0026-895X/96/030560-07$3.00/0

MOLECULAR PHARMACOLOGY, 49:560–566 (1996).

Sequestration of the Short and Long Isoforms of Dopamine D_2 Receptors Expressed in Chinese Hamster Ovary Cells

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Received July 5, 1995; Accepted November 9, 1995

SUMMARY

The short (D_{2S}) and long (D_{2L}) isoforms of dopamine D_2 receptors were stably expressed in Chinese hamster ovary cells, and dopamine-induced sequestration was examined by measuring the loss of binding of the hydrophilic ligand [^{3}H]sulpiride from the cell surface. Dopamine treatment of Chinese hamster ovary cells expressing D_{2S} for 30 min at 37° caused a 43.8 ± 3.4% decrease in [^{3}H]sulpiride binding activity measured by incubation of the treated cells with [^{3}H]sulpiride at 4° for 4 hr after the dopamine was washed out. The half-life of the decrease in binding was estimated to be 18.7 ± 1.6 min, and the concentration of dopamine giving a half-maximal effect (EC_{50}) was estimated to be 180 ± 90 nM. The decrease was reversible, and the binding activity was recovered by washing out the dopamine and incubating the cells at 37° for 30 min but was not reversible when the cells were incubated at 4°. The binding activity of [^{3}H]spiperone, a hydrophobic ligand, was not affected by the dopamine treatment under the same experimental conditions. These results indicate that approximately one half of the D_{2S} receptors undergo agonist-induced sequestration, probably endocytosis, in a reversible and temperature-dependent manner. Sequestration of D_{2L} receptors was not as apparent as that of D_{2S} receptors; the decrease in [^{3}H]sulpiride binding activity was 21.6 ± 0.9% and the rate of the decrease was delayed, with a half-life of 33.2 ± 7.8 min, although effective concentrations of dopamine were similar, with EC_{50} = 170 ± 50 nM. A D_{2S} receptor variant containing a missense mutation changing Ser^{311} in the third intracellular loop to cysteine was found to be sequestered to a significantly lesser extent than with wild-type D_{2S} receptors. This finding was discussed with respect to the report that this variant gene is found more frequently in schizophrenic patients than in control subjects.

Dopaminergic neurons are generally believed to regulate neuronal pathways in the nigrostriatum, mesocorticolimbus, and tuberoinfundibulum. The nigrostriatal pathway is involved in movement, the mesocorticolimbic pathway is involved in cognition and emotion, and the tuberoinfundibular pathway is involved in prolactin secretion from the pituitary (1). These physiological processes have been suggested to be related to Parkinson's disease (2), drug and alcohol abuse (3), and schizophrenia (4). Dopamine D_1 and D_2 receptors were pharmacologically defined on the basis of specificity for ligands and effects on second messenger systems: D_1 receptor refers to the dopamine receptor associated with dopamine-stimulated adenylate cyclase, and D_2 receptor refers to the dopamine receptor associated with dopamine-inhibited adenylate cyclase (5). cDNAs encoding five different dopamine receptors have been cloned (6–10). All of these dopamine receptors are members of the seven-transmembrane and G protein-coupled receptor family. D_1 and D_5 receptor subtypes exhibit a classic D_1 pharmacological profile (11), being functionally linked to increasing intracellular cAMP levels through activation of adenylyl cyclase. D_2, D_3, and D_4 receptors demonstrate a D_2-like pharmacology (12), bind spiperone with high affinities as a prototypic antagonist, and are likely to be the sites of action for drugs used to treat schizophrenia.

D_2 receptors are encoded by two kinds of mRNAs produced by alternative splicing. The long (D_{2L}) and short (D_{2S}) forms of D_2 receptors are composed of 444 and 415 amino acid residues, respectively, and an insertion of 29 amino acid residues exists in the third intracellular loop of D_{2L} (13). The tissue distribution of these two isoforms is similar, whereas the D_{2L} receptor appears to be expressed predominantly in most tissues. Both D_{2L} and D_{2S} receptors are linked to G_i and G_o. Some differences between D_{2L} and D_{2S} receptors have also been reported in their interaction with G proteins; in

This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan and the National Center of Neurology and Psychiatry of the Ministry of Health and Welfare, Japan.

ABBREVIATIONS: D_{2L}, long isoform of the dopamine D_2 receptor; D_{2S}, short isoform of the dopamine D_2 receptor; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; HEPES, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

GH4C1 cells transfected with D_{2L} or D_{2S}, antisense $G_{\alpha o}$ completely abolished D_{2S} receptor-mediated inhibition of calcium entry but only partially (70%) abolished D_{2L} receptor-mediated inhibition, whereas antisense $G_{\alpha i2}$ completely abolished D_{2L} receptor-mediated inhibition of adenylate cyclase but only partially (30%) abolished D_{2S} receptor-mediated inhibition (14, 15).

The D_2 receptors as well as muscarinic receptors are characterized by their long third intracellular loops. The third cytoplasmic loops of muscarinic receptors have been indicated to be involved in receptor down-regulation (16) and sequestration (17). Regarding the change in D_2 receptor density after exposure to agonists, conflicting results have been reported. These include reports of (i) no change in the density of D_2 receptor (18–20) or D_{2S} receptors (21), (ii) an increase in the density of D_2 receptors (22) or of both D_{2S} and D_{2L} receptors (23), and (iii) a decrease in the density of D_{2S} receptors and an increase in the density of D_{2L} receptors (24). In all of these reports, the receptor density was estimated by radioligand binding assay with the use of membrane preparations, and no discrimination was made between reversible sequestration (or internalization) and irreversible down-regulation.

We stably expressed cDNAs of D_{2L} and D_{2S} in CHO cells and examined their sequestration as the reversible loss of [^{3}H]sulpiride binding sites from the cell surface. At the same time, we examined the sequestration of a D_2 receptor variant (D_2 S311C, a missense variant with a change from serine to cysteine at residue 311 in the third intracellular loop of the dopamine receptor gene) (25). The D_2 S311C variant has been found more frequently in schizophrenic patients than in control subjects, and schizophrenic patients with D_2 S311C have had certain clinical features, such as less severe thought disorder, flattened affect, and psychomotor retardation, compared with schizophrenics without the variant D_2 receptor (26).

Experimental Procedures

Materials. Dopamine and haloperidol were purchased from Sigma Chemical Co.; [^{3}H]methylspiperone (specific activity, 19.0 Ci/mmol) and [^{3}H](−)-sulpiride (specific activity, 72.4 Ci/mmol) were purchased from DuPont-NEN; restriction enzymes were from obtained Toyobo Corp.; and the Transformer site-directed mutagenesis kit was obtained from Clontech. cDNA of D_{2S} receptor (pZem3) (7) was donated by Dr. D. K. Grandy; cDNA of D_{2L} receptor (pCRII) was donated by Yamanouchi Pharmaceutical Co. Ltd.; and pEF-Neo, the mammalian expression vector pEF-BOS, was donated by Dr. S. Nagata, Osaka Bioscience Institute. CHO-KI cells were obtained from Japanese Cancer Research Resources.

Vector construction and mutagenesis of D_{2L} and D_{2S} receptors. A *Hin*dIII/*Kpn*I fragment of pZem3 was inserted into the *Xba*I site of pEF-BOS after the conversion of both fragments to blunt ends (pEF-D_{2S}). An *Nsi*I fragment of pCRII was inserted into the *Xba*I site of pEF-BOS after the conversion of both fragments to blunt ends (pEF-D_{2L}). *Xba*I/*Hin*dIII fragments of pZem3 and pCRII were inserted into the *Xba*I and *Hin*dIII sites of pUC19, and site-directed mutagenesis was carried out with the Transformer site-directed mutagenesis kit. An oligonucleotide (5′-GGAGACCATGGTG-GCACGGGTCGGG-3′) was used to change Ser^{311} to cysteine. A mutant clone was sequenced, and an *Eco*RI/*Hin*dIII fragment of plasmid was inserted into the *Xba*I site of pEF-BOS after the conversion of both fragments to blunt ends. CHO cells (5×10^5 cells in a 10-cm dish) were transfected with 2 μg of pEF-Neo and 5 μg of either pEF-D_{2S}, pEF-D_{2L}, a mutant type of pEF-D_{2S}, or a mutant type of pEF-D_{2L} according to the calcium phosphate method (27). Stable transfectants were selected in the presence of 400 mg/ml geneticin and were subcloned by limiting dilution. Expression of receptors was detected with the use of [^{3}H]methylspiperone binding. The [^{3}H]methylspiperone binding sites on CHO cells transfected with pEF-D_{2S} or pEF-D_{2L} varied from 0.6 to 4 pmol/mg protein. The cell lines with similar expression levels were chosen for comparison of sequestration between D_{2S} and D_{2L} receptors and between variant and wild-type D_2 receptors. B_{max} values (pmol/5 × 10^5 cells) for [^{3}H]spiperone and [^{3}H]sulpiride binding were estimated to be 1.06 ± 0.048 and 1.06 ± 0.036 for D_{2L}, 1.17 ± 0.12 and 1.15 ± 0.035 for D_{2L} S311C, 1.03 ± 0.068 and 0.69 ± 0.093 for D_{2S}, and 0.91 ± 0.074 and 0.80 for D_{2S} S311C receptors, respectively. CHO cells expressing D_2 receptors were cultured in F-12 nutrient mixture medium (Ham's) (Life Technologies Inc.) supplemented with 10% fetal bovine serum (Cansera International Inc.), 40 units/ml penicillin G (Meiji-Seika, Kaisha Ltd.), 40 mg/ml streptomycin sulfate (Meiji-Seika, Kaisha Ltd.), and 100 mg/ml geneticin (Life Technologies Inc.) at 37° in 95% air/5% CO_2.

Assay of sequestration of D_2 receptors. CHO cells (5×10^5 cells/well) were incubated with 10^{-9}–10^{-4} M dopamine plus 0.4 mM sodium metabisulfate (antioxidant) for 15–120 min, washed with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.5; 1 ml/well) five times, and then incubated with 1.4 nM [^{3}H]sulpiride or 5.3 nM [^{3}H]methylspiperone in 0.5 ml HEPES-buffered saline (25 mM HEPES, 113 mM NaCl, 6 mM glucose, 3 mM $CaCl_2$, 3 mM KCl, 2 mM $MgSO_4$, and 1 mM NaH_2PO_4, pH 7.4) at 4° for 4 hr. After incubation, cells were washed with ice-cold PBS (1 ml/well) three times, dissolved in 0.3 ml of 1% Triton X-100 (w/v), and then mixed with 4.5 ml of Triton/Toluene mixture (3:7 v/v) containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene; then the radioactivity was measured. Each assay was carried out in triplicate. Nonspecific binding was estimated in the presence of 10^{-5} M haloperidol. For the estimation of K_d and B_{max} values for [^{3}H]sulpiride and [^{3}H]methylspiperone binding, CHO cells (2.5×10^5 cells/well) expressing D_{2S} receptors were incubated with 2–20 nM [^{3}H]sulpiride in 0.5 ml HEPES-buffered saline or 0.1–5 nM [^{3}H]methylspiperone in 2.5 ml of HEPES-buffered saline at 4° for 4 hr. K_d values were estimated to be 6.3 nM for [^{3}H]sulpiride and 0.32 nM for [^{3}H]methylspiperone, values that were equal to or a little higher than those previously reported (28).

Data analysis. Dose-response curves were fitted to the following equation: $R_{max} \times EC_{50}/(EC_{50} + [\text{dopamine}]) + (100 - R_{max})$. Time course curves were fitted to the following equation: $B \times \exp(-0.69t/t_{1/2}) + (100 - B)$. Data were analyzed with Student's t test.

Results

We expressed the D_{2S} and D_{2L} isoforms of dopamine D_2 receptors in CHO cells and examined the effect of treatment of these cells with dopamine on [^{3}H]sulpiride or [^{3}H]spiperone binding activity. The binding assay was carried out by incubation of treated cells with [^{3}H]sulpiride at 4° for 4 hr after the dopamine was washed out. Fig. 1 shows the [^{3}H]sulpiride binding activity of D_{2S} and D_{2L} receptors on CHO cells that were treated with 10^{-5} M dopamine for 0–120 min (Fig. 1A) or with 10^{-10}–10^{-4} M dopamine for 30 min (Fig. 1B). [^{3}H]Sulpiride binding activity rapidly decreased to 50% of the original value, and the half-life of the decrease was estimated to be 18.7 ± 1.6 min. The concentration of dopamine giving a half-maximal effect was estimated to be 180 ± 90 nM (Fig. 1B). A decrease in [^{3}H]sulpiride binding activity was observed for D_{2L} receptors as well as for D_{2S} receptors. However, the time course of the decrease was much slower for D_{2L} ($t_{1/2}$ = 33.2 ± 7.7 min), and the extent of the decrease

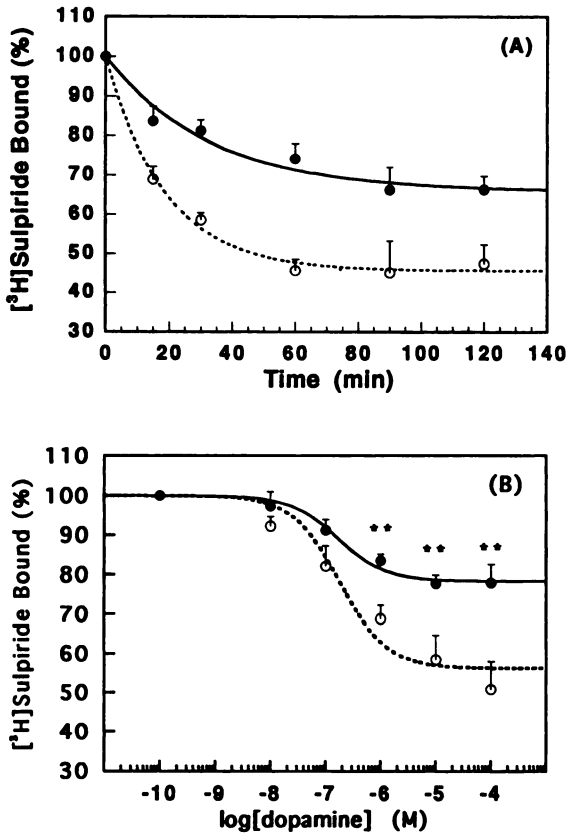

Fig. 1. Effect of treatment with various concentrations of dopamine on [^{3}H]sulpiride binding activity of CHO cells. A, CHO cells expressing D_{2S} (○) or D_{2L} (●) receptors were incubated with 10^{-5} M dopamine for indicated times, washed thoroughly with PBS, and then subjected to [^{3}H]sulpiride binding assays in the presence or absence of 10^{-5} M haloperidol at 4° for 4 hr. The difference in the binding activity in the absence or presence of haloperidol was defined as the specific binding. Results are shown as mean ± standard deviation from three independent experiments assayed in triplicate. B, CHO cells were incubated with indicated concentrations of dopamine for 30 min followed by [^{3}H]sulpiride binding assays. Results are shown as mean ± standard deviation from five independent experiments assayed in triplicate. Specific [^{3}H]sulpiride binding activity on control cells incubated without dopamine was taken as 100%, and actual counts of total and nonspecific binding were 10,000–14,000 and 500–1,000 cpm, respectively. **, Significant difference ($p < 0.001$) in [^{3}H]sulpiride binding activity between D_{2L} and D_{2S}. Curves in A and B were fitted to the following equations: $B \times \exp(-0.69t/t_{1/2}) + (100 - B)$ and $R_{max} \times EC_{50}/(EC_{50} + [\text{dopamine}]) + (100 - R_{max})$, respectively.

in the [^{3}H]sulpiride binding activity was less for D_{2L} receptors than for D_{2S} receptors; the maximal proportion of the decreased [^{3}H]sulpiride binding activity (R_{max}) was 21.6 ± 0.9% and 43.8 ± 3.4% for D_{2L} and D_{2S} receptors, respectively. Concentrations of dopamine giving a half-maximal effect (EC_{50}) were not different for D_{2L} (170 ± 50 nM) and D_{2S} (180 ± 90 nM) receptors. These differences in response between the D_{2S} and D_{2L} receptors were observed in several independent clones. A decrease in [^{3}H]sulpiride binding activity was not induced by treatment with sulpiride, a dopaminergic antagonist, and the effect of treatment with dopamine was antagonized by the addition of sulpiride in the medium (Fig. 2). This result indicates that the effect is agonist specific.

The decreased [^{3}H]sulpiride binding activity was restored by washing out the dopamine and incubating the cells at 37° but was not recovered when cells were incubated at 4°. The

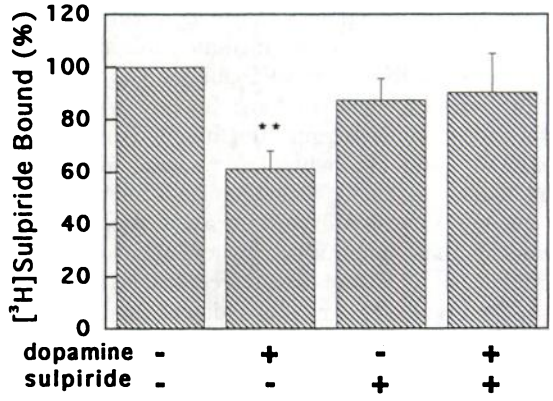

Fig. 2. Effect of treatment with dopamine and sulpiride on [^{3}H]sulpiride binding activity of CHO cells expressing D_{2S} receptor. CHO cells were cultured in the absence or presence of 10^{-6} M dopamine and 10^{-6} M sulpiride for 30 min, washed with PBS, and then subjected to [^{3}H]sulpiride binding assays at 4° for 4 hr. Results are shown as mean ± standard deviation from two independent experiments assayed in quadruplicate. [^{3}H]Sulpiride binding activity of cells treated with dopamine without sulpiride is significantly less than that of the other cells ($p < 0.001$).

time course of recovery at 37° for D_{2S} receptors is shown in Fig. 3. The rate of recovery is similar to that of the decrease, and ~30 min are required for full recovery. The restoration of [^{3}H]sulpiride binding activity was also observed for D_{2L} receptors, although ~60 min were required for full recovery (data not shown). [^{3}H]Sulpiride binding activity determined by the incubation at 4° for 4 hr for control cells was essentially the same as the binding activities determined by incubation at 37° for 1 or 2 hr followed by incubation at 4° for 2 or 3 hr (Fig. 3). These results indicate that the incubation at 4° for 4 hr is sufficient to label available [^{3}H]sulpiride binding sites.

In contrast with [^{3}H]sulpiride binding, [^{3}H]spiperone binding activity was not affected by treatment with dopamine for 2 hr under the same conditions in which [^{3}H]sulpiride binding activity decreased. A decrease in [^{3}H]spiperone binding activity was not observed even after incubation for 24 hr with dopamine (Fig. 4A). [^{3}H]Sulpiride binding activity decreased

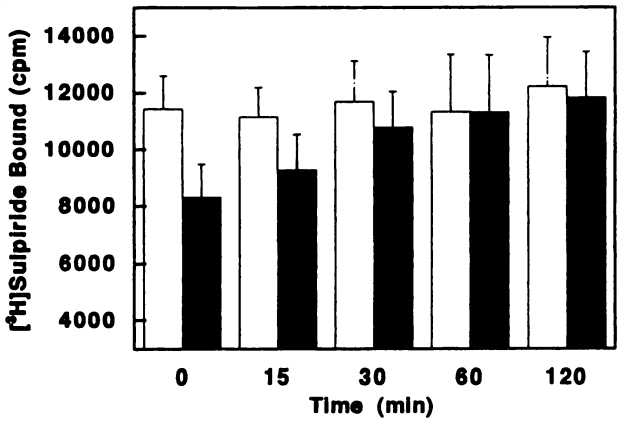

Fig. 3. Recovery of [^{3}H]sulpiride binding activity. CHO cells expressing D_{2S} receptors were incubated with (*solid bars*) or without (*open bars*) 10^{-6} M dopamine for 30 min, washed thoroughly with PBS, and then subjected to [^{3}H]sulpiride binding for the indicated times (0–120 min) at 37° and then for 240–120 min at 4° (total incubation time, 240 min). Results are shown as mean ± standard deviation from three independent experiments assayed in triplicate.

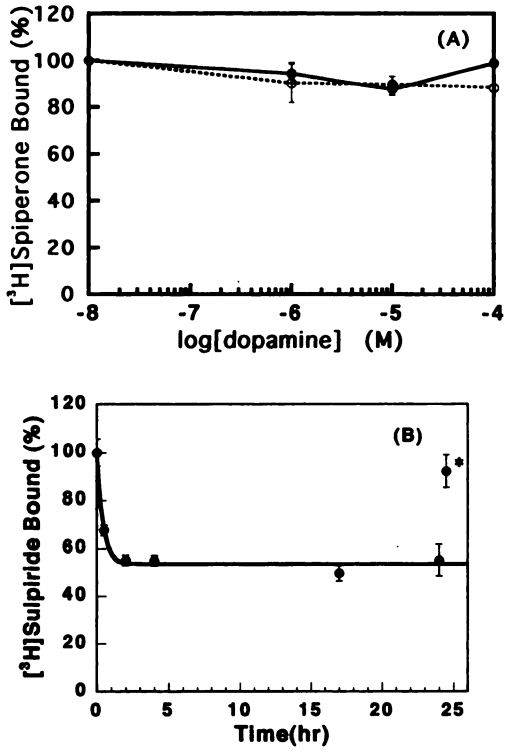

Fig. 4. Effect of treatment with dopamine for 24 hr on [^{3}H]spiperone or [^{3}H]sulpiride binding activity of CHO cells. A, CHO cells expressing D_{2L} (●) or D_{2S} (●) receptors were incubated with indicated concentrations of dopamine for 24 hr and subjected to [^{3}H]spiperone binding assays at 4° for 4 hr. Results are shown as mean ± standard deviation from an experiment assayed in triplicate. Specific [^{3}H]spiperone binding activity of control cells incubated without dopamine was taken as 100%, and actual counts of total and nonspecific binding were 12,000–14,000 and 700–1,000 cpm, respectively. B, CHO cells expressing D_{2S} receptors were incubated with 10^{-5} M dopamine for indicated times, washed thoroughly with PBS, and then subjected to [^{3}H]sulpiride binding assays in the presence or absence of 10^{-5} M haloperidol at 4° for 4 hr. CHO cells incubated with dopamine for 24 hr were washed thoroughly with PBS and then subjected to [^{3}H]sulpiride binding for 30 min at 37° and then for 210 min at 4°; results are indicated (*). The difference in binding activity in the absence or presence of haloperidol was defined as specific binding. Results are shown as mean ± standard deviation from an experiment assayed in triplicate. Specific [^{3}H]sulpiride binding activity for control cells incubated without dopamine was taken as 100%, and actual counts of total and nonspecific binding were 10,000–14,000 and 500–1,000 cpm, respectively.

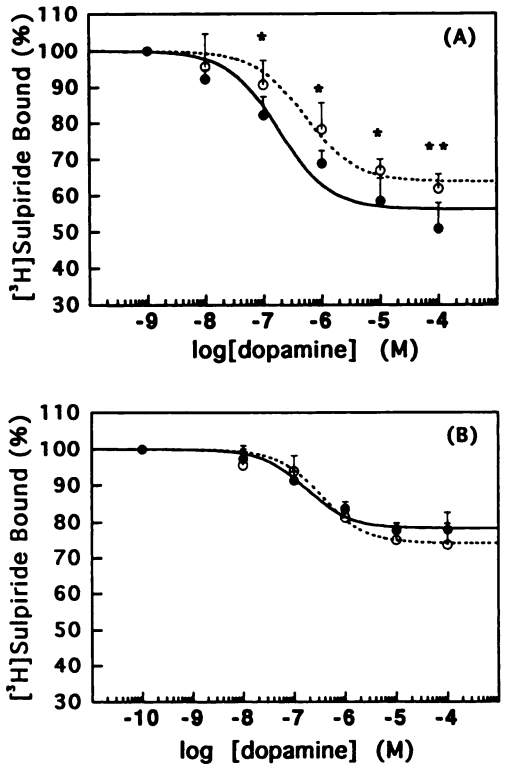

Fig. 5. Comparison between wild-type and variant D_2 receptors. CHO cells expressing wild-type (●) or S311C variant (○) D_2 receptors were incubated with the indicated concentrations of dopamine for 30 min and then subjected to [^{3}H]sulpiride binding assays at 4° for 4 hr. A, Results for D_{2S} receptors are shown as mean ± standard deviation from 10 independent experiments assayed in triplicate. Dose-response curves for averaged values were fitted to the following equation: $R_{max} \times EC_{50}/(EC_{50} + [\text{dopamine}]) + (100 - R_{max})$. Values for R_{max} were estimated to be 36.0 ± 2.3% for D_{2S} variant and 43.8 ± 3.4% for D_{2S} wild-type receptors, and values for EC_{50} were estimated to be 519 ± 190 nM for D_{2S} variant and 182 ± 89 nM for D_{2S} wild-type receptors. At the same time, curves were fitted for 10 independent data sets. R_{max} values were estimated to be 37.3 ± 3.9% and 43.2 ± 6.8% for D_{2S} variant and wild-type receptors, respectively. The difference was significant ($p < 0.05$). EC_{50} values were estimated to be 930 ± 930 and 250 ± 150 nM for D_{2S} variant and wild-type receptors, respectively ($p < 0.05$). B, Results on D_{2L} receptors are shown as mean ± standard deviation from five independent experiments. The difference was significant (*, $p < 0.01$, and **, $p < 0.005$, respectively) for [^{3}H]sulpiride binding activity between wild-type and S311C variant of D_{2S} receptors.

on incubation with dopamine for the first 2 hr and remained essentially the same during the 24-hr incubation. The decreased [^{3}H]sulpiride binding activity was restored by washing out the dopamine and incubating the cells at 37° for 30 min, even after a 24-hr exposure to dopamine (Fig. 4B). These results indicate that the decrease in [^{3}H]sulpiride binding activity is not due to a loss of D_{2S} receptors but rather to sequestration of D_{2S} receptors in the cell surface. A possible explanation is that when D_{2S} receptors on the cell surface are exposed to agonists, a certain portion of receptors are sequestered or internalized into a form that is not accessible to a hydrophilic ligand, such as [^{3}H]sulpiride, but is accessible to a hydrophobic ligand, such as [^{3}H]spiperone. Results shown in Figs. 3 and 4 indicate that this sequestration or internalization is not reversible at 4° but is reversible at 37°.

D_{2S} and D_{2L} receptor variants with a substitution of cysteine for serine at residue 311 in the third intracellular loop were also expressed in CHO cells, and their sequestration was examined. The rate of decrease in [^{3}H]sulpiride binding activity had a tendency to be slower for D_{2S} variant ($t_{1/2}$ = 24.9 ± 7.8 min) than for D_{2S} wild-type ($t_{1/2}$ = 18.7 ± 1.6 min) receptors, but the difference was not significant (data not shown). [^{3}H]Sulpiride binding activity decreased 38.2 ± 4.1% for D_{2S} variant receptors treated with 10^{-4} M dopamine for 30 min, a value that is significantly lower than the corresponding value of 49.1 ± 7.1% obtained for wild-type D_{2S} receptors ($p < 0.005$) (Fig. 5A). Significant differences in the proportion of decreased [^{3}H]sulpiride binding activity between variant and wild-type D_{2S} receptors were also detected with treatment with 10^{-5}, 10^{-6}, or 10^{-7} M dopamine. The concentration of dopamine giving a half-maximal effect was estimated to be 930 ± 930 nM for the variant D_{2S} and 250 ± 150 nM for wild-type D_{2S} receptors, a difference that is statistically significant ($p < 0.05$). This was not due to a differ-

ence in the affinity for dopamine between D_{2S} variant and wild-type receptors because there were no differences in the affinities of dopaminergic ligands, including dopamine, between these two receptors expressed in COS-7. In these experiments, binding affinity was assessed by the displacement of [^{3}H]spiperone binding to membrane preparations (data not shown). In contrast with D_{2S} receptors, there was no significant difference between wild-type and variant D_{2L} receptors with respect to the decrease in [^{3}H]sulpiride binding activity after treatment with dopamine (Fig. 5B). The half-life of decrease in [^{3}H]sulpiride binding activity for D_{2L} variant receptors was estimated to be 33.9 ± 9.4 min, which was essentially the same as that for D_{2L} wild-type receptors (33.2 ± 7.7 min).

The ratios of B_{max} values for [^{3}H]spiperone and [^{3}H]sulpiride binding were estimated to be 1.0 for D_{2L}, 0.98 for D_{2L} S311C, 0.67 for D_{2S}, and 0.88 for D_{2S} S311C receptors, suggesting that a certain portion of D_{2S}, but not of D_{2L}, receptors are in a sequestered form, even in the absence of dopamine.

Discussion

We treated CHO cells expressing D_{2S} receptors with dopamine and then measured the [^{3}H]sulpiride or [^{3}H]spiperone binding activity at 4° using intact CHO cells. We found that [^{3}H]sulpiride binding activity was decreased ≤50% after exposure to dopamine and that this decrease was restored by washing out the dopamine and incubating the cells at 37° but was not restored when the cells were incubated at 4°. In contrast, [^{3}H]spiperone binding activity was not affected by exposure to dopamine. This result indicates that the dopamine does not cause receptor degradation (down-regulation) but rather causes reversible sequestration of a portion of D_{2S} receptors into a form that is not accessible to [^{3}H]sulpiride but is accessible to [^{3}H]spiperone. The sequestration is thought to involve some kind of hydrophobic barrier because sulpiride is known to be a hydrophilic ligand and spiperone is known to be a hydrophobic ligand (29, 30). The simplest interpretation would be to assume that a portion of D_{2S} receptors undergo an agonist-induced endocytosis and that [^{3}H]sulpiride is not capable of penetrating cell membranes to access the ligand binding sites.

Consistent with the present results, Barton *et al.* (20) showed that dopamine treatment of retinoblastoma cells expressing D_2 receptors induces a decrease in B_{max} for [^{125}I]sulpiride binding and no change in B_{max} for [^{3}H]methylspiperone binding. There are significant differences, however, in the assay methods and the rate of sequestration between their system and our system. In the experiment by Barton *et al.*, the decrease in B_{max} for [^{125}I]sulpiride binding activity was measured at 22°. In these experiments, B_{max} decreased progressively during 4–24-hr incubations with dopamine. In the present system, we have not detected such a slow decrease in B_{max}. The fast sequestration (with $t_{1/2}$ of ~20 min) that we have observed is reversible at 37°, and the sequestered ligand binding sites are restored and become accessible to [^{3}H]sulpiride with a similar rate even after incubation with dopamine for 24 hr. It is not known whether the slow sequestration observed by Barton *et al.* is reversible. The loss of ligand binding sites from the cell surface may occur via different mechanisms, depending on the cell species.

The decrease in [^{3}H]sulpiride binding activity observed in the current study has many properties in common with sequestration of G protein-coupled receptors such as muscarinic acetylcholine receptors (17) and β-adrenergic receptors (31) in that all of these binding activities are (i) agonist dependent, (ii) partial in extent (sequestration of 20–50% of total receptors), (iii) reversible at 37° but not at 4° (reversible at the temperatures higher than 15° in the case of muscarinic receptors) (32), and (iv) relatively rapid ($t_{1/2}$ of ~10 min). In the case of β-adrenergic and muscarinic receptors, the vesicles containing sequestered receptors are shown to be separated from plasma membranes through sucrose density gradient centrifugation and are thought to represent receptors formed by agonist-induced endocytosis (33, 34). In preliminary experiments on D_{2S} receptors, we have found that dopamine-induced decrease in [^{3}H]sulpiride binding sites from the cell surface was accompanied by an increase in the binding sites in a "light vesicle" fraction obtained through sucrose density gradient centrifugation (data not shown).

There are significant differences between D_{2S} and D_{2L} receptors in the agonist-induced decrease in [^{3}H]sulpiride binding activity. The extent of the decrease was 22% versus 44% (D_{2L} versus D_{2S}), and the half-life of the decrease was 33 versus 19 min (D_{2L} versus D_{2S}). A decrease in [^{3}H]sulpiride binding activity from the cell surface was also observed for D_{2L} and D_{2S} receptors transiently expressed in COS-7 cells with G protein-coupled receptor kinase 2. The extent of the decrease was greater for D_{2S} receptors than for D_{2L} receptors (data not shown). These findings suggest that the difference in sequestration is due to intrinsic differences between D_{2S} and D_{2L} receptors. D_{2L} and D_{2S} receptors are identical except for an insertion of 29 amino acids in the third intracellular loop of D_{2L} resulting from alternative splicing. The third intracellular loop of D_2 receptors may be involved in the sequestration. The third intracellular loops of m1 (17, 35) and m2 (36, 37) muscarinic acetylcholine receptors are reported to be involved in sequestration. Furthermore, it is interesting to note that the β_2-adrenergic receptor is sequestered to a greater extent (60%) than the β_1-adrenergic receptor (26%), which contains an additional 24 amino acid residues in the third intracellular loop. Insertion of this 24-amino-acid peptide into the β_2 receptor or deletion of this peptide from the β_1 receptor results in changes in the sequestration of ±40–43% (38). It is reasonable to assume that an insertion of a 29-amino-acid peptide into the third intracellular loop of the D_{2L} receptors causes some conformational changes leading to attenuation of the sequestration process.

Zhang *et al.* (24) reported that treatment with dopamine of CHO cells expressing D_{2S} or D_{2L} receptors for 24 hr caused a 25% reduction in [^{3}H]methylspiperone binding sites for D_{2S} receptors and a ~2-fold increase in D_{2L} receptors. We treated CHO cells for 24 hr but did not detect any appreciable changes in [^{3}H]spiperone binding activity. The reason for the discrepancy between the results of Zhang *et al.* and our results remains unknown.

A D_2 receptor S311C variant has been detected more frequently in schizophrenic patients than in control subjects (26). The patients with the S311C variant showed less severe thought disorder, flattened affect, and psychomotor retardation and better responses to antipsychotic treatment than did

those without the variation. Some conflicting results were also reported concerning the S311C allele frequency (39–44). Recently, this variant was reported to be more frequently found in patients with alcohol dependence (45). In the present study, we have shown that the extent of sequestration is significantly less for the D_{2S} receptor variant than for the wild-type receptor, whereas no difference in the sequestration was observed between variant and wild-type D_{2L} receptors. If the sequestration of D_{2S} receptors contributes to their desensitization (31), the D_{2S} variant receptor will be impaired with regard to the ability to decrease the response when there is continuous dopaminergic stimulation. This is consistent with the dopamine hypothesis that certain dopamine pathways are overactive in schizophrenic patients (4). On the other hand, there is a possibility that the sequestration is a mechanism involved in reactivating and recycling desensitized receptors, as was proposed for β_2-adrenergic receptors (46). If this is the case, the activity of the D_{2S} variant receptor would be less than that of the wild-type receptor.

In summary, we provided evidence that D_{2S} receptors are sequestered by dopamine treatment and that there are significant differences in sequestration between D_{2S} and D_{2L} receptors and between wild-type and S311C variant D_{2S} receptors.

Acknowledgments

We thank Dr. D. K. Grandy for cDNA of D_{2S} receptor, Ymanouchi Pharmaceutical Co. Ltd. for cDNA of D_{2L} receptor, and Dr. D. W. Saffen for comments and editing of the manuscript.

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