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High Affinity Agonist Binding to the Dopamine D_3 Receptor: Chimeric Receptors Delineate a Role for Intracellular Domains

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SUMMARY

The dopamine D_3 receptor, although structurally similar to the dopamine D_2 receptor, has 100-fold higher affinity for agonists such as dopamine and quinpirole, when these receptors are expressed in 293 cells. Additionally, the D_3 receptor has generally lower affinity for several antagonists than does the D_2 receptor. To determine which regions of the receptor account for these differences, chimeras between D_2 and D_3 receptors were constructed in which intracellular loops were exchanged between the two receptors. A D_2 receptor containing the third intracellular loop (IL3) from the D_3 receptor had 10–20-fold higher affinity for dopamine and quinpirole than did the wild-type D_2 receptor. Conversely, the D_3 receptor containing the IL3 of the D_2 receptor had 15–30-fold lower affinity for agonists than did the wild-type D_3 receptor. That is, in these chimeras the IL3 shifted agonist affinity in a direction consistent with the agonist affinity of the receptor from which the IL3 was derived. In contrast, antagonist binding was not significantly altered. Chimeras in which the second intracellular loop was switched between the D_2 and D_3 receptors had essentially unchanged affinity for both agonists and antagonists. The data presented here suggest that structural differences in the IL3 of the D_2 and D_3 receptors partially account for observed differences in agonist binding to these receptors.

Dopamine receptors are members of the superfamily of G protein-coupled receptors. Receptors in this family have seven putative membrane-spanning helices connected by extra- and intracellular loops. In general, these receptors have been shown to have separate domains that perform different functions, i.e., regions that bind ligands and those that interact with G proteins. Catecholamine receptors have been shown to have the ligand binding pocket within the TM domains, whereas regions of the cytoplasmic loops, particularly at the amino- and carboxyl-terminal ends of the IL3, have been implicated in G protein interactions (for review, see Refs. 1–3).

The dopamine receptor family currently consists of five members. These five subtypes can be grouped by similarities to the classically defined D_1 and D_2 receptors (4, 5). The D_{1A} and D_{1B} (D_5) receptors are D_1-like receptors. They have similar structures and pharmacology, and both are coupled to stimulation of adenylyl cyclase (6, 7). The D_2-like receptors, the D_2, D_3, and D_4 receptor subtypes, likewise are similar in their structures and pharmacology (8–10). The D_2 receptor is coupled to a number of second messenger pathways, including inhibition of adenylyl cyclase and modulation of K^+ and Ca^{2+} currents (for review, see Ref. 11). The D_4 receptor has also been shown to inhibit adenylyl cyclase (12). The relevant coupling of the D_3 receptor has yet to be determined.

The D_2 and D_3 receptors are structurally very similar. The human D_2 and D_3 receptors share 46% overall homology and are 78% identical within their TM domains (13). Although their structures and general pharmacology define them as members of the D_2-like subfamily, the D_2 and D_3 receptors have substantially different affinities for many compounds. The affinity of agonists at the D_3 receptor is generally much higher than at the D_2 receptor, particularly for dopamine, quinpirole, and 7-hydroxydipropylaminotetralin. These differences are on the order of 20–100-fold (9, 14). Furthermore, binding of agonists to the D_3 receptor is not modulated by guanine nucleotides (9, 15, 16). The D_3 receptor also has generally lower affinity for most antagonists than does the D_2 receptor. The largest differences observed have been for spiperone, domperidone, and haloperidol, which have 10–30-fold lower affinity for the D_3 receptor in Chinese hamster ovary cells (9, 14).

Site-directed mutagenesis of the carboxyl-terminal portion of the IL3 of ARs has recently been reported to confer constitutive activity to these receptors (17–20). These mutant receptors, in addition to having increased activity in the absence of agonist stimulation, also have large increases in agonist affinity, although antagonist affinity is unchanged (17–20). This change

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ABBREVIATIONS: TM, transmembrane; IL3, third intracellular loop; AR, adrenergic receptor; IL2, second intracellular loop; PCR, polymerase chain reaction.

in agonist binding was shown to be independent of G proteins (19). These results suggest that sequences in the IL3 can influence the affinity with which agonists bind to their receptors. To test whether the intracellular loops account for the high affinity of agonists at the D_3 receptor, we constructed chimeric receptors in which these loops were switched between the D_2 and D_3 receptors. Interestingly, our results indicate that the high affinity with which the D_3 receptor binds agonists can be accounted for, at least in part, by differences in the sequences of the IL3 region between the D_2 and D_3 receptors. Reciprocal gains and losses in agonist affinity were obtained, respectively, by switching the IL3 from the D_3 receptor to the D_2 receptor and vice versa. These changes in affinity were agonist specific and also specific to the IL3, because exchanging the IL2 did not substantially change ligand binding to either receptor. These data indicate that the IL3 of the D_2 and D_3 receptors is an important determinant of agonist affinity and partially, but not completely, accounts for the guanine nucleotide-independent high affinity agonist binding properties of the D_3 receptor.

Experimental Procedures

Materials. [^{3}H]Spiperone was obtained from NEN (Boston, MA) or Amersham (Arlington Heights, IL). Dopamine, quinpirole, domperidone, haloperidol, (−)-sulpiride, and (+)-butaclamol were from Research Biochemicals International (Natick, MA). Sequenase version 2.0 was obtained from United States Biochemicals (Cleveland, OH). Cell culture reagents were from GIBCO/BRL (Gaithersburg, MD). Human D_3 receptor cDNA was kindly provided by Dr. Barbara Sahagan, DuPont Merck Pharmaceutical Co. (Wilmington, DE).

Construction of chimeric receptors. The PCR was used to construct all chimeric receptors. The mutants in which the IL2 or IL3 was transferred from one receptor cDNA to the other were constructed using an extension of the gene fusion method of Yon and Fried (21). The method used is illustrated in Fig. 1. The templates for PCR were the human $D_{2\text{-short}}$ and D_3 receptor cDNAs. After the three PCRs, the chimeric product was then subcloned into the appropriate wild-type receptor using existing restriction sites. The D_2-(D_3-IL3) chimera was constructed using the D_2 receptor restriction sites *Bst*EII and *Hinc*II. The D_3-(D_2-IL3) chimera was made with the *Sac*I and *Acc*III sites in the D_3 receptor. The D_2 receptor restriction sites *Bam*HI and *Apa*I were used to construct the D_2-(D_3-IL2) chimera, whereas *Acc*I and *Bam*HI sites in the D_3 receptor were used in making the D_3-(D_2-IL2) chimera.

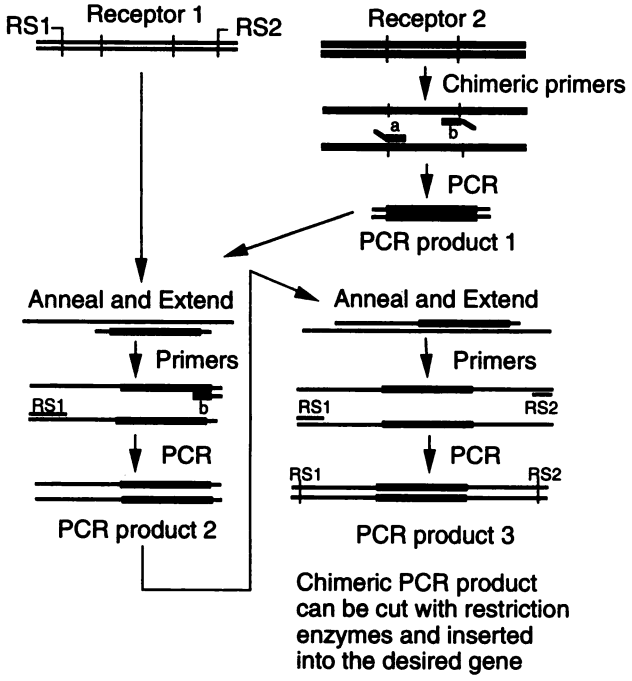

Fig. 1. Schematic diagram of the PCR method used to construct chimeric receptors. This method is a modification of that of Yon and Fried (21). RS, restriction site.

D_2 chimeras were initially constructed in pGEM3Z (Promega) and D_3 chimeras were made in pSP72 (Promega). All chimeras were then subcloned into the expression vector pCMV5 (22) for transfection in 293 cells. Each construct was sequenced using Sequenase version 2.0, to verify the sequence.

Cell culture. Human embryonic kidney 293 cells (CRL 1573; American Type Culture Collection) were grown at 37° in 5% CO_2 in minimal essential medium supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin. Transient transfection of the cells was by the calcium phosphate precipitation method (23). Cells were harvested for binding 60–70 hr after transfection.

Receptor binding assays. The 293 cells were scraped in lysis buffer (5 mM EDTA, 5 mM Tris·HCl, pH 7.4) and centrifuged at >40,000 × *g* for 20 min at 4°. The pellet was resuspended in binding buffer (118 mM NaCl, 50 mM Tris·HCl, pH 7.4, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, 5 mM KCl, 1 mM Na_2EDTA) by homogenization with a Brinkmann Polytron for 10 sec at 15,000 rpm. Saturation analysis was done using [^{3}H]spiperone at concentrations ranging from 0.04 to 2.2 nM. For competition curves, membranes were incubated with 250 pM [^{3}H]spiperone and increasing concentrations of competitor. All assays were in a final volume of 0.5 ml containing 5–20 μg of protein/tube. Nonspecific binding was determined by the addition of 1 μM (+)-butaclamol. Assays were incubated for 1 hr at 22° and terminated by rapid filtration over Whatman GF/C filters, and filters were washed with 3 × 3 ml of ice-cold wash buffer (100 mM NaCl, 50 mM Tris, pH 7.4). All assays were done in triplicate and carried out at least three times. Data analysis was performed with the nonlinear curve-fitting program EBDA/LIGAND (24).

Results

Four chimeric D_2/D_3 receptors were constructed in which the IL3 or IL2 was switched between the two receptors. The junctions between the two receptors have been made within the putative TM domains and the locations of these junctions are shown schematically in Fig. 2A. Detailed descriptions of these mutants are given in the legend to Fig. 2, and the locations of the junctions are shown in alignments of the relevant regions in Fig. 2B. All receptors were transiently expressed in 293 cells at a level of 1–5 pmol/mg of membrane protein. The K_d of each wild-type or mutant receptor for [^{3}H]spiperone was determined by saturation binding. The wild-type D_2 and D_3 receptors had a 2-fold difference in affinity for spiperone (0.16 nM and 0.31 nM, respectively). None of the chimeras showed greater than a 2–3-fold change in affinity for [^{3}H]spiperone, compared with the appropriate wild-type receptor (Table 1).

Competition binding experiments were carried out to determine the affinity with which different ligands bound to the wild-type and chimeric receptors. Both agonists and antagonists interacted at a single site, indicating that only the uncoupled state of the receptor was being measured. In 293 cells, both the D_2-(D_3-IL3) and D_3-(D_2-IL3) chimeras (IL3 chimeras) had essentially unchanged affinities, compared with the wild-type receptors, for the antagonists domperidone, haloperidol, and (−)-sulpiride. The changes in affinity for these compounds were 3-fold or less (Table 1). In contrast, the affinity of the IL3 chimeras for agonists was altered, compared with the wild-type

A

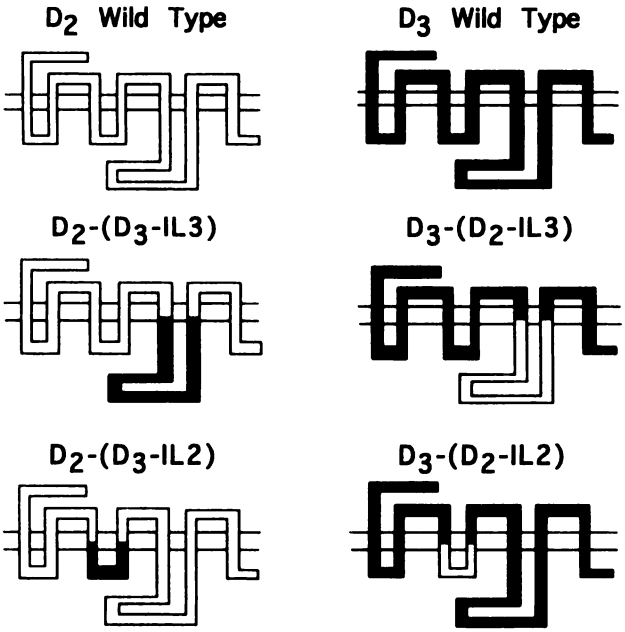

Fig. 2. A, Structure of chimeras of the human $D_{2\text{-short}}$ (□) and D_3 (■) receptors. The composition of the chimeric receptors is as follows: D_2 wild-type: amino acids 1–415; D_3 wild-type: 1–401; D_2-(D_3-IL3): D_2, 1–201; D_3, 201–343; D_2, 359–415; D_3-(D_2-IL3): D_3, 1–200; D_2, 202–358; D_3, 344–401; D_2-(D_3-IL2): D_2, 1–116; D_3, 113–162; D_2, 165–415; D_3-(D_2-IL2): D_3, 1–112; D_2, 117–164; D_3, 163–401. B, Alignment of TM domains and intervening loops from the human D_2 and D_3 receptors. *Bold amino acids*, locations of the junctions in the chimeras.

B

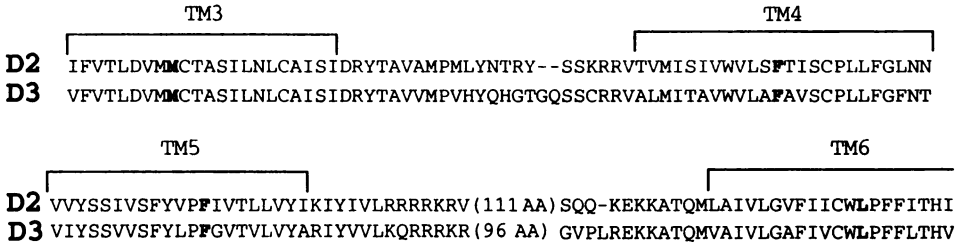

TABLE 1

Antagonist pharmacology of wild-type and chimeric receptors

Wild-type and chimeric receptors were transiently expressed in 293 cells. [^{3}H]Spiperone binding is reported as K_d values, which were determined by saturation analysis. K_i values for the other antagonists were determined by competition with [^{3}H]spiperone binding. All values are reported as mean ± standard error of the number of experiments given in parentheses.

	Spiperone K_d	K_i		
		Domperidone	Haloperidol	(−)-Sulpiride
	nM		nM	
D_2 wild-type	0.16 ± 0.04 (7)	0.26 ± 0.07 (5)	0.75 ± 0.22 (5)	22 ± 5 (3)
D_2-(D_3-IL3)	0.05 ± 0.02 (3)	0.80 ± 0.43 (4)	1.2 ± 0.5 (4)	29 ± 9 (3)
D_2-(D_3-IL2)	0.37 ± 0.10 (3)	1.0 ± 0.3 (3)	1.6 ± 0.9 (3)	19 ± 3 (3)
D_3 wild-type	0.31 ± 0.06 (7)	3.4 ± 0.9 (5)	2.2 ± 0.7 (6)	19 ± 3 (3)
D_3-(D_2-IL3)	0.20 ± 0.10 (3)	4.8 ± 1.1 (4)	2.7 ± 0.8 (3)	25 ± 7 (3)
D_3-(D_2-IL2)	0.33 ± 0.19 (3)	6.1 ± 0.9 (3)	1.9 ± 0.8 (3)	46 ± 3 (3)

receptors. The wild-type D_3 receptor had 150-fold higher affinity for dopamine and 260-fold higher affinity for quinpirole than did the wild-type D_2 receptor in 293 cells (Table 2). The D_2-(D_3-IL3) chimera had 10-fold higher affinity for dopamine and 18-fold higher affinity for quinpirole than did the wild-type D_2 receptor (Table 2). The reciprocal mutant, D_3-(D_2-IL3), had 15-fold and 28-fold lower affinity for dopamine and quinpirole, respectively, than did the wild-type D_3 receptor (Table 2). Interestingly, the affinities of agonists for both of these chimeras were shifted about halfway toward the affinities for the receptor from which the IL3 was taken (Fig. 3). That is, the difference in agonist affinity between the two wild-type

TABLE 2

Agonist pharmacology of wild-type and chimeric receptors

Wild-type and chimeric receptors were transiently expressed in 293 cells. K_i values were determined by competition with [^{3}H]spiperone binding. All values are reported as mean ± standard error of the number of experiments given in parentheses.

	K_i	
	Dopamine	Quinpirole
	nM	
D_2 wild-type	3,700 ± 600 (8)	6,400 ± 900 (6)
D_2-(D_3-IL3)	338 ± 58 (7)	352 ± 41 (5)
D_2-(D_3-IL2)	8,300 ± 4,400 (3)	10,800 ± 3,800 (3)
D_3 wild-type	25 ± 2 (10)	26 ± 4 (9)
D_3-(D_2-IL3)	381 ± 40 (6)	708 ± 67 (5)
D_3-(D_2-IL2)	62 ± 12 (3)	37 ± 3 (3)

receptors is about 2 orders of magnitude, whereas both of the IL3 chimeras have an affinity for agonists that is shifted by about 1 order of magnitude toward that of the other wild-type receptor.

The D_2-(D_3-IL2) and D_3-(D_2-IL2) chimeras (IL2 chimeras), like the IL3 chimeras, had no change in their ability to bind antagonists. However, unlike the IL3 chimeras, the IL2 chimeras also bound dopamine and quinpirole with affinities comparable to their respective wild-type receptor affinities (Table 2). In fact, both chimeras generally had slightly lower affinity for agonists, but these changes were <3-fold.

Discussion

We have constructed chimeric D_2/D_3 receptors in which the IL2 or IL3 was switched between these receptors. When the pharmacology of these receptors was examined, it was observed that sequences in the intracellular regions exerted a marked influence on the affinity of the receptors for agonists, but not for antagonists. Importantly, these changes were reciprocal. Agonist affinity for the D_3 receptor was reduced by substitution with the IL3 from the D_2 receptor, which binds agonists with relatively lower affinity. Correspondingly, an increase in agonist affinity at the D_2 receptor occurred upon substitution with the IL3 from the D_3 receptor, which has markedly higher affinity for agonists. These results suggest that the IL3 of D_2 and D_3 receptors is an important determinant of agonist affinity.

In contrast to the effects of the IL3 on agonist affinity, the IL2 did not substantially affect agonist binding. The IL2 had no greater effect on the affinities of agonists than on those of antagonists. This suggests that the IL2 exerts little influence on the affinity of these receptors for ligands and its effects are not specific for either agonists or antagonists, in contrast to the agonist-specific effects observed for the IL3.

From these data, it would appear that sequences within the intracellular domains modulate agonist affinity of the dopamine D_2 and D_3 receptors. However, based on the data presented here this conclusion must be made with an additional caveat, due to the inclusion of some TM sequence in the chimeras (Fig. 2). When IL3 chimeras were engineered with the junction at the putative cytoplasmic ends of TM domains 5 and 6, there was no specific binding of [^{3}H]spiperone when these constructs were expressed in COS-7 cells (data not shown). Therefore, to obtain functional chimeras, the junctions between receptors were made within the relatively well conserved TM domains. Thus, despite the large change of the IL3, a contribution from the included putative TM sequences cannot be ruled out. However, all chimeras in which the junctions between the two receptors were located within the TM domains were able to bind [^{3}H]spiperone with an appropriate K_d when expressed in 293 cells, suggesting that no substantial change in the binding site for the radioligand occurred in the chimeras. In the case of the IL3 mutants, three residues each were changed in TM domains 5 and 6 (Fig. 2B). These changes are generally conservative. Furthermore, in constructing the IL2 mutants, no changes were made in TM domain 2, whereas four changes were made in TM domain 3 (Fig. 2B); however, these mutants did not have altered ligand binding properties.

In mutagenesis studies of G protein-coupled receptors, most modifications in intracellular domains have been found to affect coupling of receptors to G proteins (1–3). However, a number of alterations can be found that also seem to affect agonist binding affinity for these receptors. Many but not all of these mutations are found in the IL3 of these receptors. For example,

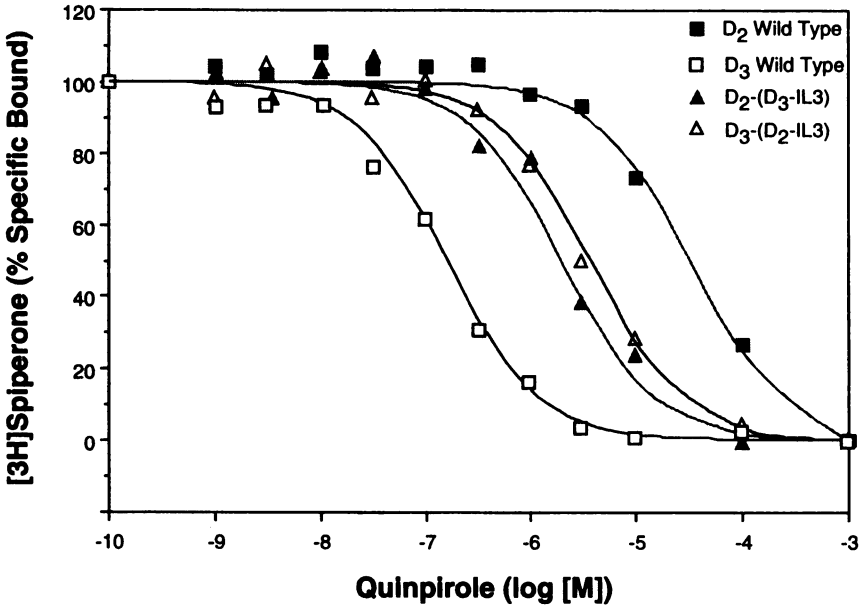

Fig. 3. Effect of IL3 substitutions on agonist binding affinity, measured as competition by quinpirole with [^{3}H]spiperone binding (250 pM). *Curves* are representative of at least three experiments performed in triplicate. K_i values are shown in Table 2. Curves were fit with the nonlinear curve-fitting package EBDA/LIGAND (23).

deletions at the amino- and carboxyl-terminal regions of the β_2-AR IL3 resulted in a receptor with increased affinity for agonists (25, 26). In addition, a deletion of residues within the IL3 of the m1 muscarinic receptor had a similar effect (27). Certain amino acid substitutions in the amino- and carboxyl-terminal portions of the IL3 of the β_2-AR and also in the first intracellular loop of this receptor caused 3–10-fold increases in affinity for agonist (28, 29). Chimeric receptors in which the IL3 or IL2 of various receptors have been switched also point out the effects of intracellular loops on ligand binding. An α_{1B}-AR containing the IL3 of the β_2-AR had higher affinity for agonist (17). Also, chimeras of the m1 muscarinic receptor containing the IL2 from the β_1-AR had 10-fold higher affinity for the muscarinic agonist carbachol (30). In addition, constitutively active ARs that contain mutations in the carboxyl-terminal portion of the IL3 gain higher affinity for agonist (17–20). These data all suggest that the intracellular loops of G protein-coupled receptors, particularly the IL3 in catecholamine receptors, exert an influence on the agonist binding properties of these receptors.

We demonstrate here that the IL3 influences agonist binding to the D_2 and D_3 receptors, because switching the entire IL3 between these receptors, which have different affinities for agonists, shifts the affinity for these compounds toward that of the receptor from which the IL3 was derived. A possible explanation for this effect of the IL3 on agonist affinity is that this region of the receptor influences the conformation of the TM domains, altering the ligand binding pocket slightly in the chimeric receptors. Because the exchange of the IL3 does not completely shift the affinity for agonists from one subtype to the other, there must be other regions of the receptor that are also involved in determining the differences in agonist binding observed between the D_2 and D_3 receptors. One possibility is that sites in the TM domains that presumably make up the ligand binding pocket (1–3) are responsible for the remainder of the differences observed in agonist affinity. Also, other intracellular regions in addition to the IL3, such as the first intracellular loop or the carboxyl-terminal tail, may play a role. Although delineation of all of the determinants responsible for ligand binding to G protein-coupled receptors may have to await the understanding of the three-dimensional structure of these proteins, the reciprocal changes observed here between the D_2 and D_3 receptors strongly imply a role for intracellular domains beyond their interaction with G proteins.

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