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Reconstitution of functional dopamine D_{2s} receptor by co-expression of amino- and carboxyl-terminal receptor fragments

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Abstract

An N-terminal dopamine D_{2s} receptor clone was constructed and coexpressed in COS-7 cells together with a separate gene fragment coding for the C-terminal sequence of the dopamine D_{2s} receptor. The truncated receptor (referred to as D_{2trunc}) contained transmembrane domains I–V and the N-terminal portion of the third cytoplasmic loop, whereas the C-terminal receptor fragment (referred to as D_{2tail}) contained transmembrane domains VI and VII and the adjacent intra- and extracellular sequences of the dopamine D_{2s} receptor. Expression in COS-7 cells of either of these two polypeptides alone did not result in any detectable [^{3}H]methylspiperone binding activity. However, specific [^{3}H]methylspiperone binding could be observed after coexpression of the D_{2trunc} and D_{2tail} gene constructs; the number of receptors present on the plasma membrane was about 10% with respect to that of the wild type. The binding properties of the coexpressed fragments were similar to those of the wild-type dopamine D_{2s} receptor for agonists and antagonists. Functional stimulation of the cotransfected D_{2trunc} and D_{2tail} fragments with quinpirole resulted in the inhibition of adenylate cyclase activity. Maximal inhibition corresponds to a 28% decrease in forskolin-stimulated adenylate cyclase. The apparent IC_{50} of quinpirole was 5.1 ± 0.3 μM. These findings confirm and extend analogous data for other G protein-coupled receptors and indicate that this phenomenon is of general importance for the entire family of these proteins.

Keywords: Dopamine receptor; Receptor fragment; COS-7 cell; Adenylate cyclase

1. Introduction

Dopamine receptors are members of the family of plasma membrane receptors that transduce their intracellular signal coupling to guanine nucleotide binding regulatory proteins (G-proteins). Molecular cloning has identified five dopamine receptor subtypes that can be classified into two categories, the D_1-like receptors, i.e., D_1 and D_5, and the D_2-like receptors, i.e., D_2, D_3 and D_4 (for review see Sokoloff and Schwartz, 1995). 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_{2l} and D_{2s} receptors (Dal Toso et al., 1989; Giros et al., 1989; Grandy et al., 1989; Monsma et al., 1989). They are composed of seven hydrophobic transmembrane α-helices connected by alternating cytoplasmic and extracellular loops, a glycosylated extracellular N-terminal domain and an intracellular C-terminal region. The seven transmembrane helices are predicted to enclose a highly conserved cavity in which the binding of dopaminergic ligands is though to occur.

Little is known about the molecular mechanism controlling the folding and assembly of G-protein coupled receptor. Pioneer studies with bacteriorhodopsin, a light-driven proton pump, have for the first time demonstrated that this protein can be functionally reconstituted from individual

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receptor fragments resulting from proteolytic cleavage of various loop regions (Popot and Engelman, 1990). Similar findings have since been described for G protein-coupled receptors. In 1988, Kobilka et al. (1988) split the β_2-adrenoceptor into two fragments, one containing transmembrane domains I to V and the other containing transmembrane domains VI and VII. The transfection of these two fragments together resulted in recovery of the binding activity and the function of the β_2-adrenoceptor. Since then, this phenomenon has been demonstrated for other G protein-coupled receptors (Maggio et al., 1993; Ridge et al., 1995; Schöneberg et al., 1996; Nielsen et al., 1998). Taken together, these findings support the notion that the folding of G-protein coupled receptors (as it has been proposed for another polytopic transmembrane protein, bacteriorhodopsin; Popot and Engelman, 1990) occurs in two consecutive steps. In step I, individual transmembrane helices are established across the lipid bilayer, which, in step II, are then assembled by specific helix–helix interactions to form a functional receptor protein that encloses the highly conserved hydrophobic core. It has been suggested that G protein-coupled receptors may ''open up'' their hydrophobic core and by domain swapping interconvert from monomers to dimers. This mechanism has been shown to be the basis of the molecular interaction between muscarinic m2 and m3 receptors (Maggio et al., 1999).

Dopamine D_2 receptors have been shown to form dimers (Ng et al., 1996; Zawarynski et al., 1998). Furthermore, single transmembrane domains have been demonstrated to disrupt dimerization. For example, transmembrane domains VI and VII dissociated dopamine D_2 receptor dimers into monomers (Ng et al., 1996), indicating that these two transmembrane helices are able to find the specific interacting amino acids in the dopamine D_2 receptor. These findings suggest that the dopamine D_2 receptor is composed of multiple interacting domains. In order to confirm this hypothesis, we split the dopamine D_2 receptor into an amino- and a carboxyl-terminal domain and we studied the binding and functional characteristics of the two fragments cotransfected in African green monkey kidney (COS)-7 cells in comparison with those of the dopamine D_{2s} wild-type receptor.

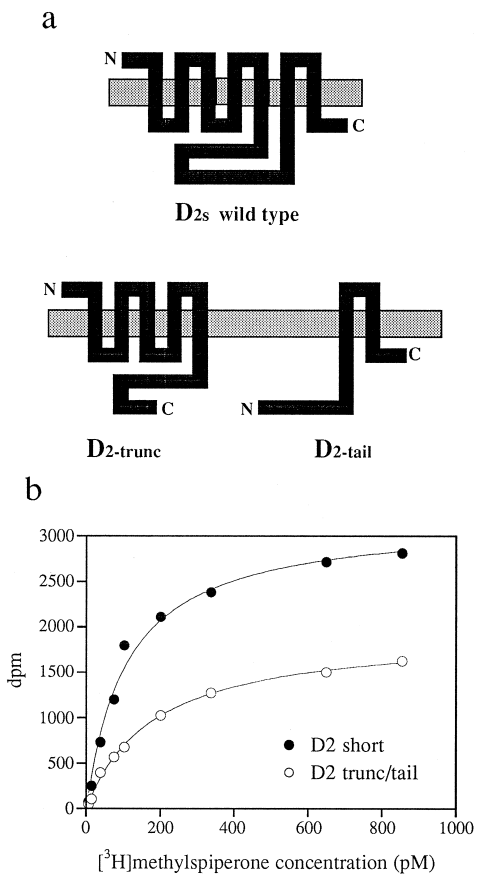

Fig. 1. (a) Schematic representation of the wild-type rat dopamine D_{2s} receptor and the D_{2trunc} and D_{2tail} fragments. The truncated fragment, D_{2trunc}, contains a STOP codon after Lys 241, while the D_{2tail} fragment contains a START codon and an Asn before Arg 245. (b) Representative [^{3}H]methylspiperone saturation curves for the wild-type dopamine D_{2s} receptor and the cotransfected D_{2trunc} /D_{2tail} fragments. The real number of binding sites for the two receptors was 183 ± 15 and 1325 ± 49 fmol/mg of protein for D_{2trunc} /D_{2tail} and D_{2s}, respectively.

2. Materials and methods

2.1. Constructs and transfection

Full-length cDNA (≈ 2.1 kb) coding for the rat dopamine D_{2s} receptor was subcloned in the pRc/CMV vector (Invitrogen) between the *Hin*dIII and the *Apa*I sites. The resulting construct, pRc/CMV-D_{2s}, was used to prepare the D_{2trunc} and D_{2tail} receptor fragments (Fig. 1a). The D_{2trunc} fragment (containing the extracellular N-terminal part of the dopamine D_{2s} receptor, transmembrane domains I–V and the proximal portion of the third cytoplasmic loop) was constructed by removing a *Bbs*I–*Apa*I fragment from pRc/CMV-D2s and inserting two overlapping oligonucleotides bearing an in frame stop codon after lysine 241 (5′-CACTCAAGTGAGTCTGCGGGCC; 5′-CGCAGACTCACTTG).

The D_{2tail} fragment (containing the distal portion of the third cytoplasmic loop, transmembrane domains VI and VII and the C-terminal part of the dopamime D_{2s} receptor) was constructed by removing a *Hin*dIII–*Sac*I fragment from pRc/CMV-D_{2s} and by inserting two overlapping oligonucleotides bearing an in frame start codon and an aspartate before arginine 246 (5′-AGCTTGCCCAATG-GATCGAGCT; 5′-CGATCCATTGGGCA).

COS-7 cells were incubated at 37°C in a humidified atmosphere (5% CO_2) and grown in Dulbecco's modified

Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum, 2% (vol/vol) L-glutamine 200 mM, 1% (vol/vol) penicillin (10,000 units/ml) and streptomycin (10 mg/ml) solution, and 1% (vol/vol) minimal essential medium non-essential amino acid solution. Cells were seeded at a density of $\sim 2 \times 10^6$ per 100-mm dish and 24 h later transiently transfected with the plasmid DNA (4 μg per dish) by the DEAE-dextran method (Cullen, 1987). In order to increase the expression of the receptors, cells were incubated with 5 mM sodium butyrate (sterilized by filtration) for 24 h before the assay.

2.2. Membrane preparation and binding assay

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 into a centrifuge tube and centrifuged at 17,000 rpm for 20 min. The crude membrane fraction was resuspended with a Polytron homogenizer in assay buffer (50 mM Tris–HCl, pH 7.4, 155 mM NaCl, 0.001% bovine serum albumin). Binding of [^{3}H]methylspiperone (83 Ci/mmol) was carried out in an assay volume of 1 ml. Dopamine 2 mM was used to define nonspecific binding. The saturation curves for D_{2s} and D_{2trunc}/D_{2tail} were made using different concentrations of protein: the ratio of proteins between the wild-type and the two cotransfected fragments was about 1/4. For competition binding studies in which agonist displacement of binding was assessed, the membranes were resuspended in assay buffer also containing 4 mM $MgCl_2$, 1 mM EDTA and 0.025% ascorbic acid. Incubation was carried out at 30°C for 1 h. The bound ligand was separated on glass-fiber filters (Whatmann, GF/B) with a Brandel Cell Harvester and the filters were counted with a β-counter.

2.3. Adenylate cyclase inhibition

Adenylate cyclase activity was routinely assayed in a crude membrane preparation obtained by homogenizing the cells in 5 ml of an ice-cold buffer containing 5 mM Tris, 1 mM EGTA, 1 mM dithiothreitol, and 10% Sucrose (pH 7.4), using a tight Teflon-glass tissue grinder. The homogenate was centrifuged at $27,000 \times g$ for 20 min at 4°C. The pellet was resuspended in homogenization buffer and 30 μl of tissue for each sample was used immediately for the adenylate cyclase assay. The enzyme activity was assayed in a 150-μl final reaction mixture containing 75 mM Tris (pH 7.4), 2 mM $MgCl_2$, 0.3 mM dithiothreitol, 0.2 mM [α-^{32}P]ATP (145 cpm/pmol), 1 mM cyclic AMP, 0.3 mM EGTA, 0.5 mM 3-isobutyl-1-methylxanthine, 5 mM phosphocreatine, 50 μg of bovine serum albumin and 0.03 mg creatine phosphokinase. The reaction was started by adding the tissue preparation and was carried out at 30°C for 10 min. The assay was stopped by adding 200 μl of a solution containing 2% sodium lauryl sulfate, 45 mM ATP and 1.3 mM cAMP. [^{32}P]Cyclic AMP was isolated according to the technique of Salomon et al. (1974). Response curves obtained with transfected cells were as follow: basal (a duplicate sample of membranes); forskolin stimulated (a duplicate sample of forskolin (50 μM)-treated membranes); forskolin stimulated + quinpirole (seven replicates of forskolin (50 μM)-treated membranes plus different concentrations of quinpirole).

Untransfected COS-7 cells did not express dopamine receptors and their cAMP content was not modified by the treatment with quinpirole.

3. Results

The dopamine D_{2s} receptor was split at the level of the third cytoplasmic loop. The two fragments, D_{2trunc} and D_{2tail} (Fig. 1a), were transfected either separately or to-

Table 1
Binding parameters of different dopamine receptor ligands for the wild type dopamine D_{2s} receptor and the split dopamine D_{2trunc}/D_{2tail} receptor. [^{3}H]methylspiperone affinity constants (K_D) were determined in direct binding assays. Inhibition constants K_i for the other two antagonists and IC_{50corr} for the four agonists were obtained in competition binding experiments. Data are presented as means ± S.E. of three separate experiments each performed in duplicate. $IC_{50corr} = IC_{50}$ corrected for the occupancy of [^{3}H]methylspiperone.

Antagonist	D_{2trunc}/D_{2tail}		D_{2s}	
	K_i (nM)	n_H	K_i (nM)	n_H
[^{3}H]methylspiperone (K_D)	0.171 ± 0.014	1.07 ± 0.04	0.128 ± 0.007	0.99 ± 0.03
Haloperidol	1.43 ± 0.12	0.91 ± 0.05	1.76 ± 0.17	0.95 ± 0.07
Clozapine	107 ± 5	0.95 ± 0.03	112 ± 9	1.03 ± 0.09
Agonist	IC_{50corr} (nM)	n_H	IC_{50corr} (nM)	n_H
Dopamine	2990 ± 50	0.59 ± 0.06	1740 ± 63	0.66 ± 0.03
Apomorphine	146 ± 11	0.74 ± 0.03	93 ± 7	0.77 ± 0.07
Pergolide	35.2 ± 4.11	0.71 ± 0.02	31.6 ± 3.07	0.62 ± 0.04
Quinpirole	1738 ± 98	0.73 ± 0.05	1152 ± 126	0.75 ± 0.06

gether in COS-7 cells and tested in a binding assay with [^{3}H]methylspiperone.

Neither of the two dopamine receptor fragments showed detectable [^{3}H]methylspiperone binding when expressed alone in COS-7 cells. In contrast, a considerable number of specific [^{3}H]methylspiperone binding sites were observed after co-expression of D_{2trunc} with D_{2tail} (B_{max}: 183 ± 15 and 1325 ± 49 fmol/mg of protein, respectively for D_{2trunc}/D_{2tail} and D_{2s} (Fig. 1b). The calculated [^{3}H]methylspiperone K_D value was similar to that found for the wild-type dopamine D_{2s} receptor (Table 1). We also tested two other antagonists: haloperidol and clozapine a typical and an atypical antipsychotic, respectively. The binding affinity of the two antagonists to the D_{2trunc}/D_{2tail} co-transfected fragments was similar to that of the wild-type dopamine D_{2s} receptor (Table 1). The Hill coefficients for all the antagonists were not significantly different from unity, indicating a single population of binding sites.

We also studied the binding affinity of the endogenous ligand dopamine and other three agonists. As shown in Table 1, dopamine, apomorphine, pergolide and the selective D_2-like agonist quinpirole bound to the split dopamine D_{2s} receptor with the same affinity as for the wild-type receptor. All the agonists showed Hill coefficients significantly different from unity, indicating that the agonists recognized a non-homogeneous population of binding sites.

The ability of the dopamine receptor fragments to mediate agonist inhibition of adenylate cyclase activity was determined. As a control, we used the human muscarinic M_2 receptor, which mediates inhibition of adenylate cyclase when transfected in COS-7 cells (Liu et al., 1996). Quinpirole stimulation of M_2-transfected COS-7 cells did not result in any detectable inhibition of the forskolin-stimulated adenylate cyclase activity; conversely, quinpirole stimulation of COS-7 cells co-transfected with the D_{2trunc}/D_{2tail} fragments or the dopamine D_{2s} receptor resulted in 28 and 30% inhibition of the forskolin-stimulated adenylate cyclase activity, respectively (Fig. 2b). The IC_{50} values were calculated from inhibition curves obtained in the presence of seven different concentrations of quinpirole. They were 5.1 ± 0.3 μM and 5.4 ± 0.3 μM for D_{2trunc}/D_{2tail} and D_{2s} respectively (Fig. 2a).

Homologous recombination between the vectors expressing the two fragments is always a possibility in COS-7 cells in which the plasmids are amplified at high copy numbers. Nevertheless, there are two reasons that exclude homologous recombination as the cause of our results. The only part that is homologous between the vectors is the pRcCMV vector itself, while the inserted DNA fragments (D_{2trunc} and D_{2tail}) are completely different. While the first one terminates at amino acid 241, the second starts at amino acid 246. Furthermore, in preparing the filling oligonucleotides we had taken care not to insert homologous sequences. Since for an efficient recombination the fragment has to be flanked on both sites by DNA sequences which are identical to the part that will be

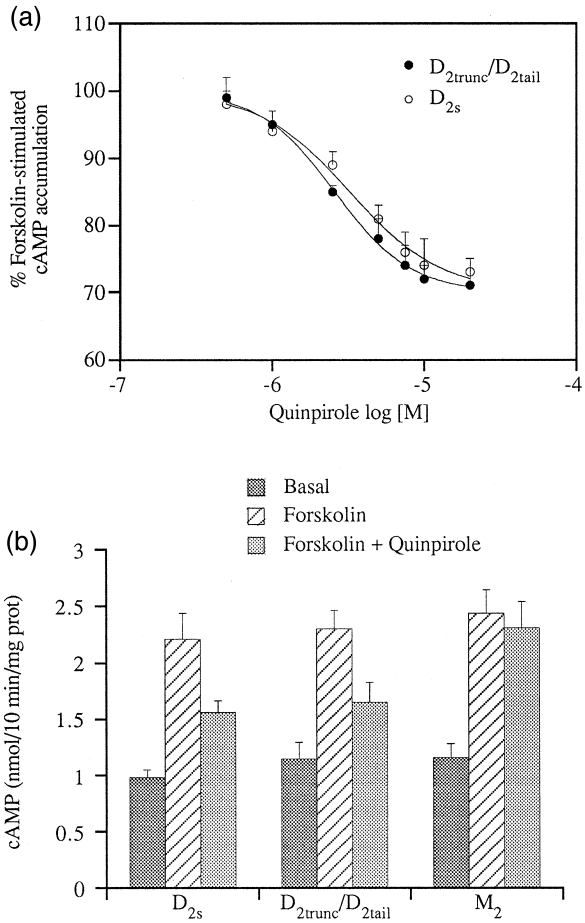

Fig. 2. (a) Concentration-dependent inhibition of adenylate cyclase by quinpirole in COS-7 cells transfected with the dopamine D_{2trunc}/D_{2tail} receptor fragments and the wild-type dopamine D_{2s} receptor. Data were transformed to percentage of forskolin (50 μM)-stimulated adenylyl cyclase. (b) Bar graph of the inhibition of forskolin (50 μM)-stimulated adenylate cyclase activity by quinpirole (10 μM). Basal represents the amount of cAMP in COS-7 cells transfected with the receptors, but not treated with forskolin. Data are the means ± S.E. of at least two experiments performed in duplicate.

replaced, it is unlikely that homologous recombination takes place under our conditions. The other and more convincing reason is that a mutant D_{2tail} fragment, in which a base deletion after the start codon (ATG-GAT-*C*GA-GCT ⇒ ATG-GAT-GAG-CTC) leads to a frame shift and the formation of a nonsense protein, did not show any binding when cotransfected with D_{2trunc}.

4. Discussion

In this work, we have shown that the expression of the dopamine D_{2s} receptor as a two separate polypeptides (one containing the transmembrane domains I–V, and the other transmembrane domains VI and VII) results in the recon-

stitution of a functional receptor complex. The lack of [^{3}H]methylspiperone binding activity observed after transfection of the two fragments alone indicates that neither fragment retains the characteristics of the dopamine D_{2s} receptor, but both are essential to form a functional protein. The split D_{2trunc}/D_{2tail} receptor displayed ligand binding and functional properties similar to those of the wild-type dopamine D_{2s} receptor. The data are in accord with those reported in the literature for the wild-type dopamine D_2 receptor (Tang et al., 1994; Perachon et al., 1999). These data suggest that the two parts of the dopamine D_{2s} receptor that are covalently linked by the third cytoplasmic loop in the native receptor have the capability to fold independently of each other, being able to adopt a conformation in the lipid bilayer that allows them to recognize each other and form a stable receptor complex.

Although the expression of the receptors in the cells cotransfected with D_{2trunc}/D_{2tail} was about 10% of that of cells transfected with the native dopamine D_{2s} receptor, the inhibition of cyclase activity was comparable. It is likely that, in our experimental setting, the amount of reconstituted receptor expressed (183 fmol/mg protein) is high enough to saturate the endogenous G-proteins and reach the maximal inhibition of adenylyl cyclase. This was also found with cotransfected fragments of muscarinic receptors (Maggio et al., 1993; Schöneberg et al., 1995).

Domain swapping has been proposed as a possible mechanism by which G protein-linked receptors interact (Gouldson et al., 1997, 1998). Inherent to the model is that receptors are composed of independent domains. Recent findings have shown that dopamine D_2 receptors can form dimers (Ng et al., 1996; Zawarynski et al., 1998). Furthermore, Ng et al. (1996) demonstrated that transmembrane peptides VI and VII can disrupt dimerization. The exact manner in which the transmembrane peptides interact with the receptor protein is not known, but there are two main possibilities. In the first one, the interaction occurs with an external face of a transmembrane α-helix of the packed receptor, while in the second one there is direct insertion of the peptide into the hydrophobic interior core of the receptor. In the second case, it is reasonable to think that the same specific helix–helix interactions that are used in the native receptor would be utilized by the transmembrane peptide and that the receptor can open up its hydrophobic core to allow the peptide to go in between. Since we have shown that the D_{2trunc} and the D_{2tail} fragments can behave as two independent folding domains, it is possible that they behave as such in the wild-type receptor, going back and forth between an assembled and a disassembled form still connected by the third cytoplasmic loop. If this is the case, receptor dimerization could occur by domain swapping, and the peptide corresponding to transmembrane domains VI and VII could disrupt dimerization by competing with the swapped domain for binding with the N-terminal part of the cognate receptor.

In conclusion, with dopamine D_{2s} receptors, we confirm the general finding that G protein-coupled receptors consist of at least two independent folding domains that are inserted into the lipid bilayer as separate units which assemble to form a functional transmembrane protein.

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