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Characterization of a Chimeric Human Dopamine D3/D2 Receptor Functionally Coupled to Adenylyl Cyclase in Chinese Hamster Ovary Cells

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SUMMARY

Dopamine D3 receptor pharmacology differs from that of the dopamine D2 receptor despite a high degree of receptor sequence similarity. The greatest divergence of the primary sequences of D3 and D2 receptors occurs in the predicted third intracellular loops of the receptors, a region implicated in G protein binding and function. To determine whether this domain specifies the distinct ligand binding and signal transduction characteristics of the D3 receptor, we developed a chimeric receptor, replacing the third intracellular loop of the human D3 receptor with the third intracellular loop of the human D2 receptor. The pharmacology of the chimeric receptor expressed in Chinese hamster ovary cells was examined and compared with that of human dopamine D2 and D3 receptors expressed in the same cell line. The chimeric receptor retained characteristic human D3 receptor binding; the D2 third intracellular loop present in the chimeric receptor did not reduce high affinity agonist binding, characteristic of the D3 receptor, or make high affinity sites sensitive to GTP analogs. Unlike the native human D3 receptor, the chimeric receptor was negatively coupled to adenylyl cyclase through a pertussis toxin-sensitive pathway, apparently mediated by the D2 third intracellular loop. The ability of D3 ligand binding domains to produce a D2 functional response implies that the third intracellular loop of the D3 receptor is unable to mediate this D2 response in Chinese hamster overy cells. The inhibition of adenylyl cyclase seen with the chimeric receptor is less than the inhibition produced by D2 receptor coupling, suggesting that additional sequences in the D2 receptor contribute to normal G protein coupling.

Dopamine is an important vertebrate neurotransmitter that has been implicated in several disorders of the central nervous system, including Parkinson's disease and schizophrenia. Molecular cloning has identified a family of receptor subtypes and splice variants for the neurotransmitter dopamine (1–6). Based on sequence similarity, gene organization, and pharmacology, these receptors can be classified into two groups represented by the two most abundant dopamine receptors, designated D1 and D2 (7, 8). The dopamine D3 receptor is a recently identified member of the dopamine D2 receptor family and represents a potential target for the development of antipsychotic drugs (9–11).

The low abundance of the D3 receptor in the brain and the lack of D3-selective compounds have hindered binding and signal transduction studies of the native receptor. To circumvent these difficulties, molecular clones of the D3 receptor have been expressed in a variety of heterologous cell types (12–15). In these systems, the D3 receptor is distinguished by a relatively high affinity for dopamine and many D2 agonists. In addition, binding affinities of predicted agonists for the D3 receptor show only minor sensitivity to guanine nucleotides (12, 15–17).

Although sequence characteristics and binding clearly place the D3 receptor in the D2-like subfamily of dopamine receptors, D3 receptor coupling to effector pathways appears to be distinct from D2 receptor signaling (15–23). Overall, results from studies comparing D2 and D3 function suggest that these receptors couple through different G protein subtypes or have different coupling efficiencies with the same G proteins.

The protein sequences of D2 and D3 receptors conform to the seven-hydrophobic transmembrane domain topology predicted for G protein-coupled receptors, and both receptors contain a large third intracellular loop, as is characteristic of the D2 family of dopamine receptors (24). The primary amino acid sequences of the D2 and D3 receptors are least similar within this region. Previous studies of G protein-coupled receptors have identified the third intracellular loop as an

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ABBREVIATIONS: CHO, Chinese hamster ovary; PCR, polymerase chain reaction; 7-OH-DPAT, 7-hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate.

important domain, mediating receptor interactions with G proteins (25–30). To determine whether the third intracellular loop of the D3 receptor is responsible for the unusual binding characteristics of the receptor and inefficient coupling of the D3 receptor to D2 second messenger pathways, we developed a chimeric D3/D2 receptor in which the third intracellular loop of the human D3 receptor has been replaced with the same region from the long form of the human D2 receptor. This approach has been used to examine the signaling and binding specificity of a variety of G protein-coupled receptors (31–33). The chimeric dopamine D3/D2 receptor was stably expressed in CHO cells, where the response of the receptor to activation by D2 receptor agonists and the effects of the third intracellular loop on receptor binding and sensitivity to guanine nucleotides were examined.

Materials and Methods

Construction of the chimeric receptor cDNA. A DNA sequence coding for the protein sequence of the chimeric receptor was constructed by PCR (34, 35), using a recombinant PCR technique (36). Three DNA sequences, representing three parts of the coding sequence for the chimeric receptor, were generated by PCR using cDNA clones of the dopamine D3 and D2 receptors as templates. The three primary PCR products represent (A) plasmid vector sequence flanking the amino terminus of the human D3 receptor through the first codon of the third intracellular loop of the receptor, representing amino acids 1–210 of the human D3 receptor, (B) the third intracellular loop sequence of the human D2 receptor, representing amino acids 212–373 of the long form of the human D2 receptor, and (C) the human D3 receptor sequence from the sixth transmembrane domain through the carboxyl terminus into the flanking plasmid sequences, representing amino acids 330–400 of the human D3 receptor. Oligonucleotide primers (representing the D3/D2 junction regions) used in the primary PCRs were designed with additional 5′ nucleotides, to generate PCR products containing overlapping complementary sequences at the D2/D3 junctions; oligonucleotide primer sequences for the primary PCRs were as follows: reaction A, 5′ primer, 5′-TAATACGACTCACTATAGGG-3′; 3′ primer, 5′-GACAATGTAGATTCTGGCATAGACAAGGACAGT-3′; reaction B, 5′ primer, 5′-TCTATGCCAGAATCTACATTGTCCTCCGCAGA-3′; 3′ primer, 5′-TGGCCACCATCTGAGTGGCTTTCTTCTCCT-3′; reaction C, 5′ primer, 5′-AGCCACTCAGATGGTGGCCATTGTGCTTGG-3′; 3′ primer, 5′-GATTTAGGTGACACTATAG-3′. PCR amplifications were performed using Perkin Elmer GeneAmp kit reagents, using recommended concentrations of primers, nucleotides, and AmpliTaq DNA polymerase. Fifty nanograms of plasmid DNA containing the appropriate receptor cDNA served as template in 100-μl reactions. A Perkin Elmer DNA thermal cycler 480 was used for PCRs. Reactions consisted of 35 cycles of 1 min at 94°, 2 min at 52°, and 3 min at 72°. The three primary PCR products were purified by acrylamide gel electrophoresis. Fifty nanograms of each of the three gel-purified primary PCR products were combined to serve as template in a secondary PCR to generate the chimeric receptor cDNA. Oligonucleotide primers in the secondary PCR, used to amplify the complete chimeric receptor gene, were the 5′ primer for primary PCR A and the 3′ primer for primary PCR C. These primers represent sequences in the plasmid vector pcDNA I/neo (Invitrogen) flanking D3 receptor cDNA sequences in the plasmid that served as template for primary PCRs A and C. The secondary PCR product was cut with the restriction endonucleases *Hind*III and *Xba*I. These recognition sites were present in the vector sequences flanking the cDNA for the human D3 receptor and were conserved in the final PCR product. The *Hind*III/*Xba*I fragment containing the chimeric receptor coding sequence was gel purified and ligated into the expression vector pcDNA I/neo (Invitrogen) at the *Hind*III and *Xba*I sites, to produce the plasmid pcDNA I/neo-hD3/hD2. In this plasmid, transcription of the chimeric receptor coding sequence is under control of the immediate early gene enhancer/promoter of cytomegalovirus isolated from humans. The plasmid vector also contains the gene for neomycin resistance and confers resistance to the antibiotic G418 in mammalian cells. The sequence of the chimeric gene in the expression vector was confirmed by DNA sequencing before transfection into CHO cells.

Transfection and cell culture. The chimeric receptor expression construct, pCDNA I/neo-hD3/hD2, was stably transfected into CHO-K1 cells by a modified calcium phosphate protocol (37). Transfected cells were selected for resistance to G418 (Gibco). Clonal cell lines arising from G418-resistant colonies were maintained in medium containing G418 and were screened for expression of the chimeric receptor by radioligand binding. Generation of CHO-K1 cell lines expressing the long form of the human D2 receptor (CHO-hD2–12A) and the human D3 receptor (CHO-hD3–6) was described previously (15). All CHO-K1 cell lines were grown at 37° in an atmosphere of 5% CO_2/95% air. All cells for this study were maintained in F-12 medium (Gibco) containing 10% dialyzed fetal bovine serum (Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin. Growth medium for transfected cells was supplemented with 400 μg/ml G418.

Radioligand binding. Cells grown to confluence were detached from culture flasks using 0.02% EDTA in phosphate-buffered saline. Cells were collected by centrifugation (700 × *g* for 5 min), suspended in ice-cold TM buffer (25 mM Tris·HCl, 6 mM $MgCl_2$, pH 7.4), and homogenized using a Brinkman Polytron homogenizer at setting 5 for 10 sec. The homogenate was centrifuged at 40,000 × *g* for 10 min at 4°. The membrane pellet was suspended in TM buffer, at a protein concentration of 1 mg/ml, and frozen at −80°. Radioligand binding reactions contained 25 μg of membrane protein in 0.5 ml of TM buffer. Competition binding reactions contained 0.2 nM [^{3}H]spiperone (107 Ci/mmol; New England Nuclear) and various concentrations of competing compounds, with or without the nonhydrolyzable GTP analog Gpp(NH)p (Calbiochem). Saturation binding assays were performed using increasing concentrations of [^{3}H] spiperone. Nonspecific binding was defined as residual binding in the presence of 1 μM haloperidol. Reaction mixtures were incubated at 22° for 1 hr, and reactions were terminated by filtration onto polyethyleniminetreated GF/B filtermats, using a Brandel harvester. The filters were washed twice with 5 ml of TB buffer (25 mM Tris·HCl, 0.01% bovine serum albumin, pH 7.4). Filters were air dried, and individual filter disks were counted in 5 ml of scintillation fluid.

cAMP accumulation assay. cAMP accumulation was measured in intact cells. Cells were grown to confluence in 24-well culture plates and washed with Earle's balanced salt solution containing 1 mM isobutylmethylxanthine, and assays were started by the addition of 0.5 ml of prewarmed Earle's balanced salt solution containing 1 mM isobutylmethylxanthine plus test drugs. After 10 min, forskolin was added to a final concentration of 10 μM in all wells, except for wells used to determine basal cAMP levels. Cells were maintained at 37° during the assay. Assays were terminated after 15 min by the addition of 0.5 ml of ice-cold 10% trichloroacetic acid to each well. Samples were acetylated (38), and cAMP levels were determined by a scintillation proximity radioimmunoassay for cAMP (Amersham), which was performed in 96-well plates and quantitated using a Microbeta scintillation counter (Wallac). In studies with pertussis toxin pretreatment, pertussis toxin (100 ng/ml; Biomol) was added to the culture medium 18 hr before drug treatment.

Data analysis. Competition and saturation curves for radioligand binding experiments and dose-response curves for the cAMP accumulation experiments were analyzed using nonlinear, least-squares, regression analysis, as provided in the program Inplot (GraphPad). Statistical differences between means were determined as described in the tables.

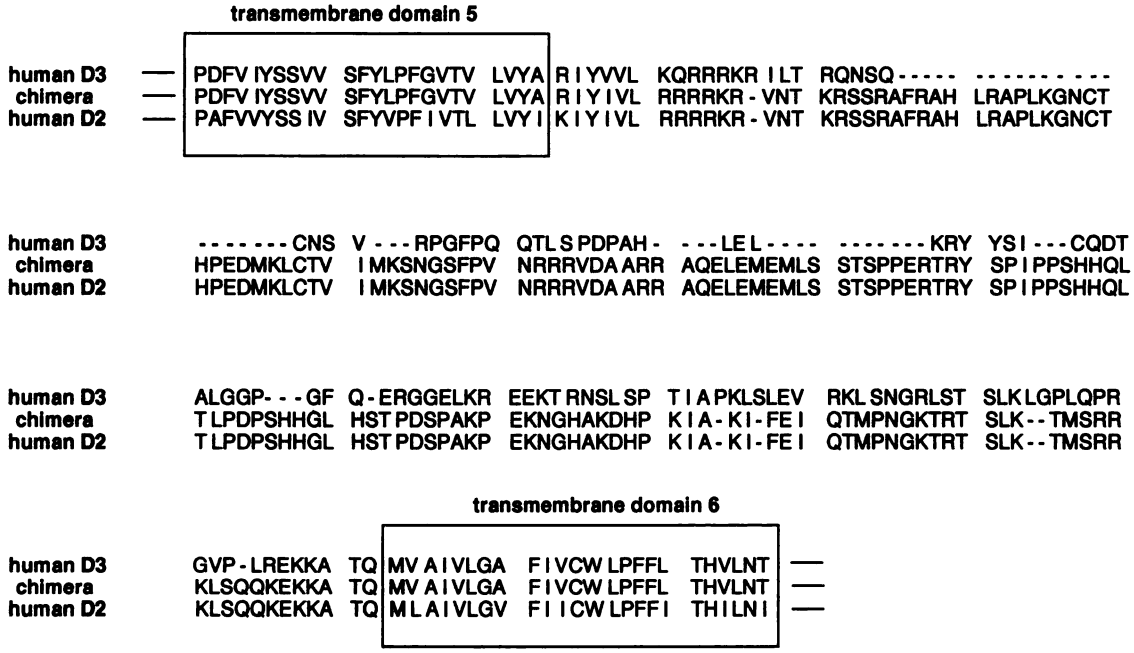

Fig. 1. Alignment of the region of the fifth and sixth transmembrane domains of the human D2, D3, and chimeric D3/D2 receptor sequences, illustrating the relationship of the sequence of the chimeric receptor to those of the D3 and D2 receptors.

Results

Chimeric receptor. Fig. 1 shows an alignment of the human D3 receptor, the long form of the human D2 receptor, and the chimeric D3/D2 receptor in the region of the fifth and sixth transmembrane domains, illustrating the relationship of the primary sequence of the chimeric receptor to those of the two parent receptors. The third intracellular loop of the chimeric receptor is identical to the third intracellular loop of the long form of the human D2 receptor, with the exception of the first amino acid residue. This amino acid represents that of the D3 receptor at this position. With the exception of sequences in the third intracellular loop, the amino acid sequence of the chimeric receptor is identical to that of the human D3 receptor.

Receptor binding studies. Membranes from two cell lines expressing the chimeric receptor, CHO-hD3hD2–5 and CHO-hD3hD2–6, were used for saturation binding studies with [^{3}H]spiperone (Table 1). CHO cell lines expressing the human D2 or D3 receptor were described previously, and the results presented here are consistent with results from that study (15). Affinities of the D2 and D3 receptors for the antagonist ligand spiperone were similar. There was a significant 3-fold difference between the affinity of the D3 re-

TABLE 1

Saturation binding of [^{3}H]spiperone to membranes from various stably transfected CHO-K1 clonal cell lines

The cell lines (and transfected receptors) were CHO-hD2–12A (human D2 receptor), CHO-hD3–6 (human D3 receptor), and CHO-hD3hD2–5 and CHO-hD3hD2–6 (chimeric human D3D2 receptor). Values are means ± standard errors of at least three independent replications. Differences between means in the same column were tested for statistical significance by analysis of variance followed by the Newman-Keuls test.

Cell line	B_{max}	K_d	n^a
	pmol/mg of protein	*nM*	
CHO-hD2–12A	0.98 ± 0.1	0.14 ± 0.02	3
CHO-hD3–6	2.3 ± 0.45	0.26 ± 0.06	4
CHO-hD3hD2–5	7.2 ± 0.9[b]	0.78 ± 0.08[c]	7
CHO-hD3hD2–6	0.79 ± 0.11	0.66 ± 0.23[d]	4

[a] *n*, number of independent experiments.
[b] Differed from the B_{max} of the other cell lines, $p < 0.01$.
[c] Differed from CHO-hD2–12A and CHO-hD3–6 K_d values, $p < 0.05$.
[d] Differed from CHO-hD3–6 K_d, $p < 0.05$.

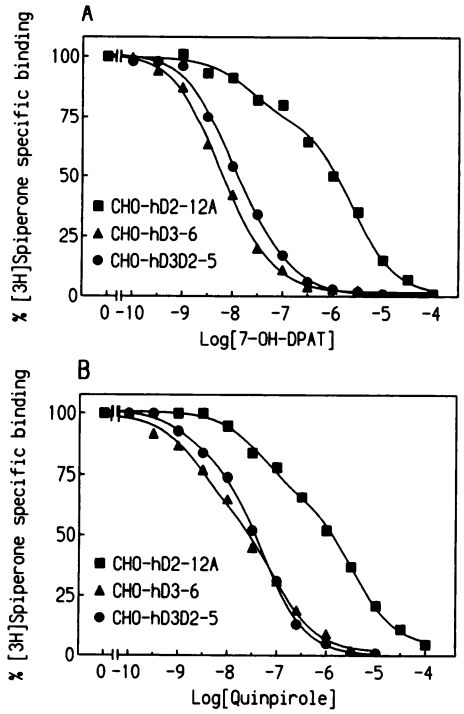

Fig. 2. Competition binding curves for inhibition of [^{3}H]spiperone binding by the D3-selective agonists 7-OH-DPAT (A) and quinpirole (B), using membranes from CHO cells transfected with the human D2 receptor (CHO-hD2–12A), the human D3 receptor (CHO-hD3–6), or the human chimeric D3/D2 receptor (CHO-hD3hD2–5). The *curves* represent results from at least three independent determinations. See Table 2 for the means ± standard errors of competitive binding parameters.

ceptor for spiperone and the affinity of chimeric receptors from both cell lines for spiperone. The K_d for spiperone of chimeric receptors from one of the two cell lines, CHO-hD3hD2–5, was also significantly different from the K_d for spiperone of human D2 receptors expressed in CHO-hD2–12A cells. The cell line CHO-hD3hD2–5 showed a significantly higher level of receptor expression than did cell lines expressing either D3 or D2 receptors.

Competition binding of [^{3}H]spiperone with the D3-selective ligands 7-OH-DPAT and quinpirole was performed with membranes prepared from the cell line CHO-hD3hD2–5 and from cell lines expressing human D2 and human D3 receptors. The data were best fit by biphasic curves for both quinpirole and 7-OH-DPAT for each of the three receptors (Fig. 2; Table 2). Overall, D2 and D3 receptors differed most in their low affinity K_i values, and the competitive binding parameters of the chimeric D3/D2 receptor most closely resembled those of the D3 receptor.

Competition binding with agonist ligands in the presence of the nonhydrolyzable GTP analog Gpp(NH)p typically produced a rightward shift in D2, D3, and D3/D2 competition curves, although the shift was more pronounced for the D2 receptor (Fig. 3). Analysis of the GTP shift of high and low affinity sites for the D2 receptor, the D3 receptor, and the chimeric receptor is shown in Table 3. In this study the high and low affinity K_i values of the D2 receptor differed from those of both the D3 and chimeric receptors.

Agonist-stimulated effects on cAMP accumulation. Fig. 4A shows dose-response curves for quinpirole-induced inhibition of forskolin-stimulated cAMP accumulation in cell lines expressing either the long form of the D2 receptor, the human D3 receptor, or the chimeric D3/D2 receptor. In the cell line expressing the long form of the D2 receptor, CHO-hD2–12A, maximal inhibition of forskolin-stimulated cAMP accumulation by quinpirole was 75%. Quinpirole showed a trend toward reversal of forskolin-stimulated cAMP accumulation in the cell line expressing the human D3 receptor, CHO-hD3–6, but we did not see a significant inhibition by dopamine agonists of cAMP accumulation in this cell line. In the cell line expressing the chimeric D3/D2 receptor, CHO-hD3hD2–5, quinpirole maximally reversed the forskolin-stimulated cAMP accumulation by 50%. Responses to quinpirole in both the D2 and chimeric receptor-expressing cell lines were completely blocked by the dopamine antagonist haloperidol at a concentration of 1 μM (data not shown). These results suggested that the substitution of the D2 third intracellular loop for the D3 third intracellular loop allowed the ligand-bound chimeric receptor to produce a functional response. However, given the differences in receptor expression levels in the various cell lines (Table 1), it was also possible that the functional response seen with the chimeric receptor in the CHO-hD3hD2–5 cell line was more reflective of its higher expression level, relative to that of the D3 receptor in the CHO-hD3–6 cell line. Therefore, an additional cell line, CHO-hD3hD2–6, which expressed the chimeric receptor at a lower level, was tested to determine whether it also showed reversal of forskolin-stimulated cAMP accumulation in response to quinpirole. This cell line expressed the chimeric receptor at a level 9-fold lower than the level of chimeric receptor expression in CHO-hD3hD2–5 cells and, more importantly, 3-fold lower than the level of expression of the D3 receptor in CHO-hD3–6 cells, but it still demonstrated a 30% reduction of forskolin-stimulated cAMP accumulation in response to quinpirole (Fig. 4B). The response was also blocked by haloperidol. The cell line with the highest level of chimeric receptor expression, CHO-hD3hD2–5, also exhibited the unusual characteristic of a greatly reduced response to forskolin, relative to the other cell lines (Fig. 5; Table 4). Although the level of expression of the chimeric receptor in CHO-hD3hD2–6 cells was equivalent to the level of expression of the D2 receptor in CHO-hD2–12A cells, the D2 cell line showed a much higher, 75% reversal of forskolin-stimulated cAMP accumulation.

To confirm that the inhibition of adenylyl cyclase in cells expressing the chimeric receptor was mediated by G protein activation, the response of forskolin-stimulated cAMP levels to quinpirole was determined after pretreatment of the cells with pertussis toxin. The parent cell line, CHO-K1, showed no response to agonist (Fig. 5A). As described previously, the CHO cell line expressing the long form of the human D2 receptor, CHO-hD2–12A, showed a significant reversal of forskolin-stimulated cAMP accumulation in response to quinpirole, and this effect was reversed by pretreatment of the cells with pertussis toxin (Fig. 5B). The cell line expressing the human D3 receptor, CHO-hD3–6, showed no response to agonist (Fig. 5C) (15). The cell line expressing the chimeric receptor at a high level, CHO-hD3hD2–5, showed a significant agonist reversal of forskolin-stimulated cAMP accumulation in response to quinpirole (Fig. 5D), and this effect was blocked by pertussis toxin. An unusual feature of this cell line

TABLE 2

Inhibition of [^{3}H]spiperone binding by dopamine agonists

Values are binding parameters (mean ± standard error) determined as described in Materials and Methods. Differences between means in the same column were tested for statistical significance by analysis of variance followed by the Newman-Keuls test.

Drug	Cell type[a]	IC_{50}	K_H	K_L	High affinity sites	n[b]
		nM	nM	nM	%	
7-OH-DPAT	D2	820 ± 134[c]	12 ± 5	817 ± 207[c]	29 ± 2[c]	4
	D3	6.5 ± 1.0	2 ± 0.2	39 ± 10	78 ± 6	5
	D3/D2	18 ± 3.0	9 ± 3.0	78 ± 20	73 ± 5	5
Quinpirole	D2	1562 ± 473[d]	101 ± 35	3153 ± 859[d]	42 ± 5	6
	D3	16 ± 5	2.9 ± 0.5	156 ± 103	54 ± 13	3
	D3/D2	39 ± 7	1.6 ± 0.8	147 ± 91	20 ± 4	3

[a] Cell type indicates which CHO cell line, transfected with the D2, D3, or D3/D2 chimeric receptor, served as the source of membranes for the competitive binding experiments.

[b] n, number of independent experiments.

[c] Differed from D3 and D3/D2, $p < 0.01$.

[d] Differed from D3 and D3/D2, $p < 0.05$.

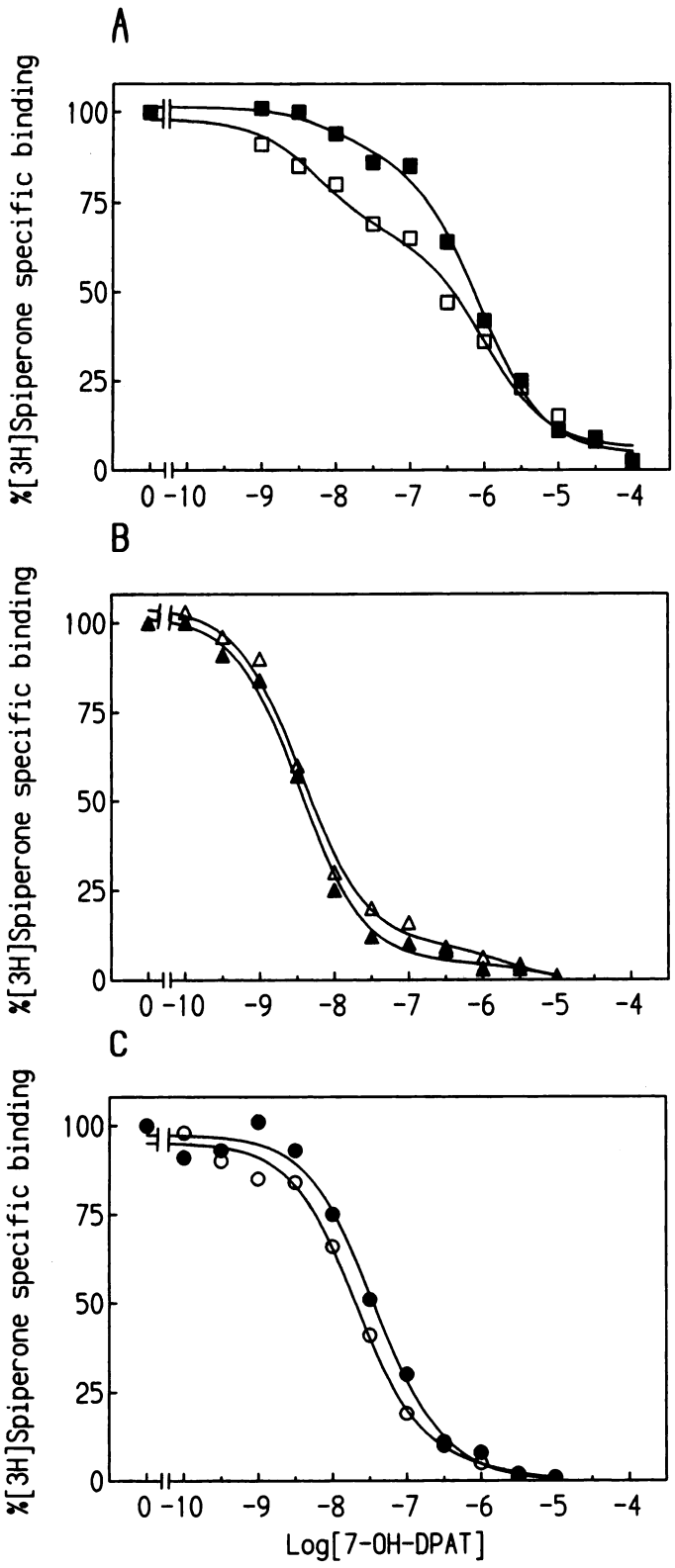

Fig. 3. Effect of Gpp(NH)p, a nonhydrolyzable analog of GTP, on the inhibition by 7-OH-DPAT of [^{3}H]spiperone binding, using membranes from the cell lines CHO-hD2-12A (A), CHO-hD3-6 (B), and CHO-hD3hD2-5 (C). *Closed symbols*, data points obtained in the presence of 100 μM Gpp(NH)p; *open symbols*, data points obtained in the absence of Gpp(NH)p. The *curves* are representative of those obtained from at least three independent determinations. See Table 3 for the means ± standard errors of competitive binding parameters.

was that forskolin-stimulated cAMP levels were typically only 20–30% of those attained with the other cell lines (Fig. 5; Table 4).

Discussion

The dopamine D3 receptor has several pharmacological properties that distinguish it from the D2 receptor. The D3 receptor typically demonstrates a higher affinity for dopamine agonists than does the D2 receptor, but D3 high affinity agonist binding also exhibits reduced guanine nucleotide sensitivity. More significantly, in response to agonist ligands the D3 receptor does not couple to several signaling pathways activated by the D2 receptor, and in systems where it was possible to detect functional coupling of the D3 receptor, such as thymidine uptake in CHO cells expressing the D3 receptor, responses were consistently severalfold less than responses to D2 receptor activation.

Many possibilities could explain these observed differences. Each subtype might couple through different G proteins, with G proteins present in heterologous cells allowing D2 receptor coupling but G proteins required for D3 receptor signaling being absent or present in low abundance. Alternately, G proteins present in these cells might bind the D3 receptor but remain bound upon receptor activation, leading to the observed stability of D3 high affinity sites in the absence of a functional response. Differences between D2 and D3 sequences might include sites in the D3 receptor that facilitated receptor down-regulation, leading to uncoupling. Additionally, factors present in neurons but absent in other cell types might be required for D3 coupling. Central to these possibilities is the variability of the intracellular domains in these two receptor subtypes.

The third intracellular loop of G protein-coupled receptors is a critical domain for receptor coupling and the interaction of receptors with G proteins. Recent studies have demonstrated that mutations in the third intracellular loop of the β_2-adrenergic receptor can modulate agonist but not antagonist binding (39). Because guanine nucleotide effects are thought to reflect changes in receptor-G protein interactions, this domain might also govern receptor sensitivity to guanine nucleotides. In this report we tested whether the distinguishing pharmacological properties of the D2 and D3 receptors were reflective of the differences in the third intracellular loops of these receptors. For this purpose, a chimeric receptor was made in which the third intracellular loop of the D2 receptor was substituted for the third intracellular loop of the human D3 receptor.

Results presented in this study showed that ligand binding of the chimeric receptor remained D3-like, based on several criteria. In competition binding studies of agonist ligands, the chimeric receptor demonstrated the same high affinity as the D3 receptor, with two highly D3-selective agonist ligands also showing high affinity for the chimeric receptor. Saturation binding indicated that replacement of the D3 third intracellular loop had only a minor effect on binding of the labeled dopamine antagonist spiperone, perhaps reflecting small changes, in the chimera, in the orientation of the D3 sequences that determine the spiperone binding site. Furthermore, exchange of the third intracellular loop did not appear to significantly increase the guanine nucleotide sensitivity of the chimera or alter the high percentage of high

TABLE 3

Effects of the nonhydrolyzable GTP analog Gpp(NH)p on the inhibition of [^{3}H]spiperone binding by the dopamine agonist 7-OH-DPAT

Values are binding parameters (mean ± standard error) determined as described in Materials and Methods.

Cell type[a]	K_H		K_L		High affinity sites		n^b
	−Gpp(NH)p	+Gpp(NH)p	−Gpp(NH)p	+Gpp(NH)p	−Gpp(NH)p	+Gpp(NH)p	
	nM		nM		%		
D2	4 ± 1[c]	45 ± 17[c,d]	556 ± 127[e]	1287 ± 310[e]	41 ± 1.5[f]	43 ± 8	5
D3	1.4 ± 0.3	1 ± 0.1	112 ± 13	193 ± 82	85 ± 3	84 ± 5	3
D3/D2	1 ± 0.6	7 ± 3	20 ± 3	150 ± 69	57 ± 14	54 ± 14	6

[a] Cell type, see Table 2.
[b] *n*, number of independent experiments.
[c] Differed from D3 and D3/D2 in the same column, $p < 0.05$, by analysis of variance followed by the Newman-Keuls test.
[d] Differed from value without Gpp(NH)p, $p < 0.05$, by *t* test.
[e] Differed from D3 and D3/D2 in the same column, $p < 0.01$, by analysis of variance followed by the Newman-Keuls test.
[f] Differed from D3 in the same column, $p < 0.05$, by analysis of variance followed by the Newman-Keuls test.

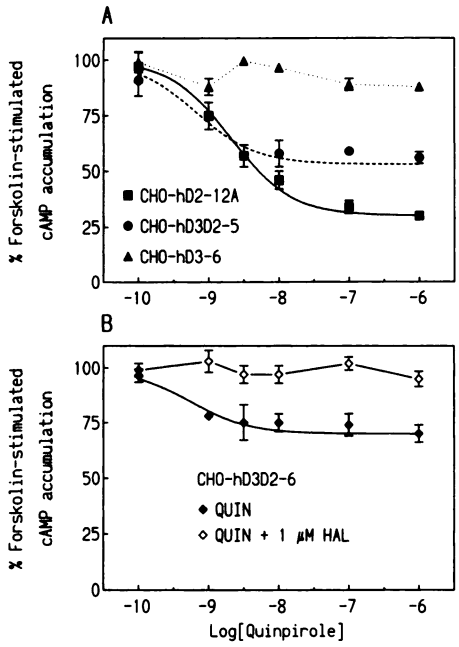

Fig. 4. A, Effect of quinpirole on 10 μM forskolin-stimulated cAMP accumulation in three different transfected cells lines. B, Effect of quinpirole (*QUIN*) on 10 μM forskolin-stimulated cAMP accumulation in another clonal D3/D2 cell line and reversal of the quinpirole effect by the dopamine receptor antagonist haloperidol (*HAL*). Each data point represents the mean ± standard error from at least four independent experiments. See Table 4 for values for basal and forskolin-stimulated cAMP levels determined from these experiments.

affinity sites seen in the D3 receptor. These results strongly suggest that differences in agonist binding properties between D2 and D3 receptors are not solely dependent upon sequence differences within the third intracellular loops of these receptors.

In contrast, the chimeric receptor exhibited D2-like properties with respect to the signaling pathway associated with inhibition of cAMP accumulation. Whereas CHO cell lines expressing the D3 receptor were not functionally coupled, dopamine agonists were able to inhibit forskolin-stimulated cAMP accumulation in cells expressing either the D2 receptor or the chimeric receptor. The agonist effects were reversed by dopaminergic antagonists and pertussis toxin treatment. These results indicate that at least part of the difference in the signaling potential of these receptors is mediated by the differences in their third intracellular loop sequences. Interestingly, in the cell line CHO-hD3hD2–5, forskolin-stimulated cAMP levels were only 20–30% of those attained in the other cell lines. An explanation for this could be constitutive activation of the D3/D2 receptor due to the structure of the chimeric receptor, high levels of expression of the receptor, or both. In this case, pertussis toxin pretreatment might serve to release forskolin-stimulated cAMP levels from constitutive inhibition, but the forskolin response in this cell line was unaffected by toxin pretreatment. At present we cannot explain the low level of forskolin stimulation of cAMP accumulation in this cell line. Two other chimeric cell lines tested (data not shown) showed normal forskolin responses, but chimeric receptor expression in these cell lines was considerably lower than that of CHO-hD3hD2–5 cells.

Although binding results in this study agree well with those seen with a similar chimeric human D3/D2 receptor characterized by McAllister *et al.* (33), results from another study using several D3 and D2 receptor chimeras, reported by Robinson *et al.* (40), suggested that third intracellular loop sequences can affect the agonist binding properties of G protein-coupled receptors. Two chimeric receptors in that study, representing D2 and D3 receptors in which the third intracellular loops were exchanged, showed that agonist binding was largely correlated with the origin of the exchanged intracellular loop. However, as noted in that study, substituted sequences also extended into the fifth and sixth transmembrane domains, resulting in five substitutions within the transmembrane domains of the chimeric D3 receptor containing the D2 third intracellular loop. Given the demonstrated importance of transmembrane domains in agonist binding to catecholamine receptors, it is possible that the discrepancies between those binding results and the results of the present study reflect the changes within the transmembrane domains of the chimeric receptors and are not the result of the exchanges of the third intracellular loops.

In contrast to the chimeric human D3/D2 receptor described by McAllister *et al.* (33), functional coupling of a chimeric D3/D2 receptor to adenylyl cyclase (presumably through G_i) was seen only in the present report. The chimeric receptors in these two studies differ by three amino acids at the D3/D2 junction sites. These substitutions represent conservative changes, and it is doubtful that they account for the difference in coupling of the two chimeric receptors to adenylyl cyclase. More likely, differences in expression levels might underlie this difference in receptor coupling. In the study by McAllister *et al.* (33), chimeric receptor expression did not

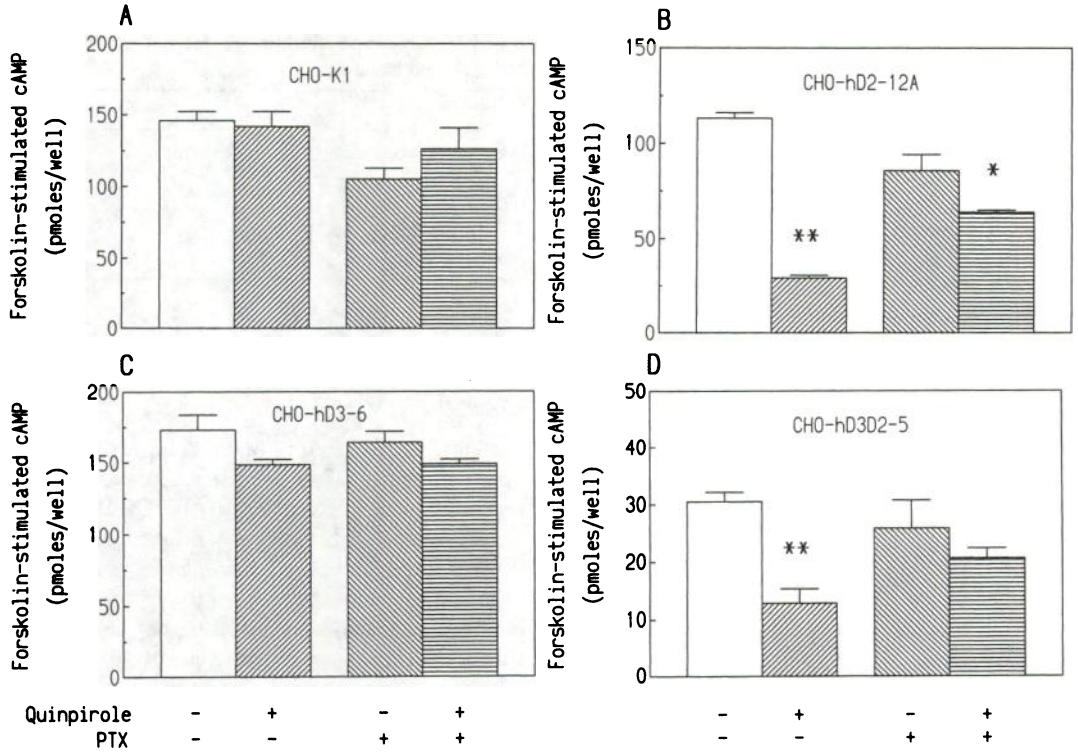

Fig. 5. Effect of 1 μM quinpirole on 10 μM forskolin-stimulated cAMP accumulation, in the presence or absence of 100 ng/ml pertussis toxin (*PTX*) (added the previous day), in untransfected CHO cells (A) or in CHO cells transfected with the cDNA for the human D2 receptor (B), the human D3 receptor (C), or a chimeric D3/D2 receptor (D). Comparisons for each cell line and for each pertussis toxin condition were made between mean values obtained in the presence or absence of quinpirole. Statistical significance of the differences between means was determined by *t* test; *, $p < 0.05$; **, $p < 0.01$.

TABLE 4

Basal and forskolin-stimulated cAMP levels

Values are means ± standard errors. Differences between means in the same column were tested for statistical significance by analysis of variance followed by the Newman-Keuls test.

Cell line	cAMP level		Fold stimulation	n^a
	Basal	+10 μM Forskolin		
	pmol/well			
CHO-hD2-12A	3 ± 0.1	74 ± 6	25 ± 2	4
CHO-hD3-6	3 ± 0.6	65 ± 4	27 ± 7	4
CHO-hD3hD2-5	3 ± 0.3	21 ± 3[b]	8 ± 1[c]	4
CHO-hD3hD2-6	2 ± 0.4	37 ± 2[d]	23 ± 4	5

[a] *n*, number of independent experiments.
[b] Differed from CHO-hD2-12A and CHO-hD3-6, $p < 0.01$; differed from CHO-hD3hD2-6, $p < 0.05$.
[c] Differed from CHO-hD3-6 and CHO-hD3hD2-6, $p < 0.05$.
[d] Differed from CHO-hD2-12A and CHO-hD3-6, $p < 0.01$.

exceed 370 fmol/mg of membrane protein, compared with 790 and 7200 fmol/mg of protein, respectively, for the CHO-hD3hD2-6 and CHO-hD3hD2-5 cell lines described in the present report. In this regard, a recent report (41) has shown that coupling efficiency can vary as a function of receptor expression level. Like other functional responses seen with the D3 receptor, the chimeric receptor described in this study was unable to match the magnitude of the functional response evoked by the D2 receptor, suggesting that D2 sequences in addition to the D2 third intracellular loop are involved in the D2 response or that D3 sequences outside the third intracellular loop inhibit the functional responses generated by the D2 third intracellular loop in the chimeric receptor. Replacement of additional D3 intracellular sequences in the chimera might identify these domains.

In summary, results from the present study indicate that substitution of the D2 third intracellular loop for the D3 third intracellular loop maintains the distinctive binding characteristics of the D3 receptor but produces D2-like coupling to adenylyl cyclase in response to receptor activation. In general terms, these results are consistent with the assignment of the ligand binding sites to membrane helices and highlight the importance of the third intracellular loop of G protein receptors in signal generation.

References

1. Bunzow, J. R., H. H. M. Van Tol, D. K. Grandy, P. Albert, J. Salon, M. Christie, C. A. Machida, K. A. Neve, and O. Civelli. Cloning and expression of a rat D_2 dopamine receptor cDNA. *Nature (Lond.)* **336**:783–787 (1988).
2. Dal Toso, R., B. Sommer, M. Ewert, A. Herb, D. B. Pritchett, A. Bach, B. D. Shivers, and P. H. Seeburg. The dopamine D_2 receptor: two molecular forms generated by alternative splicing. *EMBO J.* **8**:4025–4034 (1989).
3. Zhou, Q.-Y., D. K. Grandy, L. Thambi, J. A. Kushner, H. H. M. Van Tol, R. Cone, D. Pribnow, J. Salon, J. R. Bunzow, and O. Civelli. Cloning and expression of human and rat D_1 dopamine receptors. *Nature (Lond.)* **347**: 76–80 (1990).
4. Giros, B., M.-P. Martres, P. Sokoloff, and J.-C. Schwartz. Clonage du gène du récepteur dopaminergique D_3 humain et identification de son chromosome. *C. R. Acad. Sci. Ser. III Sci. Vie* **311**:501–508 (1990).
5. Van Tol, H. H. M., J. R. Bunzow, H.-C. Guan, R. K. Sunahara, P. Seeman, H. B. Niznik, and O. Civelli. Cloning of the gene for a human dopamine D_4 receptor with high affinity for the antipsychotic clozapine. *Nature (Lond.)* **350**:610–614 (1991).
6. Sunahara, R. K., H.-C. Guan, B. F. O'Dowd, P. Seeman, L. G. Laurier, G. Ng, S. R. George, J. Torchia, H. H. M. Van Tol, and H. B. Niznik. Cloning of the gene for a human dopamine D_5 receptor with higher affinity for dopamine than D_1. *Nature (Lond.)* **350**:614–619 (1991).
7. Kebabian, J. W., and D. B. Calne. Multiple receptors for dopamine. *Nature (Lond.)* **277**:93–96 (1979).

8. Sibley, D. R., and F. J. Monsma. Molecular biology of dopamine receptors. *Trends Pharmacol. Sci.* **13**:61–69 (1992).
9. Sokoloff, P., B. Giros, M.-P. Martres, M.-L. Bouthenet, and J.-C. Schwartz. Molecular cloning and characterization of a novel dopamine receptor (D_3) as a target for neuroleptics. *Nature (Lond.)* **347**:146–151 (1990).
10. Bouthenet, M.-L., E. Souil, M.-P. Martres, P. Sokoloff, B. Giros, and J.-C. Schwartz. Localization of dopamine D_3 receptor mRNA in the rat brain using *in situ* hybridization histochemistry: comparison with dopamine D_2 receptor mRNA. *Brain Res.* **564**:203–219 (1991).
11. Lévesque, D., J. Diaz, C. Pilon, M.-P. Martres, B. Giros, E. Souil, D. Schott, J.-L. Morgat, J.-C. Schwartz, and P. Sokoloff. Identification, characterization, and localization of the dopamine D_3 receptor in rat brain using 7-[^{3}H]hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin. *Proc. Natl. Acad. Sci. USA* **89**:8155–8159 (1992).
12. Sokoloff, P., M. Andrieux, R. Besançon, C. Pilon, M.-P. Martres, B. Giros, and J.-C. Schwartz. Pharmacology of human dopamine D_3 receptor expressed in a mammalian cell line: comparison with D_2 receptor. *Eur. J. Pharmacol.* **225**:331–337 (1992).
13. Seabrook, G. R., S. Patel, R. Marwood, F. Emms, M. R. Knowles, S. B. Freedman, and G. McAllister. Stable expression of human D_3 dopamine receptors in GH_4C_1 pituitary cells. *FEBS Lett.* **312**:123–126 (1992).
14. Freedman, S. B., S. Patel, R. Marwood, F. Emms, G. R. Seabrook, M. R. Knowles, and G. McAllister. Expression and pharmacological characterization of the human D_3 dopamine receptor. *J. Pharmacol. Exp. Ther.* **268**:417–426 (1994).
15. MacKenzie, R. G., D. VanLeeuwen, T. A. Pugsley, Y.-H. Shih, S. Demattos, L. Tang, R. D. Todd, and K. L. O'Malley. Characterization of the human dopamine D3 receptor expressed in transfected cell lines. *Eur. J. Pharmacol.* **266**:79–85 (1994).
16. Chio, C. L., M. E. Lajiness, and R. M. Huff. Activation of heterologously expressed D3 dopamine receptors: comparison with D2 dopamine receptors. *Mol. Pharmacol.* **45**:51–60 (1994).
17. Tang, L., R. D. Todd, A. Heller, and K. O'Malley. Pharmacological and functional characterization of D2, D3 and D4 dopamine receptors in fibroblast and dopaminergic cell lines. *J. Pharmacol. Exp. Ther.* **268**:495–502 (1994).
18. Vallar, L., C. Muca, M. Magni, P. Albert, J. Bunzow, J. Meldolesi, and O. Civelli. Differential coupling of dopaminergic D_2 receptors expressed in different cell types. *J. Biol. Chem.* **265**:10320–10326 (1990).
19. Hayes, G., T. J. Biden, L. A. Selbie, and J. Shine. Structural subtypes of the dopamine D2 receptor are functionally distinct: expression of the cloned $D2_A$ and $D2_B$ subtypes in a heterologous cell line. *Mol. Endocrinol.* **6**:920–926 (1992).
20. Senogles, S. E. The D2 dopamine receptor mediates inhibition of growth in GH_4ZR_7 cells: involvement of protein kinase-C_ϵ. *Endocrinology* **134**:783–789 (1994).
21. Lajines, M. E., C. L. Chio, and R. M. Huff. D2 dopamine receptor stimulation of mitogenesis in transfected Chinese hamster ovary cells: relationship to dopamine stimulation of tyrosine phosphorylations. *J. Pharmacol. Exp. Ther.* **267**:1573–1581 (1993).
22. Swarzenski, B. C., L. Tang, Y. J. Oh, K. L. O'Malley, and R. D. Todd. Morphogenic potentials of D2, D3 and D4 dopamine receptors revealed in transfected neuronal cell lines. *Proc. Natl. Acad. Sci. USA* **91**:649–653 (1994).
23. Tang, L., R. D. Todd, and K. L. O'Malley. Dopamine D2 and D3 receptors inhibit dopamine release. *J. Pharmacol. Exp. Ther.* **270**:475–479 (1994).
24. O'Dowd, B. F. Structures of dopamine receptors. *J. Neurochem.* **60**:804–816 (1993).
25. Strader, C. D., R. A. F. Dixon, A. H. Cheung, M. R. Candelore, A. D. Blake, and I. S. Sigal. Mutations that uncouple the β-adrenergic receptor from G_s and increase agonist affinity. *J. Biol. Chem.* **262**:16439–16443 (1987).
26. Kobilka, B. K., T. S. Kobilka, K. Daniel, J. W. Regan, M. G. Caron, and R. J. Lefkowitz. Chimeric α_2-,β_2-adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science (Washington D. C.)* **240**:1310–1316 (1988).
27. Kubo, T., H. Bujo, I. Akiba, J. Nakai, M. Mashina, and S. Numa. Location of a region of the muscarinic acetylcholine receptor involved in selective effector coupling. *FEBS Lett.* **241**:119–125 (1988).
28. Wess, J., M. R. Brann, and T. I. Bonner. Identification of a small intracellular region of the muscarinic m3 receptor as a determinant of selective coupling to PI turnover. *FEBS Lett.* **258**:133–136 (1989).
29. Cotecchia, S., S. Exum, M. G. Caron, and R. J. Lefkowitz. Regions of the α_1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. *Proc. Natl. Acad. Sci. USA* **87**:2896–2900 (1990).
30. Cotecchia, S., J. Ostrowski, M. A. Kjelsberg, M. G. Caron, and R. J. Lefkowitz. Discrete amino acid sequences of the α_1-adrenergic receptor determine the selectivity of coupling to phosphatidylinositol hydrolysis. *J. Biol. Chem.* **267**:1633–1639 (1992).
31. England, B. P., M. S. Ackerman, and R. W. Barrett. A chimeric D_2 dopamine/m1 muscarinic receptor with D_2 binding specificity mobilizes intracellular calcium in response to dopamine. *FEBS Lett.* **279**:87–90 (1991).
32. Wong, S. K.-F., E. M. Parker, and E. M. Ross. Chimeric muscarinic cholinergic:β-adrenergic receptors that activate G_s in response to muscarinic agonists. *J. Biol. Chem.* **265**:6219–6224 (1990).
33. McAllister, G., M. R. Knowles, S. Patel, R. Marwood, F. Emms, G. R. Seabrook, M. Graziano, D. Borkowski, P. J. Hey, and S. B. Freedman. Characterization of a chimeric hD3/D2 dopamine receptor expressed in CHO cells. *FEBS Lett.* **324**:81–86 (1993).
34. Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symp. Quant. Biol.* **51**:263–273 (1986).
35. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Washington D. C.)* **239**:487–491 (1988).
36. Higuchi, R. Recombinant PCR, in *PCR Protocols: A Guide to Methods and Applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds.). Academic Press, San Diego, 177–183 (1990).
37. Chen, C., and H. Okayama. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**:2745–2752 (1987).
38. Steffey, M. E., G. L. Snyder, R. W. Barrett, J. S. Fink, M. Ackerman, P. Adams, R. Bhatt, E. Gomez, and R. G. MacKenzie. Dopamine D_1 receptor stimulation of cyclic AMP accumulation in COS-1 cells. *Eur. J. Pharmacol.* **207**:311–317 (1991).
39. Samama, P., S. Cotecchia, T. Costa, and R. J. Lefkowitz. A mutation-induced activated state of the β_2-adrenergic receptor. *J. Biol. Chem.* **268**: 4625–4636 (1993).
40. Robinson, S. W., K. R. Jarvie, and M. G. Caron. High affinity agonist binding of the dopamine D3 receptor: chimeric receptors delineate a role for intracellular domains. *Mol. Pharmacol.* **46**:352–356 (1994).
41. Whaley, B. S., N. Nanyong, L. Birnbaumer, R. B. Clark, and R. Barber. Differential expression of the β-adrenergic receptor modifies agonist stimulation of adenylyl cyclase: a quantitative evaluation. *Mol. Pharmacol.* **45**:481–489 (1994).

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