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# Agonists and Antagonists Differentially Regulate the High Affinity State of the $D2_L$ Receptor in Human Embryonic Kidney 293 Cells

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## SUMMARY

Studies with radiolabeled antagonists have revealed that both agonists and antagonists induce up-regulation of D2 dopamine receptors in cells transfected to express $D2_L$ or $D2_S$ receptors. The regulation induced by agonists, but not antagonists, was synergistic with cAMP analogues, and differences in the time courses of the effects of agonists and antagonists have been observed. These findings have been extended by using a radiolabeled agonist to investigate agonist- and antagonist-induced regulation of the high affinity state of the $D2_L$ dopamine receptor in transfected HEK 293 cells. Exposure to agonists decreased the proportion of receptors in the high affinity, agonist-preferring state. Exposure to antagonists, however, led to an increase in the density of receptors with a high affinity for agonists. The effects of both agonists and antagonists on the agonist-preferring receptors occurred without a lag and were time and dose dependent. Inhibition of forskolin-stimulated cAMP accumulation by agonists was not affected by exposure of the cells to the antagonist (−)-sulpiride. Desensitization was seen after exposing cells to the agonist quinpirole for 1.5 hr, suggesting that the rapid loss of high affinity binding sites represents an uncoupling of the receptor from the G protein that mediates inhibition of adenylyl cyclase. Pretreatment of cells with the protein synthesis inhibitor cycloheximide did not block the quinpirole-induced loss of receptors with a high affinity for agonists. The effect of (−)-sulpiride on high affinity binding sites was blocked by cycloheximide, but only after incubation of cells for sufficient time to induce an increase in the total number of receptors. After incubation of cells with (−)-sulpiride for a short time, the increase in the number of receptors with a high affinity for agonists was unaffected by cycloheximide. These results suggest that the increase in agonist binding after brief exposure to an antagonist is due to interactions of the receptor with one or more G proteins that are not coupled to inhibition of adenylyl cyclase, whereas the increase in agonist binding at later time points is associated with the antagonist-induced up-regulation.

Two subtypes of dopamine receptors were originally classified on the basis of pharmacological and biochemical criteria (1). The D1 and D2 dopamine receptors are pharmacologically distinct, displaying differing affinities for not only the endogenous ligand dopamine but also several other compounds (for a review, see Ref. 2). D1 receptors are linked to stimulation of adenylyl cyclase (3), whereas D2 receptors are coupled to multiple effector systems, including inhibition of adenylyl cyclase activity, inhibition of phosphoinositide hydrolysis, and increases in potassium conductance (for a review, see Ref. 4). With the application of the techniques of molecular biology to the study of receptor pharmacology, at least five genes encoding dopamine receptor subtypes have been isolated and categorized as D1-like or D2-like according to their nucleotide sequences and the pharmacological profile of the expressed proteins. The D1-like receptors include the D1 (5–8) and the D5 (9), whereas the D2-like receptor family includes the two isoforms of the D2 receptor, $D2_S$ (10) and $D2_L$ (11–15), the D3 receptors (16), and the D4 receptors (17).

Within the G protein-coupled receptor family, $\beta_2$-adrenergic receptors have been the most extensively studied. Exposure of cells expressing $\beta_2$-adrenergic receptors to agonist results in stimulation of adenylyl cyclase activity, but responses to agonist desensitize rapidly. This appears to be due to phosphorylation of the receptor and its uncoupling from $G_s$. Prolonged agonist exposure results in down-regulation of

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**ABBREVIATIONS:** HEK, human embryonic kidney; PTX, pertussis toxin; CHO, Chinese hamster ovary; [$^{125}$I]7OH-PIPAT, (*R*)-*trans*-7-hydroxy-2-[*N*-propyl-*N*-3′-[$^{125}$I]-iodo-2′-propenyl)-amino]tetralin; [$^{125}$I]NCQ 298, (*S*)-3-[$^{125}$I]-iodo-2-hydroxy-5,6-dimethoxy-*N*-[(1-ethyl-2-pyrrolindinyl)-methyl]benzamide; Gpp(NH)p, guanylyl-imidodiphosphate; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; EMEM, Eagle's minimal essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; GRK, G protein-coupled receptor kinase; 5-HT, 5-hydroxytryptamine.

the receptor, whereby the receptors are sequestered from the cell surface and degraded. Recovery after desensitization occurs rapidly after removal of agonist, but recovery after receptors have been down-regulated requires protein synthesis (for a review, see Ref. 18). Exposure of cells to agonists has been reported to cause desensitization and down-regulation of other receptors, including $\alpha_1$-adrenergic (19), $\alpha_{2A}$-adrenergic (20), and 5-$HT_{2A}$ (21, 22) receptors. In contrast, agonist-induced up-regulation has been reported for $\beta_3$-adrenergic receptors (23), 5-$HT_{2A}$ receptors (24), and D2 receptors (25–29). The effect on $\beta_3$-adrenergic receptors may be a consequence of the existence of multiple cAMP response elements in the 5′ untranslated region of the gene, whereas the mechanisms responsible for up-regulation of 5-$HT_{2A}$ and D2 receptors remain unknown.

In previous work from this laboratory, Filtz *et al.* (26, 27) have reported agonist- and antagonist-induced up-regulation of $D2_L$ and $D2_S$ receptors in HEK 293 cells transfected to selectively express these receptors. Although D2 receptors are coupled to inhibition of cAMP accumulation in these cells, prolonged exposure to agonists did not lead to desensitization of this functional response. The agonist-induced up-regulation was insensitive to PTX and enhanced by coincubation of cells with agonist and analogues of cAMP. Up-regulation of D2 receptors was blocked by cycloheximide, a protein synthesis inhibitor, suggesting that protein synthesis is required for both agonist- and antagonist-induced up-regulation in 293-$D2_L$ cells. Agonist-induced up-regulation in transfected cells was also observed by Zhang *et al.* (28), who described agonist-induced up-regulation of $D2_L$ receptors in CHO-$D2_L$ cells that was blocked by cycloheximide. Zhang *et al.* also reported, however, that agonist-induced up-regulation of receptors in CHO-$D2_L$ cells was blocked by PTX and was associated with desensitization of the inhibition of adenylyl cyclase by dopamine. A recent report by Starr *et al.* (29) described agonist-induced up-regulation of $D2_L$ receptors in C6-$D2_L$ cells that was PTX insensitive, blocked by cycloheximide, and associated with desensitization of a functional response. Antagonists induced a small but significant up-regulation of receptors in C6-$D2_L$ cells.

In the present study, we characterized the agonist- and antagonist-induced regulation of the population of D2 receptors that are in a G protein-coupled, high affinity state. These experiments have used the agonist radioligand [$^{125}$I]7OH-PIPAT (30, 31), previously shown to selectively label the G protein-coupled, high affinity state of the D2 receptor in 293-$D2_L$ cells (32, 33). Differences between the effects of incubating cells with agonists and those seen with antagonists have been identified, and potential mechanisms underlying agonist- and antagonist-induced regulation of the high affinity state of the $D2_L$ receptor in transfected 293 cells have been explored.

## Experimental Procedures

**Materials.** [$^{125}$I]NCQ 298 (34) (2.2 Ci/μmol) and [$^{125}$I]7OH-PIPAT (2.2 Ci/μmol) were initially synthesized in the laboratory of Dr. Hank Kung (Department of Radiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania) and were more recently provided by DuPont/NEN (Boston, MA). $^3$H-Adenine was purchased from DuPont/NEN. (+)-Butaclamol hydrochloride, (−)-quinpirole hydrochloride, and (−)-sulpiride were purchased from Research Biochemicals Int. (Natick, MA). Gpp(NH)p and 8-bromo-cAMP were obtained from Boehringer Mannheim (Indianapolis, IN). Cycloheximide, Tris, EDTA, BSA, DMSO, and polyethylenimine were purchased from Sigma Chemical Co. (St. Louis, MO). EMEM, penicillin, streptomycin, and G418 sulfate were obtained from GIBCO-BRL (Grand Island, NY). FBS was purchased from HyClone Laboratories (Logan, UT). PTX was purchased from List Biological Laboratories (Campbell, CA). Forskolin was obtained from Calbiochem (La Jolla, CA). Isobutyl-1-methylxanthine was purchased from Aldrich Chemical Co. (Milwaukee, WI). Dowex AG50W-X4 resin and neutral alumina were purchased from Bio-Rad Laboratories (Richmond, CA).

**Tissue culture.** HEK 293 cells, a human embryonic kidney cell line (35), were grown in monolayer culture at 37° in 5% $CO_2$ and fed with EMEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). HEK 293 cells were transfected with cDNA encoding the human $D2_L$ receptor as previously described (26, 36). Cells transfected to express the $D2_L$ dopamine receptor (293-$D2_L$ cells) were grown as above and fed with EMEM supplemented with 10% FBS and 500 μg/ml of G418 sulfate. Cells were fed every third or fourth day and subcultured or harvested when confluent.

**Drug treatments.** 293-$D2_L$ cells were grown to 80% confluence in 100-mm culture dishes (Corning Costar Corp., Cambridge, MA) containing 10 ml of medium per plate. Drugs were dissolved at 100× final concentration in sterile $ddH_2O$, except for (−)-sulpiride, which was dissolved in DMSO. After dilution, the concentration of DMSO to which the cells were exposed was 0.01%. PTX was provided as a buffer-containing powder and was used after suspension in sterile $ddH_2O$. Drugs were added to cells at varying times before harvesting.

**Preparation of membranes.** 293-$D2_L$ cells were harvested at varying times after drug treatment. Cells were washed twice with isotonic PBS (138 mM NaCl, 4.1 mM KCl, 5.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, and 11.1 mM glucose, pH 7.4) and incubated at 4° for 5 min in ice-cold hypotonic lysis buffer (10 mM Tris, pH 7.4, 5 mM EDTA). Cells were removed from the plates, homogenized with a Brinkman or Omni homogenizer, and centrifuged (32,000 × *g*, 20 min, 4°). Membranes from cells previously treated with quinpirole were preincubated in 6 ml of buffer (50 mM Tris, pH 7.4, 1 mM EDTA, and 200 mM NaCl) for 30 min at 37° to dissociate residual drug from receptors and then sedimented by centrifugation (32,000 × *g*, 20 min, 4°). Membranes from cells previously treated with (−)-sulpiride were preincubated in 6 ml of buffer (50 mM Tris, pH 7.4, and 1 mM EDTA) for 30 min at 37° and then sedimented by centrifugation (32,000 × *g*, 20 min, 4°). Membranes were resuspended in 6 ml of buffer without NaCl, recentrifuged as above, and resuspended in 2 ml of 50 mM Tris buffer, pH 7.4. An aliquot was removed for assay with [$^{125}$I]7OH-PIPAT (carried out on freshly harvested tissue), and the remainder was frozen at −70° for up to 5 days for assay with [$^{125}$I]NCQ 298. When assays with [$^{125}$I]NCQ 298 were to be carried out, membranes were thawed, recentrifuged (32,000 × *g*, 20 min, 4°), and resuspended in 2 ml of 50 mM Tris, pH 7.4.

**Radioligand binding techniques.** Membrane-bound receptors were measured with a filtration binding assay as described previously (37). Assays of $D2_L$ receptors were initiated by the addition of membranes (0.3–3 μg) to [$^{125}$I]NCQ 298 (10–1000 pM) in 50 mM Tris·HCl, pH 7.4, 50 mM NaCl, and 0.1% BSA or to [$^{125}$I]7OH-PIPAT (20–2000 pM) in 50 mM Tris·HCl, pH 7.4, 2 mM $MgCl_2$, and 0.1% BSA. Nonspecific binding was defined with 2 μM (+)-butaclamol. In experiments examining the effect of guanine nucleotides, 100 μM Gpp(NH)p and 2 mM $MgCl_2$ were included. Assays were incubated at 37° for 1 hr and terminated by the addition of ice-cold wash buffer (10 mM Tris·HCl, pH 7.4, and 154 mM NaCl for experiments involving [$^{125}$I]NCQ 298 or 20 mM Tris·HCl, pH 7.4, for experiments involving [$^{125}$I]7OH-PIPAT). Samples were filtered using a Brandel cell harvester with glass-fiber filters (No. 30; Schleicher & Schuell, Keene, NH, or GF-B; Whatman Labsales, Hillsboro, OR) presoaked in 0.3% polyethylenimine. Filters were washed with a total of 15 ml of wash buffer and dried under vacuum.

Saturation binding data were transformed by the method of Scat-

chard (38). $K_d$ and $B_{max}$ values were estimated by unweighted linear regression analysis. $K_d$ values were unchanged by drug treatment as sufficient washing of cells and membranes ensured that residual drug was removed before assay. Control $B_{max}$ values (from vehicle-treated samples) were between 500 and 3000 fmol/mg of protein, and data are presented as percentage of control $B_{max}$ values. Competition curves were analyzed by nonlinear regression using an iterative curve-fitting program written with the Solver program of Microsoft Excel 5.0 for the Macintosh (Microsoft Corp., Redmond, WA). $IC_{50}$ values were converted to $K_i$ values as described by Cheng and Prusoff (39). Protein was determined by the method of Bradford (40) using BSA as a standard.

**cAMP accumulation assays.** cAMP accumulation was determined from the conversion of $^3$H-ATP to $^3$H-cAMP using a modification of the method of Jones *et al.* (41) as described previously (26, 33). Cells were incubated overnight with 1 $\mu$Ci/ml $^3$H-adenine in culture media. Cells were then rinsed twice with 10 ml PBS, removed from plates in 10 ml PBS, collected by centrifugation (1000 × *g*, 10 min, 4°), resuspended in 10 ml PBS, and recentrifuged (1000 × *g*, 10 min, 4°). Final pellets were resuspended in 5 ml EMEM at 37°. Cells were incubated in glass tubes for 10 min at 37° in EMEM containing 50 mM HEPES, pH 7.4, and 1 mM isobutyl-1-methylxanthine (a phosphodiesterase inhibitor). Quinpirole was added in EMEM containing forskolin (final concentration of forskolin was 10 $\mu$M) and incubated for 7 min at 37°. Reactions were stopped by the addition of 1 ml of 7.5% trichloroacetic acid at 4°, and samples were cooled on ice. $^3$H-cAMP was separated from $^3$H-ATP and $^3$H-ADP by sequential chromatography over Dowex and alumina columns. cAMP accumulation is expressed as the percent conversion of total radioactivity in the ATP eluate to radioactivity in the cAMP fraction. Data for inhibition of cAMP accumulation by quinpirole were normalized to percentage of forskolin-stimulated accumulation in each treatment group.

## Results

**Effects of agonists on the density of the high affinity state of the $D2_L$ receptor.** The dose-dependency of agonist-induced regulation of $D2_L$ receptors was determined after incubation of 293-$D2_L$ cells with increasing concentrations of quinpirole for 16 hr (Fig. 1). The maximum concentration of agonist used was limited by our ability to remove residual drug from the harvested cells as well as the drug's solubility in culture medium; therefore, it was not possible to determine maximal effects. Exposure of cells to quinpirole resulted in a dose-dependent decrease in the number of receptors in the G protein-coupled state. The concentration of quinpirole required to decrease the density of sites with a high affinity for [$^{125}$I]7OH-PIPAT to half-basal levels was ~7 $\mu$M (Fig. 1, ●). In the same experiments, exposure of cells to quinpirole resulted in a dose-dependent increase in the density of $D2_L$ receptors, measured with [$^{125}$I]NCQ 298. The concentration of quinpirole required to increase the total number of receptors to twice basal levels was ~4 $\mu$M (Fig. 1, ○). Under control conditions, in the absence of drug, the $K_d$ of the receptor was 0.04–0.06 nM for [$^{125}$I]NCQ 298 and 0.3–0.5 nM for [$^{125}$I]7OH-PIPAT. The $K_d$ values of the receptor in quinpirole-treated tissues was unchanged after incubation with up to 0.1 mM quinpirole.

The time-dependency of agonist-induced regulation of $D2_L$ receptors was determined after incubation of 293-$D2_L$ cells with 5 $\mu$M quinpirole for times ranging from 5 min to 15 hr (Fig. 2). A time-dependent decrease in [$^{125}$I]7OH-PIPAT binding sites to 20% of basal levels was observed within 6 hr. The loss of high affinity sites was rapid with 20% of the sites lost after only 5 min of exposure to quinpirole and almost 50% lost after 15 min

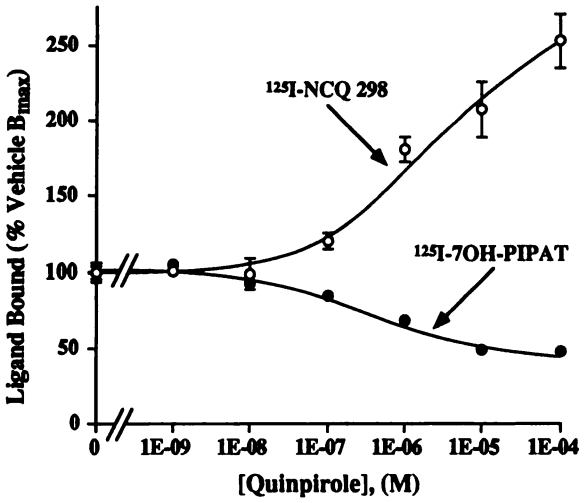


**Fig. 1.** Dose-dependency of receptor regulation by quinpirole in 293-$D2_L$ cells. Cells were exposed to the indicated concentrations of quinpirole for 16 hr. Assays with [$^{125}$I]7OH-PIPAT or [$^{125}$I]NCQ 298 were performed as described in Experimental Procedures. $B_{max}$ values were determined from Scatchard transformations of saturation binding data. Results are expressed as a percentage of the average density in vehicle-treated controls within each experiment. The average $B_{max}$ values for vehicle-treated controls were 210 ± 12 fmol/mg for [$^{125}$I]7OH-PIPAT and 615 ± 25 fmol/mg for [$^{125}$I]NCQ 298. Data shown are the mean ± standard error of four independent determinations for each data point assayed in triplicate.

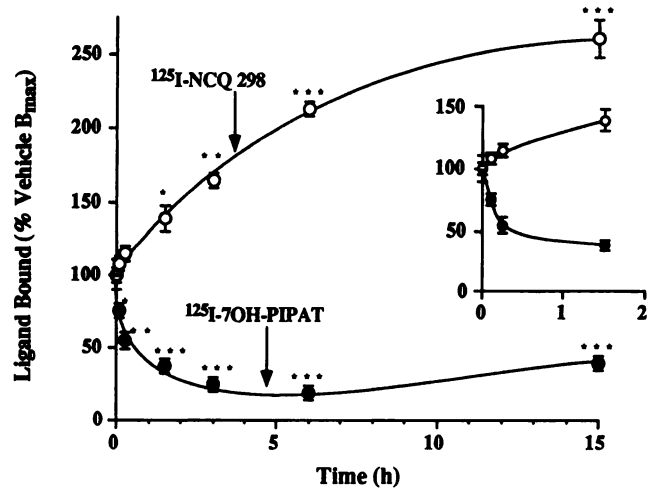


**Fig. 2.** Time course of the effect of quinpirole on 293-$D2_L$ cells. Cells were treated for times ranging from 5 min to 15 hr with 5 $\mu$M quinpirole or for 15 hr with vehicle. Saturation binding assays with [$^{125}$I]7OH-PIPAT or [$^{125}$I]NCQ 298 were performed on each sample, and $B_{max}$ values were determined from Scatchard transformations. Results are expressed as a percentage of the average density in vehicle-treated controls within each experiment. The average $B_{max}$ values for vehicle-treated controls were 389 ± 20 fmol/mg for [$^{125}$I]7OH-PIPAT and 1030 ± 106 fmol/mg for [$^{125}$I]NCQ 298. Data shown are the mean ± standard error of four independent determinations assayed in triplicate. *Inset*, expanded view of the first 2 hr of treatment. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared with vehicle controls.

of quinpirole treatment (Fig. 2, *inset*). A time-dependent increase in the density of receptors measured with [$^{125}$I]NCQ 298 was observed to 2–3-fold of basal levels within 6–15 hr (26). Exposure of 293-$D2_L$ cells to agonists affected the density of receptors measured with [$^{125}$I]NCQ 298 less rapidly than it affected receptors in the agonist-preferring state measured with [$^{125}$I]7OH-PIPAT (Fig. 2, *inset*).

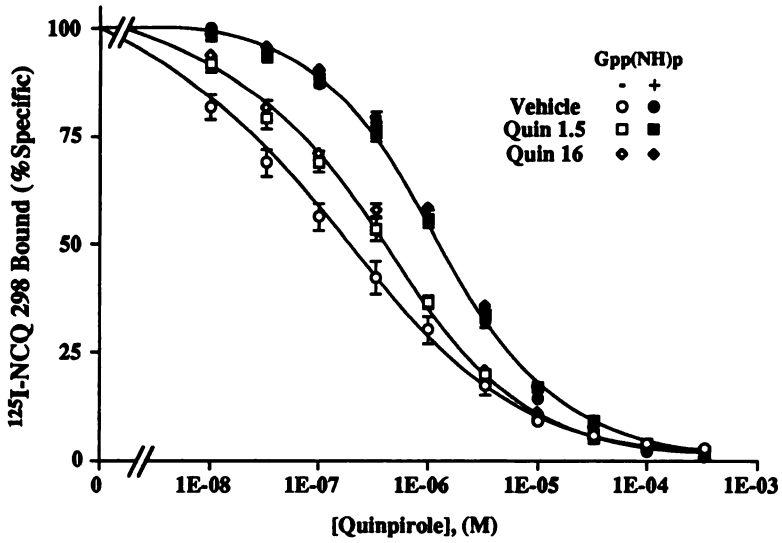


**Fig. 3.** Quinpirole inhibition of the binding of [$^{125}$I]NCQ 298 to membranes from vehicle- or quinpirole-treated 293-D2$_L$ cells. Cells were treated for 1.5 or 16 hr with 5 $\mu$M quinpirole (*Quin*) or for 16 hr with vehicle. Inhibition of [$^{125}$I]NCQ 298 binding by increasing concentrations of quinpirole was measured in the absence or presence of 100 $\mu$M Gpp(NH)p. Nonspecific binding was determined by the addition of 2 $\mu$M (+)-butaclamol. Data shown are the mean ± standard error of four independent determinations assayed in triplicate.

The effect of exposure to agonist was also examined in competition assays. Inhibition of the binding of [$^{125}$I]NCQ 298 to membranes from vehicle-treated 293-D2$_L$ cells by quinpirole revealed a shallow curve (Fig. 3, ○), which was shifted rightward and made steeper by the addition of Gpp(NH)p (Fig. 3, ●). Exposure of cells to 5 $\mu$M quinpirole for either 1.5 or 16 hr reduced the affinity of D2 receptors for the agonist (measured in the absence of guanine nucleotides), causing a small shift to the right in comparison with vehicle-treated cells (Fig. 3, □ and ◇). The addition of Gpp(NH)p to membranes from cells treated with agonist resulted in curves shifted to the right to the same extent as seen for vehicle-treated cells (Fig. 3, ■ and ◆).

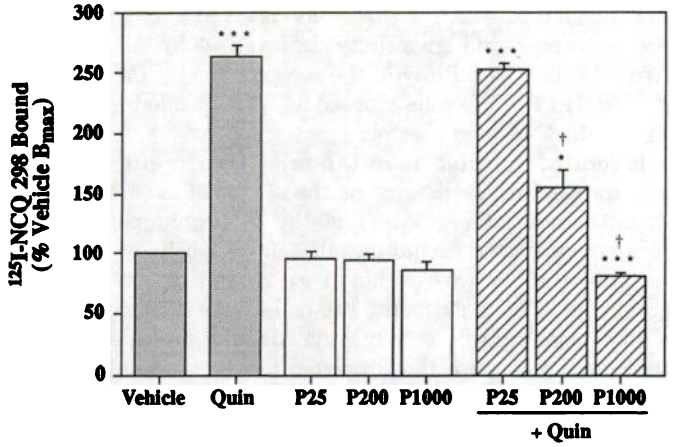


**Fig. 4.** Effect of PTX on agonist-induced regulation in 293-D2$_L$ cells. Cells were treated for 24 hr with PTX (*P25*, 25 ng/ml; *P200*, 200 ng/ml; *P1000*, 1000 ng/ml) or vehicle. Quinpirole (*Quin*) 5 $\mu$M or vehicle was then added, and incubation was continued for an additional 20 hr, after which cells were harvested and membranes were prepared. Saturation binding assays with [$^{125}$I]7OH-PIPAT or [$^{125}$I]NCQ 298 were performed on each sample, and $B_{max}$ values were determined from Scatchard transformations. Results with [$^{125}$I]NCQ 298 are expressed as a percentage of the average density in vehicle-treated controls. The average $B_{max}$ value for vehicle-treated controls was 583 ± 29 fmol/mg. Data shown are the mean ± standard error of four independent determinations for each treatment assayed in triplicate and are representative of two independent experiments. *, $p \leq 0.05$; ***, $p \leq 0.001$, compared with vehicle controls. †, $p \leq 0.001$, compared with treatment with quinpirole alone.

**Effect of PTX on agonist-induced effects.** The involvement of second messenger systems in the regulation of D2$_L$ receptors was investigated. PTX inactivates $G_i$ and $G_o$, thereby inhibiting the coupling of $G_i$- or $G_o$-linked receptors to second messengers. Agonist-induced up-regulation of D2$_L$ receptors was not blocked by pretreatment of 293-D2$_L$ cells with 25 ng/ml of PTX for 4 hr (27) or 24 hr (Fig. 4). Pretreatment of the transfected cells for 24 hr with significantly higher doses partially (200 ng PTX/ml) or completely (1000 ng PTX/ml) (see Ref. 28) blocked the agonist-induced up-regulation (Fig. 4). As expected, the binding of the agonist radioligand [$^{125}$I]7OH-PIPAT to membranes from 293-D2$_L$ cells was PTX sensitive, and thus examination of the effect of PTX on agonist-induced regulation of the high affinity state of the receptor was not possible.

**Effects of cAMP analogues on agonist-induced regulation.** Incubation of cells with cAMP analogues and forskolin has been shown to have synergistic effects with those of agonists, resulting in large increases in the density of D2$_L$ receptors in transfected 293 cells (26). The effect of cAMP analogues on agonist-induced regulation of receptors and of the G protein-coupled receptors was compared. 293-D2$_L$ cells were exposed to 5 $\mu$M quinpirole or quinpirole plus 100 $\mu$M 8-bromo-cAMP (Fig. 5). This effect was examined after both short (1.5 hr) and long (16 hr) periods of drug exposure. Synergism was seen between quinpirole and 8-bromo-cAMP on examination of the regulation of receptors with [$^{125}$I]NCQ 298 after either a long exposure (Fig. 5A, *hatched bars*), as reported previously (26), or a short exposure (Fig. 5A, *open bars*). Similarly, after short-term drug exposure, 8-bromo-cAMP acted synergistically with quinpirole to augment the decrease in the density of G protein-coupled receptors seen after treatment with 5 $\mu$M quinpirole alone (Fig. 5B, *open bars*). In the same experiments, the decrease in the density of high affinity sites seen after 16 hr of quinpirole treatment was not significantly enhanced by coincubation with 8-bromo-cAMP (Fig. 5B, *hatched bars*).

**Effects of antagonists on the density of the high affinity state of the D2$_L$ receptor.** The dose-dependency of antagonist-induced regulation of the high affinity state of the D2$_L$ receptor was determined after incubation of 293-D2$_L$ cells with increasing concentrations of (−)-sulpiride for 16 hr (Fig. 6). The

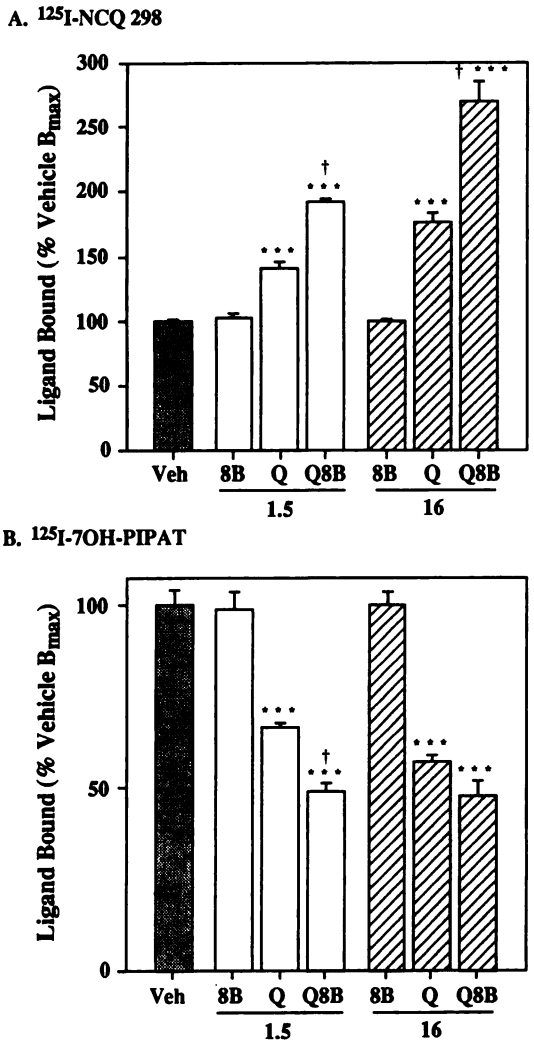


**Fig. 5.** Effect of 8-bromo-cAMP on agonist-induced regulation in 293-$D2_L$ cells. Cells were treated for 1.5 or 16 hr with 100 $\mu$M 8-bromo-cAMP (*8B*), 5 $\mu$M quinpirole (*Q*), or 5 $\mu$M quinpirole plus 100 $\mu$M 8-bromo-cAMP (*Q8B*) or for 16 hr with vehicle. Assays with [$^{125}$I]NCQ 298 (A) or [$^{125}$I]7OH-PIPAT (B) were performed as described, and $B_{max}$ values were determined from Scatchard transformations of saturation binding data. Results are expressed as a percentage of the average density in vehicle-treated controls within each experiment. The average $B_{max}$ values for vehicle-treated controls were 835 ± 118 fmol/mg for [$^{125}$I]7OH-PIPAT and 1610 ± 63 fmol/mg for [$^{125}$I]NCQ 298. Data shown are the mean ± standard error of eight independent determinations for each treatment assayed in triplicate. ***, $p \leq 0.001$, compared with vehicle controls. †, $p \leq 0.001$, compared with treatment with quinpirole alone.

maximum concentration of (−)-sulpiride used in the present study was limited by our ability to remove residual drug as well as by the solubility of the antagonist in culture medium; therefore, it was not possible to determine maximal effects. Unlike the effects seen with agonists, exposure to antagonists resulted in increases in the densities of binding sites for both the antagonist ligand [$^{125}$I]NCQ 298 and the agonist ligand [$^{125}$I]7OH-PIPAT. The concentration of (−)-sulpiride required to increase the density of receptors and high affinity sites to twice-basal levels was ~1 $\mu$M (Fig. 6).

The time-dependency of antagonist-induced regulation of the high affinity state of the $D2_L$ receptor was determined after incubation of 293-$D2_L$ cells with 1 $\mu$M (−)-sulpiride for times ranging from 15 min to 15 hr (Fig. 7). A time-dependent increase in the density of high affinity binding sites was observed to ~200% of basal level within ~15 hr. A 3–4-hr lag was seen in studies using a radiolabeled antagonist (Fig. 7, *inset*; see also Ref. 26). The increase in [$^{125}$I]7OH-PIPAT binding sites observed after exposure to an antagonist occurred, however, without a detectable lag. The increase in the number of G protein-coupled sites occurred rapidly with a 20–25% increase being seen after only 15 min of exposure to (−)-sulpiride (Fig. 7). A maximal increase (207 ± 7.6%) was observed after 6 hr. The $t_{1/2}$ for the increase in high affinity sites was ~3 hr. As in agonist-treated cells, the affinity of the receptor for [$^{125}$I]NCQ 298 or [$^{125}$I]7OH-PIPAT in cells exposed to (−)-sulpiride was the same as in vehicle-treated controls.

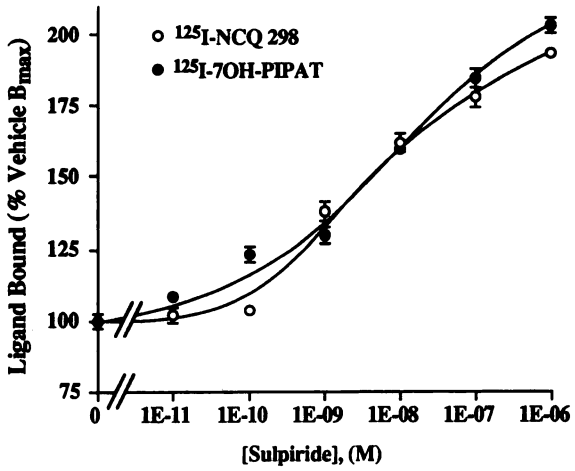


**Fig. 6.** Dose-dependency of receptor regulation by (−)-sulpiride in 293-$D2_L$ cells. Cells were exposed to the indicated concentrations of (−)-sulpiride for 16 hr. Assays with [$^{125}$I]7OH-PIPAT or [$^{125}$I]NCQ 298 were performed as described, and $B_{max}$ values were determined from Scatchard transformation of saturation binding data. Results are expressed as a percentage of the average density in vehicle-treated controls. The average $B_{max}$ values for vehicle-treated controls were 1060 ± 8 fmol/mg for [$^{125}$I]7OH-PIPAT and 2220 ± 52 fmol/mg for [$^{125}$I]NCQ 298. Data shown are the mean ± standard error of four independent determinations for each data point assayed in triplicate and are representative of two independent experiments.

**Effect of agonist or antagonist treatment on cAMP accumulation.** To determine the effects of agonist- and antagonist-induced regulation of the G protein-coupled $D2_L$ receptor on cAMP accumulation, 293-$D2_L$ cells were treated for 1.5 or 16 hr with either 5 $\mu$M quinpirole or 1 $\mu$M (−)-sulpiride. After treatment, the cells were washed, and the ability of quinpirole to inhibit cAMP accumulation was determined. Exposure to quinpirole for 16 hr resulted in increases in both basal and forskolin-stimulated levels of cAMP accumulation (Fig. 8B, *inset*). The $IC_{50}$ of quinpirole for inhibition of forskolin-stimulated cAMP accumulation was not changed (Fig. 8B; see also Ref. 27). Short-term exposure to agonist (5 $\mu$M quinpirole, 1.5 hr) resulted in desensitization seen as an increase in the $IC_{50}$ of quinpirole for inhibition of cAMP accumulation (Fig. 8A). The $IC_{50}$ values (five experiments) for quinpirole inhibition of cAMP accumulation were 3.8 ± 0.5 nM in vehicle-treated cells, 4.1 ± 0.5 nM in cells treated with quinpirole for 16 hr, and 38.5 ± 6.9 nM in cells treated with quinpirole for 1.5 hr. Maximal inhibition of cAMP accumulation by quinpirole was not changed by exposure of 293-$D2_L$ cells to agonist for 16 hr. Exposure of 293-$D2_L$ cells to (−)-sulpiride for 1.5 or 16 hr had no effect on the ability of quinpirole to inhibit cAMP accumulation (Fig. 8C).

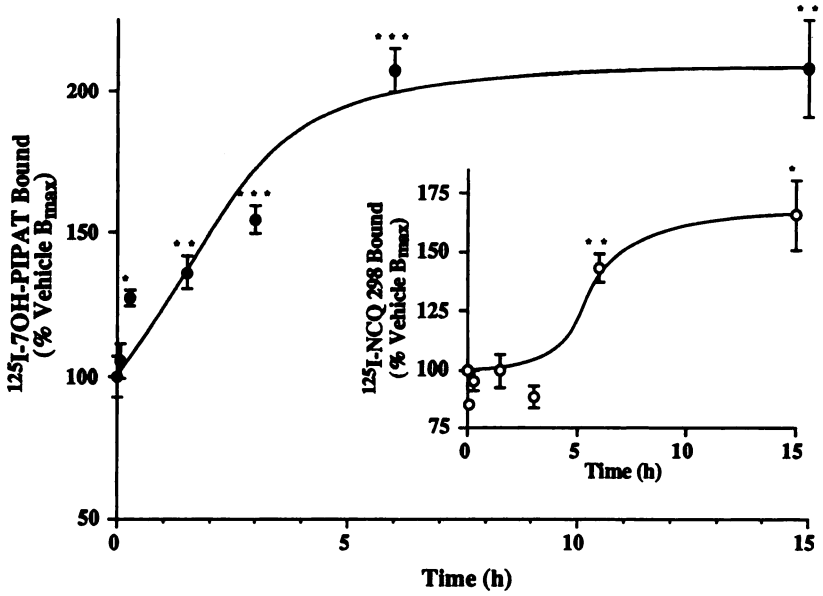


**Fig. 7.** Time course of the effect of (−)-sulpiride on 293-$D2_L$ cells. Cells were treated for 5 min to 15 hr with 1 μM sulpiride or for 15 hr with vehicle. Saturation binding assays with [$^{125}$I]7OH-PIPAT or [$^{125}$I]NCQ 298 (*inset*) were performed on each sample, and $B_{max}$ values were determined from Scatchard transformations. Results are expressed as a percentage of the average density in vehicle-treated controls within each experiment. The average $B_{max}$ values for vehicle-treated controls were 813 ± 58 fmol/mg for [$^{125}$I]7OH-PIPAT and 2210 ± 34 fmol/mg for [$^{125}$I]NCQ 298. Data shown are the mean ± standard error of four independent determinations assayed in triplicate. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared with vehicle controls.

**Effects of cycloheximide on agonist- and antagonist-induced regulation.** The protein synthesis inhibitor cycloheximide has been shown to attenuate the increase in receptors seen after incubation of 293-$D2_L$ cells with agonists or antagonists (27). The effects of cycloheximide on agonist- and antagonist-induced regulation of receptors and of G protein-coupled receptors were compared using 293-$D2_L$ cells pretreated for 30 min with either vehicle or 14 μM cycloheximide and subsequently treated for various times with vehicle, 5 μM quinpirole, or 1 μM (−)-sulpiride (Fig. 9). Exposure to 14 μM cycloheximide did not change basal levels of $D2_L$ receptors (Fig. 9, A through D, *open bars*). Cycloheximide blocked the agonist-induced up-regulation of receptors measured with [$^{125}$I]NCQ 298 when cells were exposed to drugs for 12 hr (Fig. 9A, *hatched bars*; see also Ref. 27) or for 1.5 hr (Fig. 9A, *open bars*). Cycloheximide had no effect, however, on the quinpirole-induced loss of high affinity sites (Fig. 9B). The up-regulation of receptors and the increase in high affinity binding sites seen after treatment with (−)-sulpiride for 12 hr were blocked by cycloheximide (Fig. 9, C and D, *hatched bars*). In contrast, after 1.5 hr of exposure to (−)-sulpiride, when the total receptor population is not yet up-regulated, the increase in high affinity binding sites was not affected by cycloheximide (Fig. 9D, *open bars*).

## Discussion

Exposure of transfected 293 cells expressing $D2_L$ dopamine receptors to agonists decreased the density of $D2_L$ receptors that are in a G protein-coupled, high affinity state. The complete loss of [$^{125}$I]7OH-PIPAT binding in cells that were pretreated with PTX confirms our earlier findings that [$^{125}$I]7OH-PIPAT is labeling G protein-coupled receptors (32, 33) and suggests that the high affinity state of the receptor involves a PTX-sensitive G protein such as $G_i$ or $G_o$.

The loss of sites with high affinity for agonists was seen not only directly with the binding of [$^{125}$I]7OH-PIPAT but also indirectly in studies of the inhibition of [$^{125}$I]NCQ 298 binding by agonists. The concentration of quinpirole required to see this loss of high affinity binding sites was similar to the concentration required to cause an up-regulation of total sites. The time courses of these two regulatory events, however, were distinct. Up-regulation of $D2_L$ receptors occurred with a $t_{1/2}$ of ~4 hr, whereas the decrease in the density of receptors with a high affinity for agonists occurred with a $t_{1/2}$ of 10–15 min. The rapidity of the loss of high affinity sites suggests that the receptors are uncoupling from G proteins. Results of studies of the inhibition of cAMP accumulation also support the hypothesis that receptors are rapidly uncoupling as desensitization was seen after exposure to 5 μM quinpirole for 1.5 hr. The observed effects may be due to phosphorylation of the agonist-occupied receptor by a GRK. Phosphorylation of sites in the third intracellular loop of the $\alpha_{2A}$-adrenergic receptor by a GRK has been shown to confer agonist-mediated desensitization (42). Similarly, GRK phosphorylation sites in the central part of the third intracellular loop of the m2 muscarinic acetylcholine receptor have been identified (43). Although the carboxyl terminus of the D2 receptors contains no serine or threonine residues, there are 10 or 12 threonine and 13 or 15 serine residues in the third intracellular loops of $D2_S$ or $D2_L$ receptors, respectively. The enhancement of the quinpirole-induced loss of high affinity binding sites by 8-bromo-cAMP might be due to the involvement of cAMP-dependent protein kinase. There are five possible sites for phosphorylation by cAMP-dependent protein kinase in the third intracellular loop of the D2 receptors.

We previously reported that agonist-induced up-regulation of $D2_L$ receptors in 293-$D2_L$ cells did not involve a PTX-sensitive G protein as a concentration of PTX sufficient to block agonist-induced inhibition of forskolin-stimulated cAMP accumulation did not block the up-regulation (27). Zhang *et al.* (28) reported up-regulation of the $D2_L$ receptor in CHO cells to be sensitive to high doses of PTX (1000 ng/ml). In C6 glioma cells expressing the $D2_L$ receptor, agonist-induced up-regulation was not blocked by pretreatment with 100 ng PTX/ml (29). It was originally suggested that these differences in sensitivity to PTX may be attributable to host cell differences (27). The ability of high concentrations of PTX to block the up-regulation of $D2_L$ receptors in 293 cells as well as in CHO cells may involve ribosylation of a G protein with a sensitivity for PTX that is different from that of the G

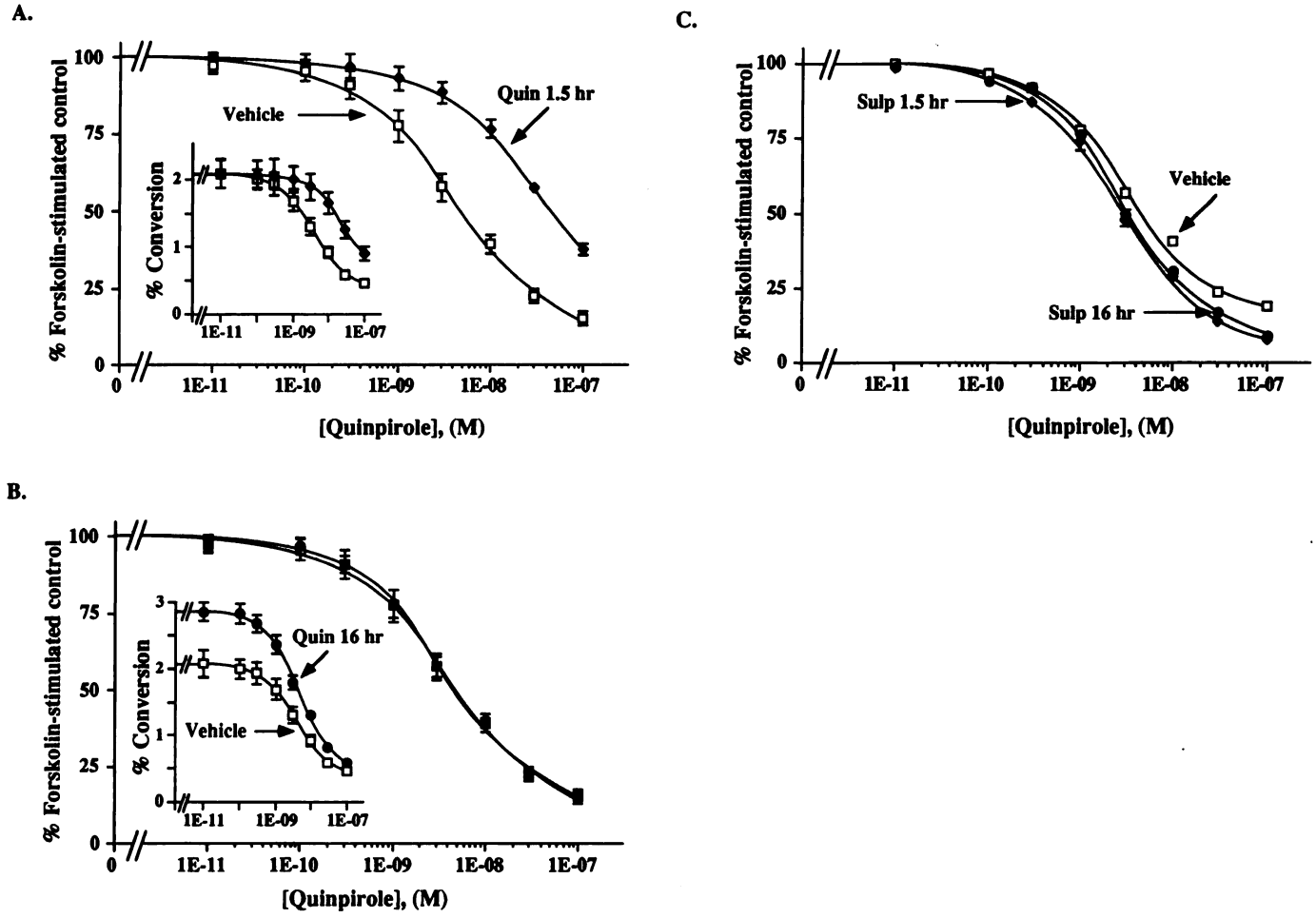


**Fig. 8.** Inhibition of cAMP accumulation by quinpirole in 293-$D2_L$ cells treated with quinpirole or (−)-sulpiride. Cells were incubated with 5 μM quinpirole or 1 μM (−)-sulpiride for 1.5 or 16 hr. 293-$D2_L$ cells were assayed for inhibition of cAMP accumulation as described. Forskolin (10 μM) was added to each sample to stimulate cAMP accumulation. Increasing amounts of quinpirole were added to cells before incubation for 7 min. 293-$D2_L$ cells were pretreated with vehicle (□) or 5 μM quinpirole (♦) for 1.5 hr (A) or with vehicle (□) or 5 μM quinpirole (●) for 16 hr (B). *Insets*, the same data are expressed as percentage of conversion of $^3$H-ATP to $^3$H-cAMP. C, 293-$D2_L$ cells were pretreated with vehicle for 16 hr (□) or 1 μM (−)-sulpiride for 1.5 hr (♦) or 16 hr (●). Results shown are the mean ± standard error of three (C) or five (A and B) independent determinations for each data point assayed in triplicate.

proteins linked to the inhibition of adenylyl cyclase. Differential PTX sensitivity of multiple effects associated with the same receptor has been described by Felder *et al.* (44). They demonstrated that the concentration of PTX necessary to maximally inhibit m2 muscarinic receptor-mediated augmentation of ATP-stimulated arachidonic acid release was 100-fold less than that required to maximally block m2 receptor-mediated inhibition of cAMP accumulation.

As Filtz *et al.* (27) reported, neither the potency nor the efficacy of quinpirole to inhibit cAMP accumulation stimulated by forskolin was changed by pretreatment with quinpirole for 16 hr. Basal and forskolin-stimulated levels of cAMP accumulation were increased, however, in 293-$D2_L$ cells pretreated with quinpirole for 16 hr. The sensitization of basal and forskolin-stimulated cAMP levels after pretreatment with dopamine without desensitization of the response to dopaminergic agonists was also seen in SUP1 cells, which endogenously express D2 receptors (25). Agonist-induced up-regulation of D2 receptors accompanied by a time-dependent desensitization of cAMP accumulation was seen in CHO-$D2_L$ cells (28) and, more recently, in C6-$D2_L$ cells (29). In the present study with 293-$D2_L$ cells, desensitization was seen after the cells were exposed to agonist for relatively short periods but not after longer periods of exposure. These differences in regulation of the functional response may be attributable to the different cell lines used to express the receptors.

The density of receptors with a high affinity for [$^{125}$I]7OH-PIPAT was reduced after exposure of cells to quinpirole for 1.5 or 16 hr. Desensitization was seen, however, only after the short term agonist treatment. A maximal decrease in the density of high affinity binding sites was seen after exposure of the cells to quinpirole for 6 hr. After 15 hr of agonist exposure, the density of receptors in the high affinity state appeared to be returning toward control levels. It is possible that some of the binding sites for [$^{125}$I]7OH-PIPAT observed even in untreated cells involve G proteins that are not linked to adenylyl cyclase and that the rapid uncoupling that takes place in the first hour after exposure to agonist selectively uncouples the adenylyl cyclase-linked G proteins. Although a decrease in high affinity sites was still observed after exposure to agonist for 16 hr, a decrease in functional response was no longer seen. A change in either the type of G protein associated with the receptor or in the properties of an already associated G protein could explain these results.

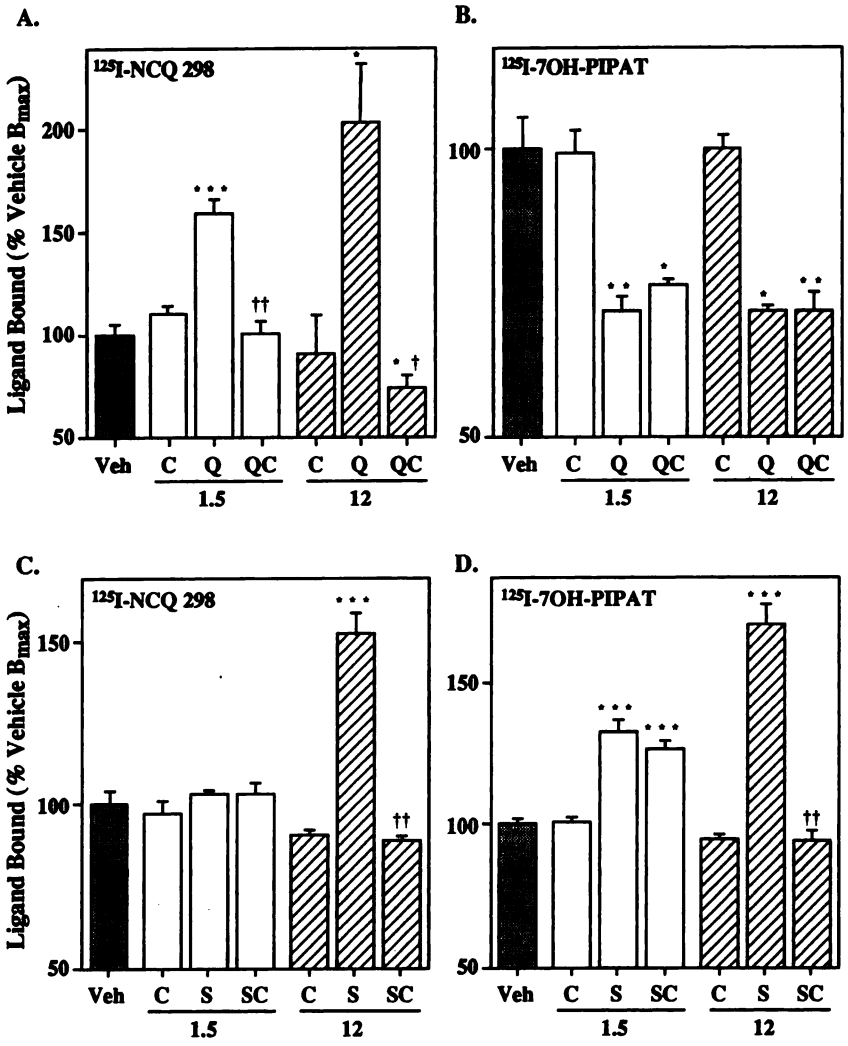


**Fig. 9.** Effect of cycloheximide on regulation of receptors in 293-$D2_L$ cells. Cells were harvested and assays carried out with [$^{125}$I]NCQ 298 (A and C) or [$^{125}$I]7OH-PIPAT (B and D) performed as described. $B_{max}$ values were determined from Scatchard transformation of saturation binding data. Results are expressed as a percentage of the average density in vehicle-treated controls within each experiment. A and B, Cells were treated for 1.5 or 12 hr with 14 $\mu$M cycloheximide (*C*), 5 $\mu$M quinpirole (*Q*), or cycloheximide plus quinpirole (*QC*), or for 12 hr with vehicle. The average $B_{max}$ values for vehicle-treated controls were 896 ± 56 fmol/mg for [$^{125}$I]7OH-PIPAT and 2850 ± 174 fmol/mg for [$^{125}$I]NCQ 298. C and D, Cells were treated for 1.5 or 12 hr with 14 $\mu$M cycloheximide (*C*), 1 $\mu$M (−)-sulpiride (*S*), or cycloheximide plus (−)-sulpiride (*SC*) or for 12 hr with vehicle. The average $B_{max}$ values for vehicle-treated controls were 949 ± 193 fmol/mg for [$^{125}$I]7OH-PIPAT and 2510 ± 233 fmol/mg for [$^{125}$I]NCQ 298. Indicated times are for vehicle, agonist or antagonist treatment; cycloheximide was added to the cells 30 min before the beginning of drug treatment. Data shown are the mean ± standard error of four to eight independent determinations assayed in triplicate. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, compared with vehicle controls. †, $p \leq 0.01$; †† $p \leq 0.001$, compared with treatment with quinpirole or sulpiride alone.

Treatment of 293-$D2_L$ cells with an antagonist resulted in an increase in the density of receptors labeled by [$^{125}$I]NCQ 298 and agonist-preferring receptors labeled by [$^{125}$I]7OH-PIPAT without changing the affinity of the receptors for either radioligand. The increase in the number of $D2_L$ receptors in the agonist-preferring state was not accompanied by a change in the functional response. Pretreatment of 293-$D2_L$ cells with (−)-sulpiride had little or no effect on either the potency or the efficacy of quinpirole-inhibition of cAMP accumulation. This suggests that the increase in coupling involves G proteins that are not able to inhibit adenylyl cyclase. The dose responses for the antagonist-induced up-regulation of receptors and for the increase in the density of high affinity sites were almost identical. The time courses of these two antagonist-induced effects were, however, entirely distinct. The lag seen for up-regulation of the binding of [$^{125}$I]NCQ 298 was not seen for the increase in the number of high affinity sites. The rapidity of the effect of antagonists on high affinity sites suggests that antagonists induce an interaction of uncoupled receptors with available G proteins.

The roles of protein synthesis and degradation were studied with the use of cycloheximide. Exposure to cycloheximide did not significantly affect basal levels of $D2_L$ receptors, but up-regulation of receptors seen after exposure of cells to quinpirole or (−)-sulpiride was blocked. Cycloheximide had no effect on agonist-induced loss of high affinity binding sites. Cycloheximide blocked the increase in [$^{125}$I]7OH-PIPAT binding seen after 12 hr of antagonist exposure (when total receptor density is up-regulated) but not the increase seen after only 1.5 hr of treatment (before the onset of receptor up-regulation). Protein synthesis is therefore required to achieve the elevated number of receptor/G protein complexes present after 12 hr of antagonist exposure but not after 1.5 hr. The increase in high affinity sites after prolonged exposure to an antagonist may be due in part to the elevated total receptor number, whereas the increase in agonist-preferring receptors after short term exposure to an antagonist is due entirely to an increase in coupling. The antagonist-induced changes in high affinity binding, like the agonist-induced changes, suggest that the agonist-preferring receptors present at 1.5 and 16 hr may involve different populations of G proteins.

In summary, exposure of 293-$D2_L$ cells to either agonists or antagonists differentially regulates the G protein-coupled D2 receptors. Agonists and antagonists induce opposite effects on the density of $D2_L$ receptors with high affinity for agonists. Agonist- and antagonist-induced up-regulation of the density of $D2_L$ receptors is also seen in these cells. Although the time- and dose-dependencies of the agonist-induced effects are similar, the agonist-induced up-regulation and regulation of the G pro-

tein-coupled D2 receptor appear to be mechanistically distinct. On the other hand, results obtained with cycloheximide suggest that the changes in high affinity binding and the up-regulation seen after prolonged exposure to antagonists are not entirely independent. These data suggest that multiple mechanisms are involved in the regulation of both the expression and coupling of D2 receptors in 293-$D2_L$ cells.

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