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D_2/D_3 Dopamine Receptor Heterodimers Exhibit Unique Functional Properties*

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Evidence for heterodimerization has recently been provided for dopamine D_1 and adenosine A_1 receptors as well as for dopamine D_2 and somatostatin $SSTR_5$ receptors. In this paper, we have studied the possibility that D_2 and D_3 receptors interact functionally by forming receptor heterodimers. Initially, we split the two receptors at the level of the third cytoplasmic loop into two fragments. The first, containing transmembrane domains (TM) I to V and the N-terminal part of the third cytoplasmic loop, was named D_{2trunk} or D_{3trunk}, and the second, containing the C-terminal part of the third cytoplasmic loop, TMVI and TMVII, and the C-terminal tail, was named D_{2tail} or D_{3tail}. Then we defined the pharmacological profiles of the homologous (D_{2trunk}/D_{2tail} and D_{3trunk}/D_{3tail}) as well as of the heterologous (D_{2trunk}/D_{3tail} and D_{3trunk}/D_{2tail}) cotransfected receptor fragments. The pharmacological profile of the cross-cotransfected fragments was different from that of the native D_2 or D_3 receptors. In most cases, the D_{3trunk}/D_{2tail} was the one with the highest affinity for most agonists and antagonists. Moreover, we observed that all of these receptor fragments reduced the expression of the wild type dopamine D_2 and D_3 receptors, suggesting that D_2 and D_3 receptors can form complexes with these fragments and that these complexes bind [^{3}H]nemonapride less efficiently or are not correctly targeted to the membrane. In a second set of experiments, we tested the ability of the split and the wild type receptors to inhibit adenylyl cyclase (AC) types V and VI. All of the native and split receptors inhibited AC-V and AC-VI, with the exception of D_3, which was unable to inhibit AC-VI. We therefore studied the ability of D_2 and D_3 to interact functionally with one another to inhibit AC-VI. We found that with D_2 alone, R-(+)-7-hydroxydypropylaminotetralin hydrobromide inhibited AC-VI with an IC_{50} of 2.05 ± 0.15 nM, while in the presence of D_2 and D_3 it inhibited AC-VI with an IC_{50} of 0.083 ± 0.011 nM. Similar results were obtained with a chimeric cyclase made from AC-V and AC-VI. Coimmunoprecipitation experiments indicate that D_2 and D_3 receptors are capable of physical interaction.

G protein-coupled receptors are seven-transmembrane domain proteins that mediate a variety of signaling processes. Dopamine receptors are members of this family and are important for a variety of functions in the nervous system, including drug reward, learning, motor activity, and neuropsychiatric disorders. Molecular cloning has revealed the existence of five different receptor subtypes that have been grouped into two different classes based on pharmacological and biochemical profiles: (i) the D_1-like receptors, D_1 and D_5, are positively coupled to adenylyl cyclase (AC),[1] and (ii) the D_2-like receptors, D_2, D_3, and D_4, are negatively coupled to this enzyme (for a review, see Ref. 1). An alternative splicing of 29 amino acids at the level of the third cytoplasmic loop of the dopamine D_2 receptor leads to the generation of two molecular forms: the dopamine D_{2long} (D_{2l}) and D_{2short} (D_{2s}) receptors (2–4). Analogous splice variants have been observed in the mouse for the D_3 receptor (5).

Traditionally, the interaction of G protein-coupled receptors has been described by models that assume that the receptor exists as a monomer coupled to G protein in a 1:1 stoichiometry. However, these classical models of receptor/G protein coupling may be oversimplified. It has now been shown that many G protein-coupled receptors can form dimers or higher order oligomers and that this phenomenon has relevance to receptor function (for a review, see Ref. 6). Dopamine receptors have also been shown to form dimers and higher order oligomers. Evidence has been provided for D_1, D_2, and D_3 homodimers in transfected cell lines (7–9), and D_2 receptors have been shown to exist as dimers in human and rat brain tissues (10). Moreover, Rocheville *et al.* (11) have recently shown that the dopamine D_2 receptors not only form homodimers but also form heterodimers with somatostatin $SSTR_5$ receptors. In addition, Gines *et al.* (12) have shown that the dopamine D_1 receptor forms hetero-oligomers with the adenosine A_1 receptor.

As the issue of G protein-coupled receptor homo- and heterodimerization is becoming more and more important, it is crucial to define the mechanism(s) of receptor-receptor interactions in order to predict which receptors can interact with one another. The results obtained until now suggest that more than one mechanism exists and that one receptor can interact with another in more than one way.

One of the mechanisms that have been proposed to explain receptor dimerization is the phenomenon of domain swapping

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[1] The abbreviations used are: AC, adenylyl cyclase; FS, forskolin; 7-OH-DPAT, R-(+)-7-hydroxydypropylaminotetralin hydrobromide; TM, transmembrane domain.

proposed by Gouldson *et al.* (13, 14). Domain swapping is a very efficient method for forming dimers, since the interactions within the monomers are being preserved in the dimer. We have previously shown that this mechanism occurs between two chimeras of α_2-adrenergic and m_3-muscarinic receptors (15); more recently, using a pharmacological approach, we have shown that domain swapping also occurs between the wild type m_2- and m_3-muscarinic receptors (16).

Since different dopamine receptors (including D_2 and D_3) are often physiologically coexpressed in the same cells (17, 18), we decided to use this same domain swapping approach to identify the interaction between the D_2- and D_3-dopamine receptors. As mentioned above, these two receptors belong to the family of D_2-like receptors, and they share a high degree of amino acid homology and are therefore suitable for an interaction through domain swapping.

We have previously shown that the dopamine D_2 receptor can be split at the level of the third cytoplasmic loop into two fragments (D_{2trunk} and D_{2tail}) and that the mixture of the two fragments retains the binding and functional activity of the wild type receptor (19). In the present study, we extended this analysis to the D_3 receptor, and in addition, we cross-cotransfected the fragments originating from the two dopamine receptors in order to study the pharmacology of the mixed D_{2trunk}/D_{3tail} or D_{3trunk}/D_{2tail} receptor fragments. We further investigated whether the wild type D_2 and D_3 receptors can functionally interact to inhibit AC. The results show that, indeed, the cotransfection of fragments originating from D_3 was able to rescue in full the pharmacology and the functional activity of this receptor. Moreover, the mixed D_{2trunk}/D_{3tail} and D_{3trunk}/D_{2tail} receptor fragments were able to bind dopamine ligands and to inhibit AC activity. The D_2 receptor was able to rescue the ability of the dopamine D_3 receptors to inhibit AC type VI in conditions under which the D_3 receptor by itself does not communicate with this AC isozyme (20). Coimmunoprecipitation experiments provide further confidence that D_2 and D_3 receptors are able to physically interact. Together, these results demonstrate the formation of functional, active D_2-D_3 heterodimers.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Adenine and [^{3}H]nemonapride were from PerkinElmer Life Sciences; forskolin (FS), pertussis toxin, clozapine, (−)-quinpirole, R-(−)-apomorphine, R-(+)-7-hydroxydypropylaminotetralin hydrobromide (7-OH-DPAT), dopamine, and haloperidol were from Sigma; Ro-20–1724 was from Calbiochem. Olanzapine and pergolide were from Lilly; domperidone and BP897 were kindly provided by Janssen Pharmaceuticals (Beerse, Belgium) and C. G. Wermuth (Université Louis Pasteur, Strasbourg, France), respectively. Tissue culture media and sera were from Sigma and Life Technologies, Inc.

Plasmids and Preparation of Mutant Dopamine Receptor Constructs—Full-length cDNA (~2.1 kilobase pairs) coding for the rat dopamine D_{2s} receptor was subcloned between the *Hin*dIII and *Apa*I sites of the pRc/CMV vector (Invitrogen). The resulting construct, pRc/CMV-D_{2s}, was used to prepare the D_{2trunk} and D_{2tail} receptor fragments (Fig. 1; see Ref. 20 for details).

A full-length cDNA (~1.4 kilobase pairs) encoding the rat D_3 receptor was subcloned in the pRc/CMV vector between the *Eco*RI and *Sal*I sites of the pRc/CMV vector (Invitrogen). For the construction of the truncated receptor fragments, polymerase chain reaction was utilized. The D_{3trunk} fragment (containing the extracellular N-terminal part of the D_3 receptor, TMI–TMV and the proximal portion of the i3 loop until Ser-315) was obtained using two primers: the sense primer, containing a *Hin*dIII site and spanning the start codon (underlined) of the dopamine D_3 receptor (CCCAAGCTTCC<u>ATG</u>GCACCTCTGAGCCAG), and the antisense primer, containing a stop codon and an *Xba*I site and spanning the region of Ser-315 (GCTCTAGATCAGCTTGGGTGTCTCAAGGCA). The amplified polymerase chain reaction fragment was digested with *Hin*dIII and *Xba*I and introduced into the polylinker region of the pRc/CMV vector. The D_{3tail} fragment (containing the distal portion of the i3 loop starting from Glu-317, TMVI and TMVII, and the C-terminal part of the D_3 receptor) was constructed using two primers: the sense primer, containing a *Hin*dIII site and an initiation ATG codon and spanning the codon for Glu-317 of the dopamine D_3 receptor (CCCAAGCTTCCATGGAAGGCGGGGCAGGGATGA), and the antisense primer, containing an *Xba*I site and spanning the region of the stop codon (GCTCTAGAGTGCGGTCTCTTCTCCTC). The amplified polymerase chain reaction fragment was digested with *Hin*dIII and *Xba*I and introduced into the polylinker region of the pRc/CMV vector. Mouse D_{2l} and D_{3l} subcloned into the eukaryotic expression vector pcDNA/Neo (Stratagene) were kindly provided by Dr. Sara Fuchs. Binding experiments were performed with rat dopamine receptors, while functional experiments were performed with mouse and rat dopamine receptors.

Descriptions of the cDNAs for rabbit AC-V in pXMD1 and rat AC-VI in pCMV-neo were given previously (21). The cDNA encoding the AC-V/VI chimera was prepared in pXMD1 by joining the cDNA encoding amino acids 1–856 of rabbit AC-V with that encoding amino acids 774–1166 of rat AC-VI at an *Afl*III site. The junction was made at an area of homology between AC-V and AC-VI in the carboxy part of the ninth transmembrane domain of the AC molecules.

Cell Cultures and Transfection—COS-7 cells were incubated at 37 °C in a humidified atmosphere (containing 5% CO_2) and grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded at a density of 2×10^6/100-mm dish. 24 h later, they were transiently transfected with the plasmid DNA encoding the dopamine receptors and, when indicated, also AC types V and VI, by the DEAE-dextran chloroquine method (22). The total amount of DNA used for each transfection was completed to 4 μg by the addition of an appropriate amount of vector DNA. For the binding assays, in order to increase the expression of the receptors, cells were incubated with 5 mM sodium butyrate (sterilized by filtration) for the last 24 h before membrane preparation.

Membrane Preparation and Binding Assay—Three days after transfection, confluent plates of cells were lysed by replacing the medium with ice-cold hypotonic buffer (1 mM Na-HEPES, 2 mM EDTA). After 20 min, the cells were scraped off the plate and centrifuged at 17,000 rpm for 20 min. The lysed cell pellet was homogenized with a Polytron homogenizer in the binding assay buffer (50 mM Tris HCl, pH 7.4, 155 mM NaCl, 0.01 mg/ml bovine serum albumin). Binding of [^{3}H]nemonapride (82 Ci/mmol) was carried out at 30 °C for 1 h in a final volume of 1 ml. Dopamine (2 mM) was used to define nonspecific binding. For saturation experiments, seven different concentrations of the radioligand [^{3}H]nemonapride were used. Displacement experiments were performed with 12 concentrations of the unlabeled competitor in the presence of a fixed concentration of the radioligand. In competition binding studies in which agonists were used, the membranes were resuspended in assay buffer containing 4 mM $MgCl_2$, 1 mM EDTA, and 0.025% ascorbic acid. The bound ligand was separated from the unbound ligand using glass fiber filters (Whatman; GF/B) with a Brandel Cell Harvester, and the filters were counted with a scintillation β-counter.

AC Assay—24 h after transfection, the cells were trypsinized and recultured in 24-well plates, and after an additional 24 h, the cells were assayed for AC activity. The assay was performed in triplicate as described by Avidor-Reiss *et al.* (23). In brief, the cells in the 24-well plates were incubated for 2 h with 0.25 ml/well of fresh growth medium containing 5 μCi/ml [^{3}H]adenine, and this medium was replaced with 0.5 ml/well of Dulbecco's modified Eagle's medium containing 20 mM HEPES, pH 7.4, 0.1 mg of bovine serum albumin, and the phosphodiesterase inhibitors 1-methyl-3-isobutylxanthine (0.5 mM) and RO-20–1724 (0.5 mM). AC activity was stimulated by the addition of 1 μM FS in the presence or absence of the indicated dopamine receptor ligands. After a 10-min incubation at 30 °C, the medium was removed, and the reaction was terminated by the addition of perchloric acid containing 0.1 mM unlabeled cAMP, followed by neutralization with KOH. The amount of [^{3}H]cAMP formed was determined by a two-step column separation procedure, as described by Avidor-Reiss *et al.* (23).

Immunoprecipitation and Immunoblotting—Immunopreceipitation and immunoblotting were carried out essentially as described by Karpa *et al.* (24). Briefly, FLAG-tagged human D_2 and HA-tagged monkey D_3 dopamine receptors were expressed either singly or together in HEK 293 cells. Immunoprecipitation was carried out using anti-HA antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and immunocomplexes were captured using Protein A/G resin (CytoSignal, Irvine, CA). Following separation on SDS-polyacrylamide gels and transfer to nitrocellulose filters, proteins were detected using the anti-FLAG M2 monoclonal antibody (Sigma) and horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA).

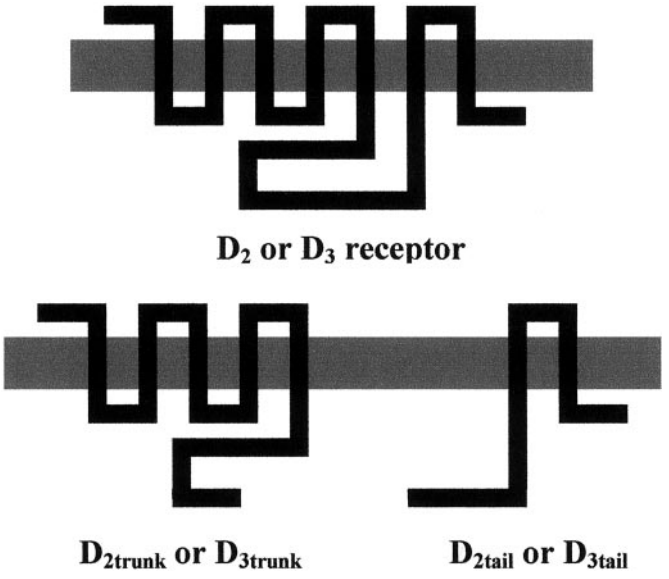

FIG. 1. **Schematic representation of wild type dopamine D_2 and D_3 receptors and the derived receptor fragments.** The truncated D_2 and D_3 receptors (D_{2trunk} and D_{3trunk}) contain an in frame STOP codon after Lys-241 and Ser-315, respectively, while the tail fragments (D_{2tail} and D_{3tail}) contain a START codon before Asn-245 and Glu-317, respectively.

RESULTS

We had previously shown that the dopamine D_2 receptor can be split at the level of the third cytoplasmic loop and retains its pharmacological and functional characteristics (19). In this work, we extended this analysis to the dopamine D_3 receptor. The mixture of D_{3trunk} and D_{3tail} fragments (defined as D_{3trunk}/D_{3tail}; Fig. 1) was coexpressed in COS-7 cells, and the cells were tested for binding. The radioligand [^{3}H]nemonapride bound with high affinity to the split dopamine D_3 receptor, and the K_D was not significantly different from that of the wild type receptor (Table I). Neither of the two fragments, when transfected alone, bound [^{3}H]nemonapride (data not shown). The pharmacological profile of the split D_{3trunk}/D_{3tail} receptor perfectly superimposed over that of the dopamine D_3 receptor (Table I). These results show that the two halves of the D_3 receptor recognize each other to form an active complex.

In a second set of experiments, we tested whether or not the cross-cotransfection of fragments originating from D_2 and D_3 results in the formation of protein complexes that are able to bind [^{3}H]nemonapride. We found that D_{2trunk} mixed with D_{3tail} (D_{2trunk}/D_{3tail}) and D_{3trunk} mixed with D_{2tail} (D_{3trunk}/D_{2tail}) receptor complexes were both able to bind [^{3}H]nemonapride with high affinity (Table I). We next defined the pharmacological profile of these mixed receptors. The two antipsychotic drugs, clozapine and olanzapine, showed the highest affinity for the split D_{3trunk}/D_{2tail} receptor complex, with a 3-fold gain in affinity with respect to D_2 and about 8-fold gain in affinity with respect to D_3 (Table I). Furthermore, the D_{3trunk}/D_{2tail} receptor fully retained a high affinity for the dopamine D_2 receptor-preferring antagonist domperidone, while the split D_{2trunk}/D_{3tail} receptor complex showed an intermediate affinity (between the values for the D_2 and D_3 receptors) for this compound (Table I).

Among the dopamine agonists, pergolide and apomorphine showed a high affinity for the split D_{3trunk}/D_{2tail} receptor, with a 5-fold gain in affinity with respect to D_2, while dopamine and quinpirole had intermediate affinities for the split heteroreceptors, between those of the wild type D_2 and D_3 receptors (Table I). We also tested two highly selective agonists for the D_3 receptor, 7-OH-DPAT and BP897. Both of them displayed high affinity for the split D_{3trunk}/D_{2tail} receptor similar to that of the wild type D_3 receptor and a gain in affinity with respect to D_2 of 40- and 24-fold for 7-OH-DPAT and BP897, respectively (Table I). All of the agonists gave inhibition curves that were best fitted by a one-site model. The high degree of scatter in the binding data, together with the small proportion of receptors in the high affinity state has precluded a two-site fit. However, since some curves had a Hill coefficient significantly lower than 1, we preferred to express the data as IC_{50} corrected for the radioligand occupancy (IC_{50corr}).

Receptor fragments were also cotransfected with the wild type D_2 and D_3 receptors, and the cells were tested for binding, in order to see if an eventual interaction between fragment and receptor could result in a modification of the binding affinity to selected compounds. To this end, we cotransfected the cells with either dopamine D_2 receptor together with the D_{3trunk} fragment or with D_3 receptor together with the D_{2tail} fragment, and we tested the binding affinity of 7-OH-DPAT and of domperidone. In both cases, we found that none of the fragments modified the affinity of 7-OH-DPAT and domperidone for the wild type receptors (data not shown). Despite this apparent lack of interaction in terms of binding affinity, all of the fragments interfered with the expression level of the wild type receptors. As shown in Table II, all of the four receptor fragments reduced the number of D_2 and D_3 receptor binding sites. As shown here for the D_{2trunk} mixed with the D_2 receptor, this phenomenon could be directly correlated with the amount of D_{2trunk} cDNA used in the transfection (Table II). As control, we showed that expression of the truncated muscarinic m_2 receptor, m_{2trunk}, did not modify expression of full-length dopamine D_2 or D_3 receptors (Table II).

In the second part of the work, we tested whether agonist activation of the split dopamine receptors has the ability to regulate the activity of AC and thus to inhibit the FS-induced increase in cAMP production. For this purpose, we cotransfected COS-7 cells with dopamine receptors, together with AC-V. As shown in Table III, both wild type D_2 and D_3 as well as the split dopamine receptors were able to inhibit AC-V when activated by quinpirole. The calculated IC_{50} values for inhibition by quinpirole were in the nanomolar range. However, the wild type D_3 receptor showed a 3-fold higher potency than D_2 (IC_{50} values were 0.68 ± 0.24 and 2.23 ± 0.34 nM for the D_3 and D_2 receptors, respectively). Moreover, the extent of inhibition was different among the receptors; the largest inhibition was observed with the dopamine D_2 receptor (61.4 ± 5.9%), while the weakest was seen with the D_3 receptor, 25.2 ± 9.5% (Fig. 2*A*). The split D_2 and D_3 receptors (D_{2trunk}/D_{2tail} and D_{3trunk}/D_{3tail}) gave an inhibition of 35.1 ± 7.5 and 40.7 ± 8.1%, respectively. The two mixed split receptors, D_{2trunk}/D_{3tail} and D_{3trunk}/D_{2tail}, gave a percentage inhibition of 32.5 ± 4.4 and 37.4 ± 2.1%, respectively.

7-OH-DPAT is known to be a selective ligand for D_3 (see Refs. 25 and 26 and Table I). This agonist was able to inhibit AC-V in COS-7 cells transfected with the different dopamine receptors to the same extent as quinpirole (data not shown). However, in agreement with the binding data of Table I, there was a marked difference in its potency toward the different dopamine receptors (Table III). The D_2 and the split D_{2trunk}/D_{3tail} receptors showed an IC_{50} of 3.91 ± 1.01 and 4.32 ± 1.32 nM, respectively, while the D_3 and D_{3trunk}/D_{2tail} receptors showed a much lower IC_{50} of 0.074 ± 0.011 and 0.121 ± 0.016 nM, respectively. In contrast, AC-VI activity was inhibited by quinpirole only when the enzyme was cotransfected in COS-7 cells with D_2, the split D_{2trunk}/D_{2tail} or D_{3trunk}/D_{2tail} receptors but not D_3 receptors (Fig. 2*B*).

We also tested the efficacy of 7-OH-DPAT to inhibit AC-VI

TABLE I

Comparison of the binding profile of the dopamine D_2 and D_3 receptors with the split trunk/tail dopamine receptors

Saturation and displacement binding experiments were performed in membranes of COS-7 cells transiently transfected with the indicated receptors. The amount of DNA transfected for each wild type receptor or receptor fragment was 2 μg. The data represent the means of two or three experiments, each performed in duplicate. The IC_{50corr} represents the concentration of the unlabeled ligand that displaces 50% of the labeled sites, corrected for the radioligand occupancy.

	D_2	D_{2trunk}/D_{2tail}	D_{2trunk}/D_{3tail}	D_{3trunk}/D_{2tail}	D_{3trunk}/D_{3tail}	D_3
[^{3}H]Nemonapride binding parameters						
K_d (nM)	0.014	0.016	0.095	0.010	0.060	0.064
B_{max} (fmol/mg)	1331	162	159	303	82	1125
Antagonists (displacement, K_i (nM))						
Clozapine	64.2	63.1	134	22.4	188	161
Olanzapine	12.6	15.3	90	4.80	46.6	40.6
Haloperidol	1.01	1.63	2.39	2.59	5.57	5.04
Domperidone	0.52	0.57	3.08	1.02	14.4	11.7
Agonists (displacement, IC_{50corr} (nM))						
Dopamine	3140	3260	582	483	124	111
Quinpirole	4784	4321	454	1362	29	25
Pergolide	32.0	27.5	5.59	5.85	2.41	5.12
Apomorphine	118	135	69.5	24.3	27.2	29.4
7-OH-DPAT	192	180	59.0	4.84	1.11	1.25
BP897	24.2	28.3	27.5	1.02	0.72	0.41

TABLE II

Effect of receptor fragments on the expression (in binding capacity, in fmol/mg of protein) of D_2 and D_3 in transfected COS cells

Dopamine receptor density (B_{max}) was detected by radioligand binding assay in membranes of COS-7 cells transiently cotransfected with 1 or 2 μg of the D_2 or D_3 receptor and the indicated amounts of receptor fragments (in μg). The data represent the means ± S.E. of two experiments, each performed in duplicate.

Fragment (μg)	D_2 receptor, B_{max}		D_3 receptor, B_{max}	
	1 μg	2 μg	1 μg	2 μg
	fmol/mg protein		*fmol/mg protein*	
Vector (2–3)	735 ± 54	1125 ± 68	632 ± 41	1258 ± 112
m_{2trunk} (1)	672 ± 47	1262 ± 101	662 ± 75	1210 ± 92
D_{2trunk} (1)	251 ± 29	710 ± 35	312 ± 37	769 ± 48
D_{2trunk} (2)	145 ± 17			
D_{2trunk} (3)	83 ± 9			
D_{2tail} (1)	301 ± 41	840 ± 42	295 ± 32	758 ± 32
D_{3trunk} (1)	331 ± 33	815 ± 62	187 ± 18	819 ± 39
D_{3tail} (1)	340 ± 69	765 ± 25	211 ± 26	806 ± 47

TABLE III

Relative potency of quinpirole and 7-OH-DPAT on the inhibition of AC-V and AC-VI via D_2, D_3, and various combinations of split dopamine receptors

The amount of DNA transfected for each fragment or wild type receptor was 1 μg, while the amount of DNA for AC-V or AC-V1 was 2 μg. The data represent the mean ± S.E. of three experiments, each performed in quadruplicate. ND, not detected; NI, no inhibition.

Receptor	Quinpirole AC-V, IC_{50}	7-OH-DPAT AC-V, IC_{50}	7-OH-DPAT AC-VI, IC_{50}
		nM	
D_2	2.23 ± 0.34	3.91 ± 1.01	3.58 ± 1.14
D_{2trunk}/D_{2tail}	0.93 ± 0.294	ND	ND
D_{2trunk}/D_{3tail}	1.06 ± 0.27	4.32 ± 1.32	6.72 ± 2.39
D_{3trunk}/D_{2tail}	0.82 ± 0.24	0.121 ± 0.016	0.093 ± 0.014
D_{3trunk}/D_{3tail}	1.59 ± 0.14	ND	ND
D_3	0.68 ± 0.24	0.074 ± 0.011	NI

via activation of the various dopamine receptors. In cells transiently transfected with the D_3 receptor, 7-OH-DPAT was not able to inhibit AC-VI (see Fig. 3 and Table III). Conversely, the activation of all of the other receptors (*i.e.* D_2, D_{2trunk}/D_{3tail}, and D_{3trunk}/D_{2tail}) by 7-OH-DPAT resulted in AC-VI inhibition (Table III and Fig. 3). The calculated IC_{50} values were similar to those observed with AC-V, but the extents of inhibition were lower: 30.8 ± 4.0% for D_2, 25.2 ± 7.0% for D_{2trunk}/D_{3tail}, and 31.8 ± 8.1% for D_{3trunk}/D_{2tail}.

Employing a transient transfection assay, we assessed the ability of D_2 and D_3 receptors to interact functionally with one another. Using AC-VI as the effector, we examined whether the cotransfection of D_3 with D_2, or with D_2 fragments, results in the rescue of the capacity of D_3 to inhibit AC-VI activity. As described in Table III and Fig. 4*A*, D_2 activation (by 7-OH-DPAT) leads to inhibition of FS-induced AC-VI activity, while no inhibition of AC-VI was observed in cells transfected with D_3. Interestingly, AC-VI is inhibited by 7-OH-DPAT in cells cotransfected with D_2 plus D_3, with an IC_{50} much lower than that observed in cells transfected with D_2 alone. The shift in IC_{50} of about 25-fold (Fig. 4*A* and Table IV) is similar to that observed for D_{3trunk}/D_{2tail} *versus* D_2 (see Table III). This change in AC-VI regulation when D_3 is cotransfected with D_2 suggests that a heterodimer is being formed by domain swapping between D_2 and D_3 to allow high affinity binding and communication with AC-VI.

We also examined the ability of D_2 and D_3 receptors to couple to chimeras in which amino acid 856 of AC-V was conjugated with amino acid 774 of AC-VI. This chimera, in similarity with AC-VI, was not inhibited by the D_3 receptor activated with 7-OH-DPAT (Fig. 4*B* and Table IV). Conversely, D_2 and the cotransfected D_2 plus D_3 receptors were able to strongly inhibit this AC-V/VI chimera (Fig. 4*B*). The inhibition curve of 7-OH-DPAT in cells cotransfected with AC-V/VI and D_2 plus D_3 receptors was biphasic, with a high potency IC_{50} of 0.064 ± 0.025 nM and a low potency IC_{50} of 3.55 ± 0.98 nM (Table IV). The inhibition curve of AC activity in cells transfected with AC-V/VI and D_2 was monophasic with an IC_{50} of 1.88 ± 0.07 nM (Table IV). These data further support that a complex is being formed between the dopamine D_2 and D_3 receptors and that domain swapping is probably the most likely explanation for the shift in potency of 7-OH-DPAT.

To determine whether D_2 and D_3 dopamine receptors are

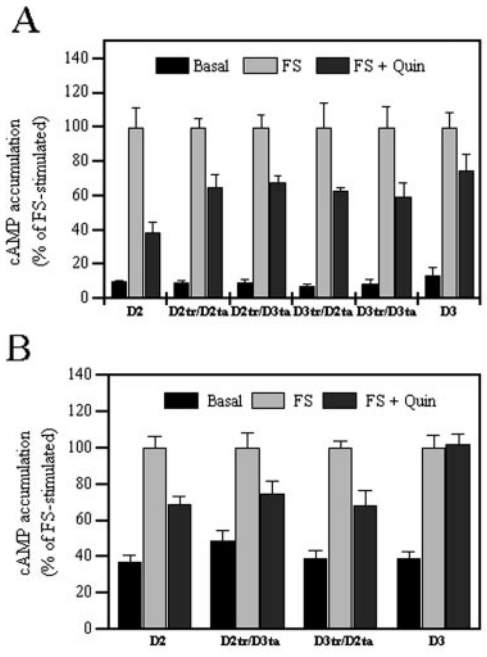

FIG. 2. **Inhibition of FS-stimulated AC activity by quinpirole in COS-7 cells transfected with different wild type and split dopaminergic receptors.** COS-7 cells were transfected with the wild type or the split dopamine receptors, together with AC-V (*A*) or VI (*B*). AC activity was determined by FS stimulation in the presence of quinpirole (*Quin*). The increase in FS-stimulated cAMP level over the basal level ranged between 6- and 9-fold in cells transfected with ACV and between 2- and 3-fold in cells transfected with ACVI. Data represent the mean ± S.E. of three experiments, each performed in quadruplicate. The mixtures of split receptors are indicated by a *slash*. The trunk and tail fragments of each receptor mixture are specified on the *left* and *right side* of the *slash*, respectively.

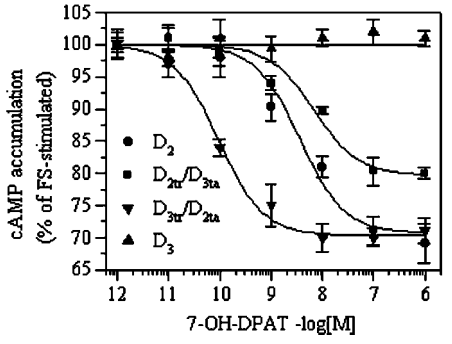

FIG. 3. **Inhibition by 7-OH-DPAT of FS-stimulated AC-VI activity in COS cells transfected with wild type D_2 and D_3 and with D_{2trunk}/D_{3tail} and D_{3trunk}/D_{2tail} mixed split receptors.** COS-7 cells transfected with AC-VI and the wild type or the split receptor combinations were stimulated with FS in the presence of the indicated concentrations of 7-OH-DPAT. The increase in FS-stimulated cAMP level over the basal ranged between 2- and 3-fold. Values are the average of quadruplicate determinations, with *error bars* corresponding to S.E., of a representative of three experiments.

able to functionally interact, we coexpressed FLAG-tagged D_2 receptors with HA-tagged D_3 receptors. HA-tagged D_3 receptors were immunoprecipitated with anti-HA antibodies, and FLAG-tagged D_2 receptors in the immunoprecipitate were visualized with anti-FLAG antibodies. We found that a band of ~55 kDa, corresponding to the long form of the D_2 receptor, was present in the immunoblot only when D_2 and D_3 receptors were expressed together, while no D_2 receptor was present in the immunoblot when the D_3 receptor was absent from the immunoprecipitate (Fig. 5, *lanes 1* and *3*). Under our conditions for transfection and immunoprecipitation, we found that ~17% (±1.53%; $n = 3$) of FLAG-tagged D_{2l} receptors were coimmunoprecipitated with the HA-tagged D3 receptors (data not shown). Similar to the long form, the short form of the D_2 receptor was immunoprecipitated together with the D_3 receptor (Fig. 5, *line 5*). Treatment of transfected cells with 10 μM dopamine appeared to decrease the level of D_2/D_3 heterodimers that were detectable by coimmunoprecipitation (Fig. 5, *lane* 2).

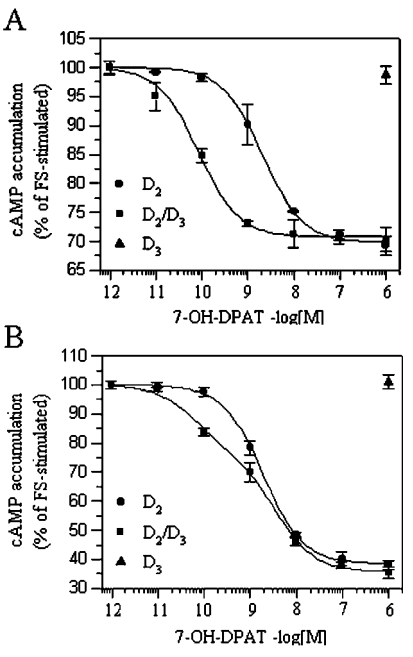

FIG. 4. **Differential inhibition of AC-VI and AC-V/VI chimera by D_2, D_3, and a mixture of D_2 and D_3 receptors.** COS-7 cells were transfected with the indicated receptor(s), together with AC-VI (*A*) or with the AC-V/VI chimera (*B*), and the cells were assayed for AC activity by stimulation with FS in the presence of the indicated concentrations of 7-OH-DPAT. The increase in FS-stimulated cAMP level over the basal ranged between 2- and 3-fold in cells transfected with ACVI and between 6- and 8-fold in cells transfected with AC-V/VI chimera. Values are the average of quadruplicate determinations, with error bars corresponding to S.E., of a representative of five experiments.

TABLE IV
Inhibition by 7-OH-DPAT (IC_{50} values) of AC-VI and AC-V/VI chimera: COS-7 cells were cotransfected with the indicated receptor(s) and AC molecules

The amount of DNA transfected was 1 μg for each receptor and 2 μg for the adenylyl cyclase. The data represent the mean ± S.E. of five experiments, each performed in quadruplicate. NI, no inhibition.

Receptor	7-OH-DPAT AC-VI, IC_{50}	7-OH-DPAT AC-V/VI, IC_{50}
	nM	
D_2	2.05 ± 0.15	1.88 ± 0.07
D_2/D_3	0.083 ± 0.011	0.064 ± 0.025[a] 3.55 ± 0.98[b]
D_3	NI	NI

[a] High potency IC_{50}.
[b] Low potency IC_{50}.

DISCUSSION

In a previous paper (19), we showed that the dopamine D_2 receptor can be split at the level of the third cytoplasmic loop and retain its binding and functional activity. In this paper, we have extended this analysis to the dopamine D_3 receptor and tested the ability of fragments originating from the two receptors to interact with one another. We find that fragments of the D_3 receptor are able to fully reconstitute the binding activity and the selectivity observed for the wild type D3 receptor. These data clearly indicate that the integrity of the third cytoplasmic loop is not important, at least when the receptor is on

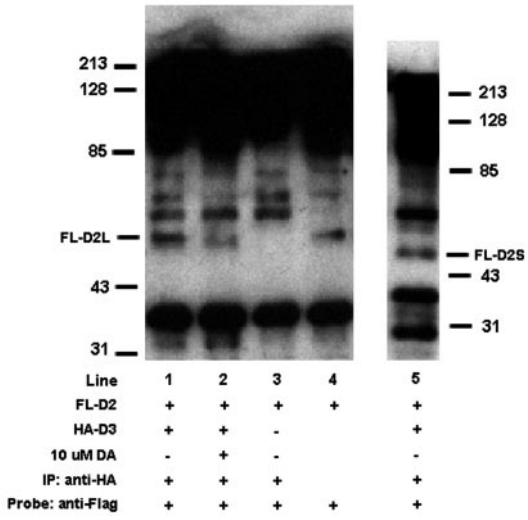

FIG. 5. **Coimmunoprecipitation of D_2 and D_3 receptors.** FLAG-tagged D_2 and HA-tagged D_3 receptors were transfected either singly or together into HEK 293 cells. Complexes were immunoprecipitated (*IP*) with anti-HA antibodies and detected with anti-FLAG antibodies. Positions of D_{2l} and D_{2s} receptors on the blot are indicated. The *left panel* (*lines 1–4*) shows results obtained with FLAG-tagged D_{2l}, while the *right panel* (*line 5*) shows results obtained with D_{2s}. The *fourth lane* just shows the position of authentic FLAG-tagged D_{2l}.

the membrane, to maintain correct folding and function of the dopamine receptors. Rather, as shall be discussed below, it is likely that the integrity of this loop is needed for the correct trafficking of the receptor to the membrane. Additional G protein-coupled receptors, including the α_2-adrenergic (27), m_2- and m_3-muscarinic (28), rhodopsin (29), vasopressin V_2 (30), gonadotropin-releasing hormone (31), and neurokinin NK_1 (32) receptors, have been shown to retain their characteristics when they were split at the third cytoplasmic loop, suggesting that this is a general feature of this receptor family.

In this paper, we show that fragments originating from D_2 and D_3 receptors can interact with each other to form ligand-binding D_{2trunk}/D_{3tail} and D_{3trunk}/D_{2tail} receptors. Binding to mixed receptor fragments has already been observed for the m_2- and m_3-muscarinic receptors, but in this case, only the m_{2trunk} fragment was able to interact with the m_{3tail} fragment (to yield ligand binding) and not vice versa (28). The pharmacological profile of the cross-cotransfected dopamine receptor fragments was different from that of the wild type D_2 or D_3 receptors. In most cases, the D_{3trunk}/D_{2tail} receptor was the one with the highest affinity for most agonists and antagonists; moreover, clozapine and olanzapine showed the highest affinity for this receptor, among all of the receptors tested. Interestingly, selective D_3 receptor ligands, such as BP 897, have high affinity for the D_{3trunk}/D_{2tail} receptor, suggesting that the trunk and not the tail of the D_3 receptor contributes the most for the binding affinity. This is in agreement with the fact that the trunk has five TM domains, two extracellular loops, and an extracellular N terminus, while the tail has a lesser area into which the ligand can bind. Moreover, D_2 and D_3 amino acid sequences diverge more in TMI and TMIV than in TMVI and TMVII.

As we have recently shown for muscarinic receptors (16), receptor fragments originating from m_2 or m_3 receptors were able to interact with the heterologous wild type m_3 or m_2 receptor and rescue the high affinity binding for selected compounds. In this work, we used the same approach to study the pharmacological interaction of the dopamine D_2 and D_3 receptor fragments with the wild type D_3 and D_2 receptors, respectively. No changes in the affinities of the wild type receptors were observed in the presence of receptor fragments. Nevertheless, the cotransfected fragments (homologous and heterologous) markedly reduced the B_{max} of the wild type D_2 and D_3 receptors. This phenomenon has been previously described in experiments in which wild type receptors were cotransfected with homologous truncated receptor fragments (24, 33, 34). It is possible that the reduction in binding observed in our experiments represents fragment-induced mistargeting of the wild type receptor. Karpa *et al.* (24) have shown that D_{3nf} (a truncated splice variant of the D_3 receptor) causes mislocalization of the D_3 receptor to an intracellular compartment.

The split dopamine receptors were also tested in functional experiments to determine if they can couple to and inhibit AC activity. For this purpose, we cotransfected the receptors with AC-V or AC-VI. These two AC types belong to the same AC subfamily and share a high degree of amino acid homology but were found to be differentially regulated by the D_2 and D_3 dopamine receptors. In this regard, Robinson and Caron (20) have shown that activation of the D_2 receptor inhibits both AC-V and AC-VI, while activation of D_3 inhibits only AC-V and does not affect the activity of AC-VI. We initially showed that the two wild type and all of the mixtures of split dopamine receptors, when stimulated with quinpirole or 7-OH-DPAT, inhibit AC-V (although to different extents). Of the two compounds tested, the latter showed a greater potency and selectivity between the different receptors, with a 32-fold higher potency for D_{3trunk}/D_{2tail} compared with the D_2 receptor. The same ratio of potency was also observed between D_{3trunk}/D_{2tail} and D_2 in experiments in which AC-VI was used. Similar to results described previously by Robinson and Caron (20), 7-OH-DPAT was not able to induce D_3 inhibition of FS-stimulated AC-VI.

Our mixed split receptors are very similar in terms of structure to the $D_{2/3}$ and $D_{3/2}$ chimeric receptors reported by Lachowicz and Sibley (35). These two chimeras were constructed by swapping the C-terminal receptor fragments (containing the last two transmembrane regions and the half-terminal part of the third cytoplasmic loop) between the D_2 and D_3 receptors. These chimeras, stably transfected in Chinese hamster ovary cells, were able to inhibit AC to the same extent as the wild type D_2 receptor. However, activation of the wild type D_3 receptor did not lead to AC inhibition. It is interesting to note that mRNA measurements showed that AC-VI is a major AC isozyme in Chinese hamster ovary cells (36), and this could explain why D_3 activation cannot regulate AC in these cells. Our pharmacological and functional results are in accord with these findings, although a clear difference exists in the relative ratio of the B_{max} between the chimeras and the wild type receptors. Previous experiments showed that the two $D_{2/3}$ and $D_{3/2}$ chimeras were expressed in the membrane roughly at the same level as the wild type D_2 and D_3 receptor (35). Our experiments, in contrast, indicate that the various mixtures of split receptors were expressed at a lower level than that of the wild type receptors. This implies that the integrity of the third cytoplasmic loop, while not important for keeping the correct folding of the receptor when the protein is in the membrane, probably plays a role during the folding itself. Alternatively, it is possible that the two fragments achieve correct folding by themselves but are transported to the plasma membrane less efficiently than the intact receptor. It is interesting to note that a functional rescue of the D_3 receptor activity was also obtained by Filteau *et al.* (37) with reciprocal chimeras between the C-terminal portion of the third intracellular loops of the dopamine D_2 and D_3 receptors. The reciprocal construct generates a chimeric D_3 receptor that was fully coupled to the second messenger pathway with a higher potency compared with D_2.

The fact that the D_3 receptor does not inhibit AC-VI and that 7-OH-DPAT has a high potency for D_3 and for the split D_{3trunk}/D_{2tail} receptor, compared with D_2, opened the possibility of examining a possible functional interaction between the dopamine D_2 and D_3 receptors. As shown under "Results," when D_2 and D_3 receptors were coexpressed together with AC-VI, lower concentrations (25-fold) of 7-OH-DPAT were needed to induce inhibition of cAMP accumulation, as compared with cells transfected with D_2 and AC-VI. The extent of inhibition in both cases was about 30%. In another set of experiments, we used a chimera of AC-V and AC-VI; the activity of this AC molecule was not inhibited by D_3 activation but was inhibited by D_2 activation by about 60%, with an IC_{50} similar to that obtained for AC-VI. However, in cells cotransfected with D_2 and D_3, the dose-response curve of 7-OH-DPAT was substantially different from that observed in cells transfected with D_2 alone, since 7-OH-DPAT began to inhibit the FS-induced accumulation of cAMP in the picomolar range (~100 pM), in agreement with the picomolar range of IC_{50} observed for D_{3trunk}/D_{2tail} for the inhibition of AC-V and AC-VI.

There are two potential explanations that would account for our results. It is possible that domain swapping occurs between the "trunk" and the "tail" portion of the coexpressed wild type D_2 and D_3 receptors and the formation of a D_2/D_3 swapped heterodimer. This could be looked upon as a tetrameric arrangement consisting of D_2/D_3 and D_3/D_2 "mixed" receptors and should have pharmacological and functional characteristics similar to those obtained following mixing of the mixed split receptors (D_{2trunk}/D_{3tail} together with D_{3trunk}/D_{2tail}). If this is the case, it is not difficult to imagine why the potency of 7-OH-DPAT is higher when D_3 is cotransfected with D_2. Alternatively, it is possible that the D_2 receptor could in some way sensitize the AC-VI and AC-V/VI chimera to be more strongly inhibited by D_3 activation. For instance, sustained inhibition of AC by the D_2 receptor's constitutive activity (38) could upregulate the enzyme. The coimmunoprecipitation experiments we performed strongly suggest that D_2 and D_3 receptors form functional heterodimers. In cells transfected with D_3 receptors alone, no detectable decrease in AC-VI activity was observed in response to dopamine agonists. In contrast, cells expressing D_2 and D_3 receptors exhibited 7-OH-DPAT-mediated reduction in AC-V activity far above that produced by wild type D_2 receptors. The formation of D_2/D_3 heterodimers may therefore give rise to novel receptors with unique pharmacological and physiological properties that are different from the activities produced by D_2 or D_3 receptors alone.

The phenomenon of heterodimerization could explain some unexpected results involving dopaminergic activities in animal models. For example, Millan *et al.* (39) found that hypothermia is induced in the rat by 7-OH-DPAT at doses that should activate only the D_3 receptors. However, this hypothermic effect of 7-OH-DPAT could be inhibited not only by the selective D_3 antagonists S33084 and GR218,231, but also by the highly selective D_2 receptor antagonist L741,626. They proposed that this effect could be explained by the formation of dopamine D_2/D_3 receptor heterodimers.

In conclusion, our data indicate that wild type dopamine D_2 and D_3 receptors can interact with each other to form a functional heterodimer that exhibits unique functional properties.

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