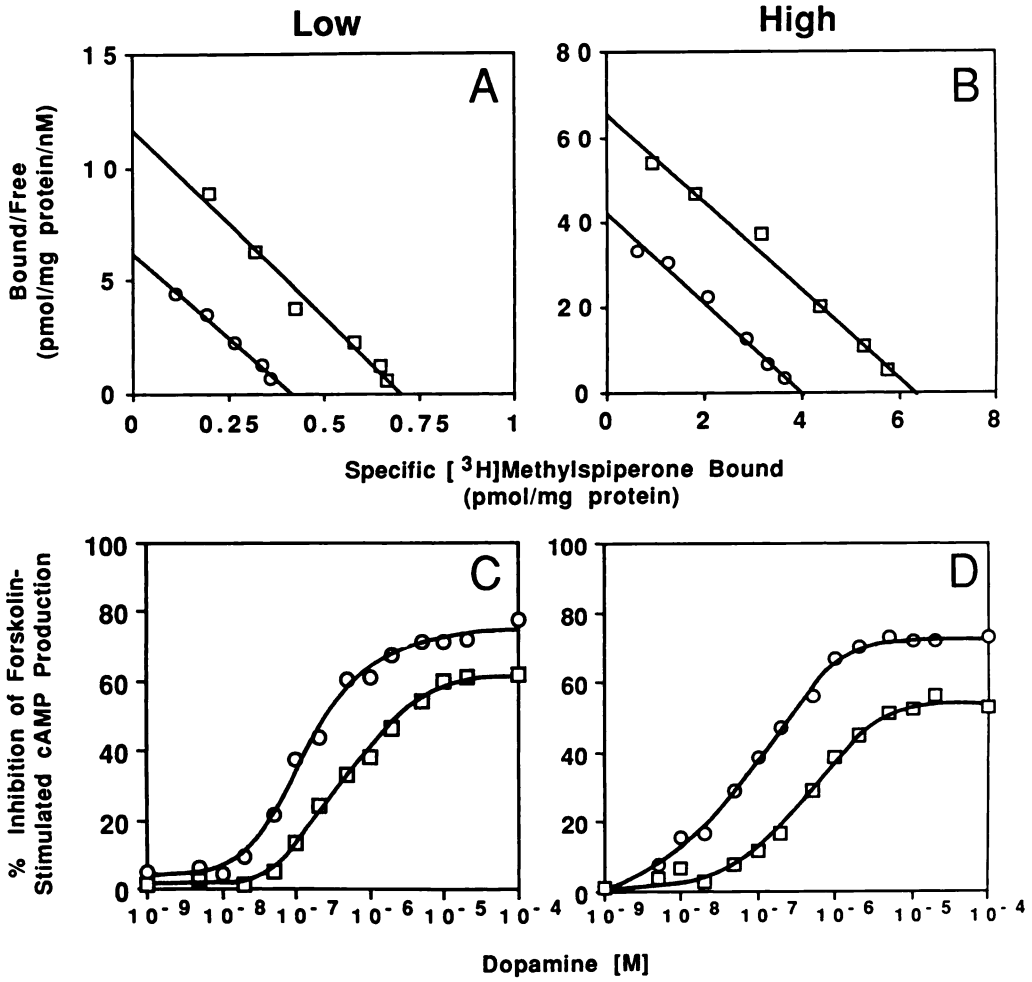

Fig. 5. Dopamine regulation of D_{2L} receptor activity in CHO cells with different levels of receptor expression. CHO cells expressing different levels of receptors were cultured in the presence of 0.2 mM sodium metabisulfite (control) (○) or 0.2 mM sodium metabisulfite plus 100 μM dopamine (□) for 24 hr. After extensive washing with EBSS, the cells were harvested and prepared for radioligand binding and cAMP analyses as described in Experimental Procedures. The data shown are from a single representative experiment, which was performed three times with similar results. A, Saturation binding isotherm for a lower level receptor-expressing CHO cell line. In this experiment, the K_d values were as follows: control, 67 pM; dopamine-treated, 60 pM. The B_{max} values were as follows: control, 0.41 pmol/mg; dopamine-treated, 0.7 pmol/mg. B, Saturation binding isotherm for a higher level receptor-expressing CHO cell line. In this experiment, the K_d values were as follows: control, 96 pM; dopamine-treated, 97 pM. The B_{max} values were as follows: control, 4.0 pmol/mg; dopamine-treated, 6.4 pmol/mg. C, Dopamine dose-response curves for inhibition of forskolin-stimulated cAMP accumulation. The experiment was performed using the same cells as in A, and the data were calculated as described for Fig. 1. In this experiment, the estimated EC_{50} values were as follows: control, 150 nM; dopamine-treated, 500 nM. D, Dopamine dose-response curves for inhibition of forskolin-stimulated cAMP accumulation. The experiment was performed using the same cells as in B, and the data were calculated as described for Fig. 1. In this experiment, the estimated EC_{50} values were as follows: control, 100 nM; dopamine-treated, 500 nM.

pletely attenuate the dopamine-induced increase in D_{2L} receptor mRNA levels. These data indicate that the dopamine-induced increase in D_2 receptor mRNA levels is selectively mediated by D_2 receptor activation. Interestingly, (−)-sulpiride treatment alone resulted in a small but significant reduction in the amount of D_{2L} receptor mRNA (Fig. 11). This latter effect might be due to blockade of low levels of catecholamines known to be present in fetal bovine serum, which was used in the culture medium.

Discussion

The present investigation indicates that D_2 receptors are subject to multiple forms of regulation when expressed in CHO cells. Our initial hypothesis was that the D_2 receptor isoforms would be subject to agonist-induced desensitization with a loss in receptor binding activity, as we had previously observed using human Y-79 retinoblastoma cells, which endogenously express the D_2 receptor subtype (23). Indeed, we found that agonist treatment of CHO cells expressing D_2 receptors resulted in functional desensitization, as manifested by a reduction in both the potency and efficacy of dopamine inhibition of cAMP production. This response was time dependent and was observed for both the short and long isoforms of the D_2 receptor. The dose dependence and pharmacological specificity suggest that the desensitization response is tightly coupled to occupancy of the receptor by agonists. In addition to a functional desensitization response, a loss in receptor binding activity was observed for the D_{2S} isoform, whereas D_{2L} receptor binding was paradoxically increased upon agonist exposure. Although the decrease in receptor number might contribute to the D_{2S} receptor desensitization, this cannot fully explain the loss in functional activity, because the D_{2L} receptors were desensitized to

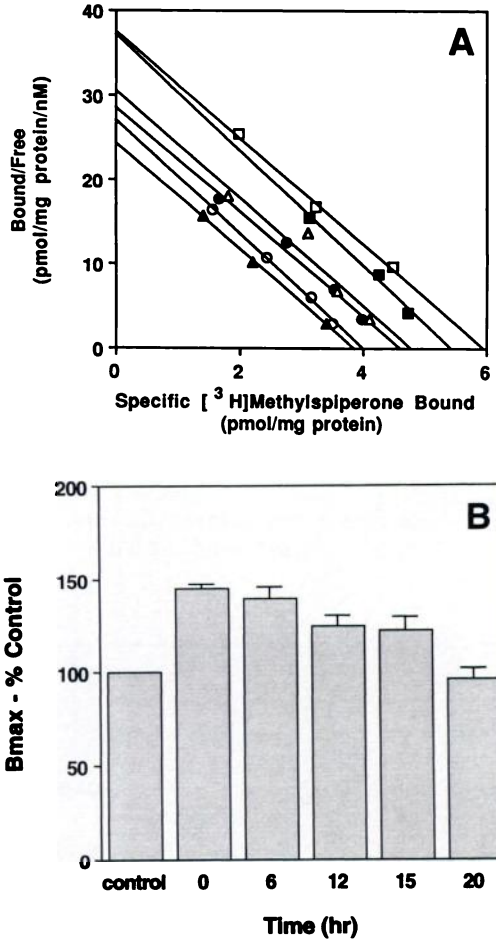

Fig. 6. Time course for recovery of D_{2L} receptor binding after dopamine treatment. CHO cells were cultured with 0.2 mM sodium metabisulfite (control) (○) or 0.2 mM sodium metabisulfite plus 100 μM dopamine for 24 hr. After extensive washing with EBSS, the cells were either harvested and prepared for radioligand binding or, for the dopamine-treated groups, further cultured for either 6 hr (■), 12 hr (△), 15 hr (●), or 20 hr (▲). In this experiment, the time 0 data group (□) indicates dopamine-treated cells that were harvested immediately after 24 hr of treatment, with no time allowed for recovery. A, Saturation isotherms are presented from a single experiment that was replicated three times with similar results. In this experiment, the K_d values were as follows: control, 140 pM; 0 hr, 150 pM; 6 hr, 145 pM; 12 hr, 150 pM; 15 hr, 160 pM; 20 hr, 150 pM. The B_{max} values were as follows: control, 3.9 pmol/mg; 0 hr, 6.0 pmol/mg; 6 hr, 5.4 pmol/mg; 12 hr, 4.8 pmol/mg; 15 hr, 4.6 pmol/mg; 20 hr, 3.8 pmol/mg. B, Plot of the average B_{max} values from three separate experiments, expressed as a percentage of the control value, versus time of recovery. *Error bars*, standard error values.

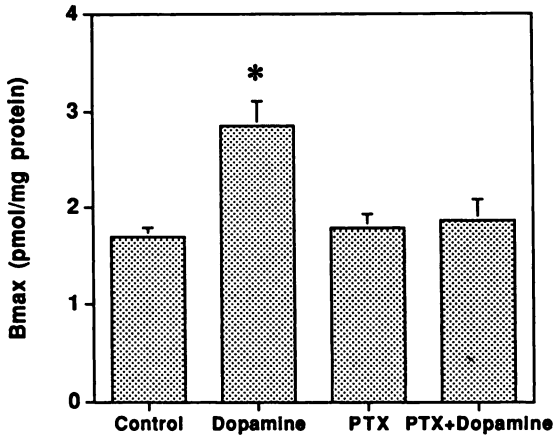

Fig. 7. Effect of pertussis toxin pretreatment on dopamine regulation of D_{2L} receptor binding activity. CHO cells were cultured in the absence (*Control*) or presence of 1 μg/ml pertussis toxin (*PTX*) for 24 hr, followed by further culture with either 0.2 mM sodium metabisulfite (*Control* or *PTX*) or 0.2 mM sodium metabisulfite plus 100 μM dopamine (*Dopamine* or *PTX + Dopamine*) for 24 hr. After extensive washing with EBSS, the cells were harvested and prepared for radioligand binding and cAMP analyses as described in Experimental Procedures. The maximum binding (B_{max}) parameters were calculated from the saturation isotherms, which were performed for each treatment group. The data shown represent the mean ± standard error values for six independent experiments. *, The B_{max} value for the dopamine treatment group is significantly different from all other groups, as determined using analysis of variance with Student-Newman-Keuls *post hoc* test ($p < 0.001$).

a similar extent despite an increase in binding activity. This suggests that the desensitization response is predominantly due to a functional modification of either the D_2 receptor itself or perhaps a downstream effector protein.

Although our data indicate that both D_2 receptor isoforms are subject to agonist-induced desensitization in CHO cells, the precise mechanism underlying this response remains unclear. Our finding that cycloheximide treatment of the CHO cells does not block the desensitization suggests that protein synthesis is not required for this response. Similarly, it appears that a D_2 receptor-mediated reduction in intracellular cAMP is not involved, because we found that increasing the cAMP levels resulted in a similar desensitization and a loss of ligand-binding activity. It is interesting to note that both isoforms of the D_2 receptor contain consensus sequences for phosphorylation by the cAMP-dependent protein kinase (29). Conceivably, the observed cAMP-induced desensitization and/or the loss of ligand-binding activity could be due to direct phosphorylation of the D_2 receptor by cAMP-dependent kinase. However, modification of the receptor by this kinase is unlikely to account for the agonist-induced desensitization, for the reasons noted above. Instead, we favor the hypothesis that agonist occupancy of the receptor promotes its direct phosphorylation and functional modification by a yet to be identified "receptor kinase," as has been demonstrated recently for a number of G protein-linked receptors, including the adrenergic and muscarinic receptor families (34). In support of this hypothesis, the dose-response relationship for dopamine-induced desensitization (EC_{50} = 2 μM) correlates better with the affinity of dopamine for occupying the D_2 receptor (K_i = 2 μM) (data not shown) than with the potency for inhibiting adenylyl cyclase activity (EC_{50} = 100 nM). Obviously, direct testing of this hypothesis will involve additional experimentation.

In addition to an agonist-induced desensitization response, we found that the D_{2L} receptor isoform was paradoxically up-regulated in response to agonist exposure. Conceivably, this could be attributed to a number of different events, primarily involving either an increase in the synthesis or a decrease in the degradation of the receptor protein. This was investigated initially by the addition of cycloheximide, which blocks protein synthesis at the level of mRNA translation. Co-treatment with cycloheximide was found to completely block the agonist-induced increase in D_{2L} receptor binding activity, suggesting that an increase in receptor synthesis is primarily responsible for this effect. This was investigated further by examination of the D_2 receptor mRNA levels during agonist treatment of the cells. Dopamine was found to produce an elevation in D_{2L} receptor mRNA levels of about 2-fold, which is similar in magnitude to

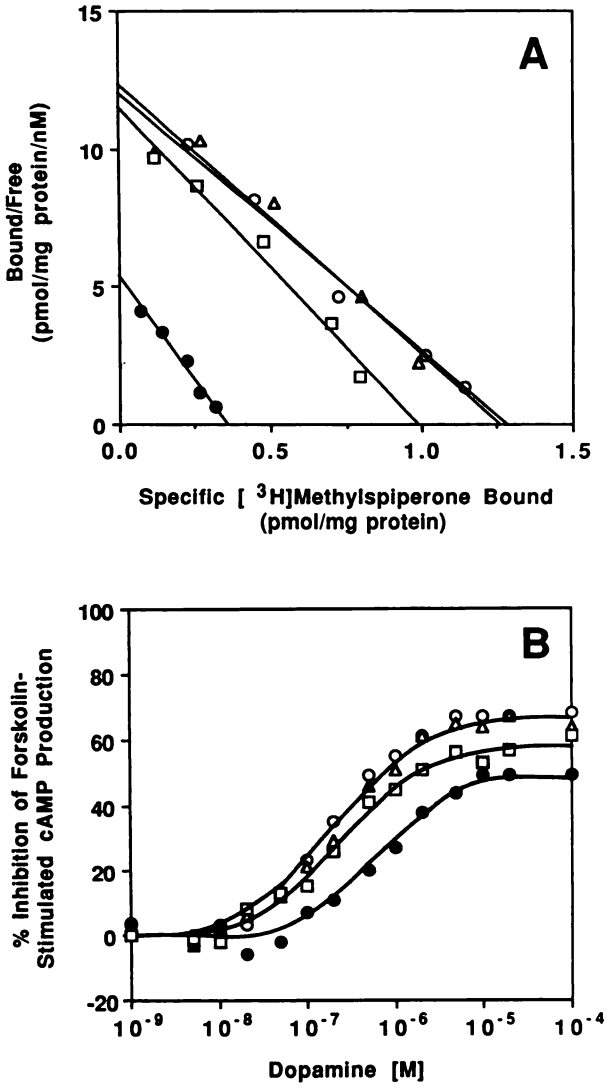

Fig. 8. Regulation of D_{2L} receptor activity in CHO cells by cAMP analogs. CHO cells were cultured for 24 hr in the absence (control) (○) or presence (△) of 100 μM levels of the phosphodiesterase inhibitor RO-20–1724, with or without 1 mM levels of the cAMP analogs Sp-cAMPS (●) and Rp-cAMPS (□). After extensive washing with EBSS, the cells were harvested and prepared for radioligand binding and cAMP analyses as described in Experimental Procedures. The data shown are from a single representative experiment, which was performed four times with similar results. A, Saturation binding isotherms for control and treated cells. In this experiment, the K_d and B_{max} values were as follows: control, 102 pM and 1.2 pmol/mg, respectively; RO-20–1724-treated, 107 pM and 1.28 pmol/mg, respectively; RO-20–1724 plus Sp-cAMPS-treated, 67 pM and 0.34 pmol/mg, respectively; RO-20–1724 plus Rp-cAMPS-treated, 86 pM and 1.0 pmol/mg, respectively. B, Dopamine dose-response curves for inhibition of forskolin-stimulated cAMP accumulation. In this experiment, which was conducted using a membrane preparation (see Experimental Procedures), the estimated EC_{50} values were as follows: control, 0.2 μM; RO-20–1724-treated, 0.2 μM; RO-20–1724 plus Sp-cAMPS-treated, 1.0 μM; RO-20–1724 plus Rp-cAMPS-treated, 0.3 μM. The basal and forskolin-stimulated cAMP levels for the control cells were 1.4 pmol/tube and 31 pmol/tube, respectively.

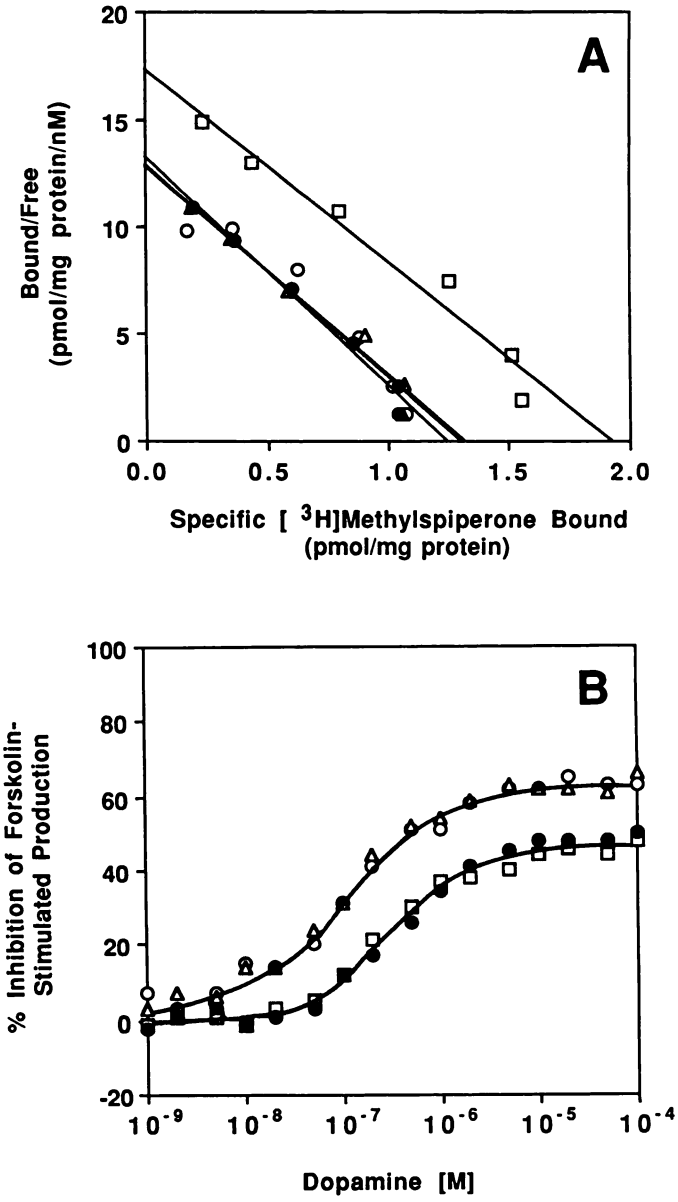

Fig. 9. Effect of protein synthesis inhibition on dopamine regulation of D_{2L} receptor activity. CHO cells were cultured in the absence (○, □) or presence (△, ●) of 5 μg/ml cycloheximide for 2 hr and then further cultured with 0.2 mM sodium metabisulfite in the absence (○, △) or presence (□, ●) of 100 μM dopamine for 9 hr. After extensive washing with EBSS, the cells were harvested and prepared for radioligand binding and cAMP analyses as described in Experimental Procedures. The data shown are from a single experiment, which was performed four times with similar results. A, Saturation binding isotherms from control and treated cells. In this experiment, the K_d and B_{max} values were as follows: control, 100 pM and 1.3 pmol/mg, respectively; dopamine-treated, 110 pM and 1.9 pmol/mg, respectively; cycloheximide-treated, 100 pM and 1.3 pmol/mg, respectively; dopamine plus cycloheximide-treated, 94 pM and 1.2 pmol/mg, respectively. B, Dopamine dose-response curves for inhibition of forskolin-stimulated cAMP accumulation. In this experiment, the estimated EC_{50} values were as follows: control, 0.1 μM; dopamine-treated, 0.5 μM; cycloheximide-treated, 0.1 μM; dopamine plus cycloheximide-treated, 0.5 μM.

the increase observed for receptor binding. It is also notable that the time course for the increase in D_{2L} receptor mRNA was found to precede that for the increase in receptor binding activity. Thus, the increased D_{2L} receptor synthesis is presumably due to an increase in the steady state levels of its mRNA.

Surprisingly, we found that the D_{2S} receptor mRNA was also increased in response to dopamine treatment, although the magnitude of the effect was lower than that observed for the D_{2L} receptor mRNA and appeared transient in nature. This increase in D_{2S} receptor mRNA levels was not accompanied by an increase in receptor expression, however, as was observed

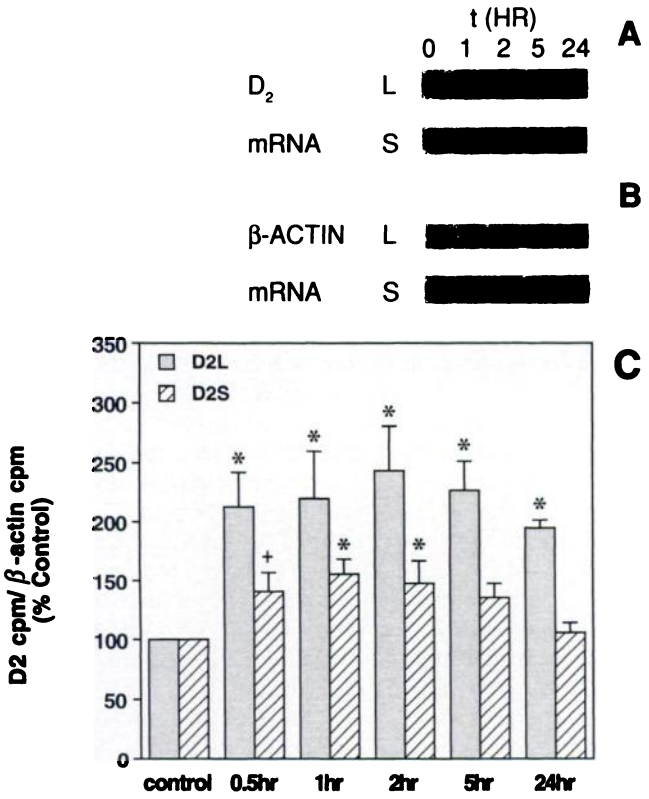

Fig. 10. Dopamine regulation of D_{2L} and D_{2S} receptor RNA levels in CHO cells. CHO cells expressing either isoform of the D_2 receptor were cultured in the absence (control) or presence of 100 μM dopamine for 1–24 hr as indicated. The cells were then washed and harvested for RNA dot-blot hybridization analyses as described in Experimental Procedures. A, Autoradiograms of total cellular RNA blots from both D_{2L} (*L*) and D_{2S} (*S*) receptor-expressing cells hybridized with a D_2-specific oligonucleotide probe. A single experiment is shown, which was performed four times with similar results. B, Duplicate blots from A, which were hybridized with an oligonucleotide probe for β-actin mRNA. A single experiment is shown, which was performed four times with similar results. C, Quantitation and normalization of dot blot hybridization data. The data were calculated by dividing the cpm incorporated in D_2 receptor RNA by the cpm incorporated in β-actin RNA and expressing this ratio as a percentage of the ratio for the control group. The data shown represent the mean ± standard error for four experiments. The groups that showed significant elevations in RNA levels, compared with control, are indicated as follows: *, $p < 0.01$; +, $p < 0.05$, as determined using analysis of variance followed by Dunnett's *post hoc* test.

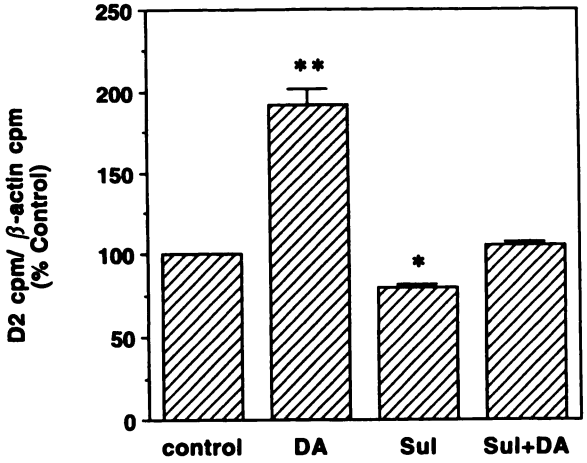

Fig. 11. Blockade of the dopamine-induced elevation of D_{2L} receptor RNA levels in CHO cells. CHO cells expressing the long isoform of the D_2 receptor were cultured in the absence (*control*) or presence of 100 μM dopamine (*DA*) and/or 50 μM (−)-sulpiride (*Sul*) for 24 hr. In the dopamine plus sulpiride treatment group, sulpiride was added 1 hr before the addition of dopamine. The cells were then washed and harvested for RNA dot-blot hybridization analyses, as described in Fig. 10. The data shown represent the mean ± standard error from five separate experiments. The groups that showed significant differences in D_{2L} receptor RNA levels, compared with control, are indicated as follows: **, $p < 0.01$; *, $p < 0.05$, as determined using analysis of variance followed by Dunnett's *post hoc* test.

with the D_{2L} receptor. Our interpretation of these data is that, despite the small increase in mRNA levels, the D_{2S} receptor is predominantly subject to an agonist-induced down-regulation event, presumably occurring at the level of the receptor protein. Conversely, the D_{2L} receptor appears less subject to down-regulation, if at all, with the net result being up-regulation of receptor expression due to increased mRNA levels. Future experimentation will thus be directed at determining the mechanisms underlying the differential responses of these receptor isoforms with respect to both agonist-induced down-regulation and increased mRNA levels.

Conceivably, there are at least two mechanisms by which the levels of the D_2 receptor mRNA could be elevated in the CHO cells. First, the stability of the mRNA could be increased upon exposure of the cells to dopamine. In this scenario, the D_2 receptor mRNA might contain sequence elements that interact with cellular factors to regulate its stability (35). Dopamine treatment would result in either an enhancement of stabilization or a reduction in destabilization, producing an increase in mRNA levels. A second possibility is that the D_2 receptor cDNA could contain regulatory elements that modulate its transcriptional rate, despite the presence of a strong viral promoter in the pCD-SRα vector. Presumably, these could be enhancer or silencer elements that interact with specific transcription factors and would be either activated or attenuated, respectively, by dopamine. In either mechanism, the D_2 receptor mRNA levels would rise and, at least in the case of the D_{2L} receptor isoform, result in an increase in receptor synthesis. It should be noted that the cDNAs encoding the short and long isoforms of the D_2 receptor used to create the stably transfected cell lines contain identical lengths of 5′ and 3′ untranslated sequences (see Experimental Procedures). Thus, if any sequence elements exist that differentially regulate the transcription and/or stability of the D_{2L} and D_{2S} receptor mRNAs, they must be contained within the 87-base pair casette exon that defines the long isoform of the receptor (1–3, 29).

It is interesting to note that if the D_2 receptor mRNA levels are increased through stabilization, with the longer isoform showing a greater response, then this would result in differential regulation of the short and long isoforms of the D_2 receptor in endogenous receptor-expressing tissues. This might explain the observation that D_{2L} receptor mRNA is 2–10-fold more abundant than D_{2S} receptor mRNA in various tissues and brain regions (36–38). Conversely, if enhanced transcription of the D_2 receptor cDNA in the CHO cells is responsible for the increase in receptor mRNA levels, then this would not result in differential expression of the D_2 receptor isoforms in endogenous receptor-expressing tissues. Rather, this would imply that dopamine is simply capable of increasing the transcription of the D_2 receptor gene, which can give rise to either receptor isoform through subsequent alternative RNA splicing. Recently, dopamine was shown to increase D_2 receptor mRNA

levels in dispersed rat pituitary cells, although the issue of the different isoforms was not addressed (39).

Whatever the mechanism for the increase in D_2 receptor mRNA levels, an obvious question is how dopamine might elicit this response. It seems clear that the D_{2L} receptor up-regulation occurs through a mechanism distinct from that of desensitization. This is suggested by the observation that the two responses are induced by dopamine with different time courses and dose-response relationships. Moreover, cycloheximide completely dissociated these responses, by blocking the up-regulation event without affecting receptor desensitization. Our data further suggest that simple occupancy of the receptor by dopamine is not sufficient to produce up-regulation and that functional coupling of the receptor is required. This is indicated by the fact that inactivation of pertussis toxin-sensitive G proteins in the CHO cells was able to completely abolish the ability of dopamine to increase D_{2L} receptor binding. In addition, the potency for the dopamine-induced receptor up-regulation more closely matched the potency for inhibition of adenylyl cyclase activity, rather than occupancy of the receptor. These data might suggest that a reduction in intracellular cAMP levels is the stimulus for D_{2L} receptor up-regulation. Our observation that increasing intracellular cAMP levels promote down-regulation of the receptor is consistent with this hypothesis. However, it might be expected that treatment of the cells with a cAMP antagonist such as Rp-cAMPS would also promote receptor up-regulation, although this was not observed. It should be noted that the D_2 receptor is known to be linked to multiple second messenger systems in CHO cells (8). Additional experimentation will be required to determine whether alterations in cAMP levels or modulation of a distinct signaling pathway mediates the dopamine-induced up-regulation of the D_{2L} receptor.

Recently, similar regulatory phenomena have been demonstrated for the serotonin 5-HT_2 receptor, which is linked to activation of phospholipase C. Pretreatment of cerebellar granule cells in culture with serotonin was found to promote functional desensitization of serotonin-stimulated phosphatidylinositol turnover; however, there was an increase in 5-HT_2 receptor binding activity (40). The increase in 5-HT_2 receptor expression was found to correlate with an increase in the levels of 5-HT_2 receptor mRNA (40). Similarly, in cultured rat myometrial smooth muscle cells serotonin treatment was found to increase 5-HT_2 receptor mRNA levels (41). These results suggest that agonist-promoted increases in receptor mRNA levels are not unique to the D_2 receptor system but may be common among a variety of receptor subtypes.

Our present data may also partially explain some of the disparate results that have been obtained in studies examining agonist regulation of D_2 receptor systems. As noted above, numerous investigations have found that agonist exposure of D_2 receptors can result in either negative, positive, or no effects on D_2 receptor expression and functional activity. If D_2 receptors are indeed subject to multiple agonist-induced regulatory phenomena that can be either stimulatory (increased expression) or inhibitory (decreased expression or functional uncoupling), then the net result depends upon which mode of regulation is predominant in the cell type or tissue under study. In the present case, using D_{2L} receptor-expressing CHO cells, however, it is clear that despite the increase in receptor expression the net result is a decline in functional activity.

While this manuscript was in preparation, a report appeared describing findings similar to ours using human embryonic kidney 293 cells transfected with human D_{2S} and D_{2L} receptor cDNAs (42). Those authors found that exposure of the cells to dopamine could increase the receptor binding activity of both the D_{2S} and D_{2L} receptor isoforms. The functional activity of the D_2 receptors after agonist treatment was not investigated, however (42). We believe that the mechanism leading to the receptor up-regulation observed by those authors is fundamentally different from that described for the D_{2L} receptor in our present report. In contrast to our findings, Filtz *et al.* (42) showed that the agonist-induced receptor up-regulation was potentiated by elevation of intracellular cAMP levels and did not involve increases in receptor mRNA levels, suggesting that enhanced receptor synthesis was not involved. Moreover, the effect was observed only at saturating concentrations of dopamine (>10 μM) and was mimicked by exposure of the receptors to antagonist ligands. Our interpretation of those data is that the receptor up-regulation observed by those authors is due to stabilization of the receptor protein by ligand occupancy, which results in decreased receptor turnover. In preliminary experiments we have found that incubation of our CHO cells with ultra-high concentrations of dopamine (1 mM) can result in enhanced receptor binding of both the D_{2S} and D_{2L} receptor isoforms and this effect is not blocked by cycloheximide or pertussis toxin pretreatment.[1] These data suggest that additional mechanisms can come into play at high dopamine concentrations to further up-regulate D_2 receptor expression, illustrating the complexity involved in the regulation of this receptor system.

Acknowledgments

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