A Transmembrane Domain-derived Peptide Inhibits D1 Dopamine Receptor Function without Affecting Receptor Oligomerization*

Susan R. George**, Samuel P. Lee‡, George Varghese‡, Peter R. Zeman‡, Philip Seeman‡**, Gordon Y. K. Ng**, and Brian F. O’Dowd‡‡

From the Departments of Pharmacology, Medicine, and Psychiatry, University of Toronto, Toronto, Ontario M5S 1A8, Canada and the Addiction Research Foundation, Toronto, Ontario M5S 2S1, Canada

In this study, we show that a peptide based on the sequence of transmembrane domain 6 of the D1 dopamine receptor (D1DR) specifically inhibited D1DR binding and function, without affecting receptor oligomerization. It has been shown that an analogous peptide from the β2-adrenergic receptor disrupted dimerization and adenylyl cyclase activation in the β2-adrenergic receptor (Hebert, T. E., Moffett, S., Morello, J. P., Loisel, T. P., Bichet, D. G., Barret, C., and Bouvier, M. (1996) J. Biol. Chem. 271, 16384–16392). Treatment of D1DR with the D1DR transmembrane 6 peptide resulted in a dose-dependent, irreversible inhibition of D1DR antagonist binding, an effect not seen in D1DR with peptides based on transmembrane domains of other G protein-coupled receptors. Incubation with the D1DR transmembrane 6 peptide also resulted in a dose-dependent attenuation of both dopamine-induced [3H]Dopamine 5'-3-O-(thio)triphosphate (GTPγS) binding and receptor-mediated dopamine stimulation of adenylyl cyclase activity. Notably, GTPγS binding and cAMP production were reduced to levels below baseline, indicating blockade of ligand-independent, intrinsic receptor activity. Immunoblot analyses of the D1DR revealed the receptor existed as monomers, dimers, and higher order oligomers and that these oligomeric states were unaffected after incubation with the D1DR transmembrane 6 peptide. These findings represent the first demonstration that a peptide based on the transmembrane 6 of the D1DR may represent a novel category of noncompetitive D1DR antagonists.

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Dopamine receptors belong to the family of G protein-coupled receptors (GPCRs)† and mediate the many biological actions of the neurotransmitter dopamine in brain and periphery. The distinct biological functions of dopamine are mediated by the five dopamine receptor subtypes, that are divided into the D1-like (D1 and D5) and D2-like (D2, D3, and D4) subfamilies. We have previously provided evidence for the existence of dimers and oligomers of the D1 and D2 dopamine receptors (1–4). The occurrence of receptor oligomers may be an universal phenomenon in the GPCR family, as suggested by observations for serotonin 5HT-1B (5), β2-adrenergic and V2 vasopressin (6), metabotropic glutamate (7), δ opioid (8), H2 histamine (9), and D3 dopamine (10) receptors. A survey of receptor families (other than the GPCRs) reveals that these receptors are often organized into functional units that involve dimerized or oligomerized proteins. Dimerization of receptor monomers is a critical and necessary mechanism for signal transduction in tyrosine kinase receptors (11) such as the epidermal growth factor (12) and insulin receptors (13), the transferrin receptor (14), and steroid receptors (15). However, the functional significance of GPCR oligomers remains unknown. Intriguing insights from studies using receptor chimeras have suggested that both intramolecular and intermolecular interactions occur in GPCRs (16), but the role of such interactions in signal transduction is not known.

Peptide sequences derived from specific regions of large proteins have been successfully studied to elucidate the function of these regions. The use of such peptide "probes" has enabled the mapping of the functional domains of certain proteins and the identification of domains that promote interactions with other proteins. This strategy has also been used in the study of GPCRs. For instance, studies have used peptides corresponding to the third cytoplasmic loops of GPCRs to elucidate the importance of this region of the receptor for interaction with G proteins. (17–19).

Recently, a peptide derived from transmembrane (TM) domain 6 of the β2-adrenergic receptor (β2AR) was shown to disrupt dimers of that receptor in parallel with inhibition of agonist-induced adenylyl cyclase activity (6). It was hypothesized that these observations were the result of an interference with an amino acid motif in TM6 of the β2AR postulated to be important for dimerization. However, the D1 dopamine receptor (D1DR) does not contain a dimerization motif similar to the β2AR, and whether dimer disruption by TM6-derived peptide occurs in other GPCRs is not known. Therefore, in the present study, we examined the effect of a peptide derived from TM6 of the D1DR on the oligomeric state and function of the D1DR, despite the lack of a dimerization motif as proposed for the β2AR.

GPCRs are considered to have a central core consisting of the seven TMs aligned as α-helices that is important in maintaining the three-dimensional structure of the receptor and the pocket created for agonist binding (20). Therefore, we postulated that disruption of the TM core may alter agonist interaction and signal transduction. We also hypothesized that the interaction between helical TMs within a GPCR could be manipulated by the addition of exogenous peptides containing a particular TM sequence, which would compete for the sites of interaction of the native TM region of
the receptor and disrupt receptor architecture. Furthermore, a model based on amino acid sequence analysis and computer simulations predicts that GPCR dimers have multiple sites of interaction (21). Thus, it is possible that circumscribed disruption of receptor structure by a TM peptide will not coincide with a loss of oligomeric structure. Hence, these observations provided the scientific basis for the speculation that TM s of GPCRs may be possible targets for inhibition of receptor activity but have no effect on oligomerization.

EXPERIMENTAL PROCEDURES

Construction of Recombinant c-myc-tagged D1DR Baculovirus—Details of the construction of the recombinant baculovirus encoding the c-myc epitope-tagged human D1DR have been reported previously (1, 3).

Cell Culture and Membrane Preparation—All culture media, antibiotics, and supplements were purchased from Life Technologies, Inc. Sf9 cells were maintained at 27 °C in Grace’s insect media supplemented with 10% (v/v) fetal bovine serum, 0.5% antibiotic-antimycotic, and 1% (v/v) Phorun F-68, a surfactant. The cells were grown either as monolayer cultures in T flasks or as suspension cultures in spinner flasks. Suspension cultures were infected with baculovirus when cell density was 1–5 x 10⁶ cells/ml with a multiplicity of infection of approximately 5. Membranes were prepared from the cells 48 h after infection unless otherwise noted.

Membrane Preparation—Cells were washed with phosphate-buffered saline, resuspended in hypotonic lysis buffer (5 mM Tris-HCl, 2 mM EDTA, 5 μg/ml leupeptin, 10 μg/ml benzamide, 5 μg/ml soybean trypsin inhibitor, pH 7.4), and homogenized by Polytron (Brinkmann Instruments). The homogenate was centrifuged to pellet unbroken cells and nuclei. The supernatant was collected and centrifuged at 40,000 x g for 20 min, and the resulting pellet was washed and resuspended in lysis buffer.

Peptides—Peptides were synthesized using standard solid state methodology by Quality Control Biochemicals (Hopkinton, MA) and stored at −80 °C. Typically for experiments, 5 μg of peptide was dissolved in 200 μl of pH 7.4 Tris-buffered solution of 50% (v/v) dimethylsulfoxide and 2.5% (w/v) digitonin. The peptide solution was then diluted to a volume of 1 ml with 800 μl of Tris-HCl/NaCl/EDTA buffer for a final composition of 1 mg/ml peptide, 10% dimethylsulfoxide, 0.5% digitonin, 100 mM NaCl, 10 mM Tris-HCl, 2 mM EDTA, pH 7.4. Peptide sequences and the regions from which they were derived are as follows: D1DR TM6, SYMVPVFCWLPFFILNCIL (amino acids 275–295); D2DR TM5, PAFVVYSSIVSFYVPFIVTL (amino acids 187–206); D2DR intracellular loop domain 3, LSSTPERRYSPIPPSHH (amino acids 284–303); and β2AR TM6, G1HMGTFTLCLWLPFFIVNIH (amino acids 276–295).

Radioligand Binding Assays—Saturation binding and competition binding assays were performed as described previously (1, 9). Briefly, for saturation experiments, 20–25 μg of membrane protein was used, and the equilibrium binding was determined for each ligand. Competition binding assays were performed as described previously (1, 9). The membrane preparation was incubated with increasing concentrations of [3H]SCH 23390 with a Ki of 552.9 ± 27 pm (n = 6). Incubation of membranes with increasing concentrations of D1DR TM6 peptide resulted in a dose-dependent reduction of [3H]SCH 23390 binding (Fig. 1). No effect on binding was observed when membranes were incubated with a peptide derived from TM5 of the D2 dopamine receptor (D2DR) or a peptide based on a portion of the D2DR third intracellular loop domain. The IC50 of peptide inhibition of [3H]SCH 23390 binding to D1DR was 1.7 ± 0.2 mm (n = 6). The Ki was determined to be 460 ± 39 μM.

We have previously characterized c-myc-tagged D1DR expressed in Sf9 cells (1, 3) and demonstrated ligand binding, G protein coupling, and function assessed by adenylyl cyclase activation to be identical to those of D1DR in endogenously expressing tissues. All results obtained from Sf9 cell membranes are from cells expressing ~5–10 pmol of receptor 48 h after infection (cellular viability, ~90%).

Effect of D1DR TM6 Peptide on Antagonist Binding to the D1DR—D1DR in Sf9 membranes bound the antagonist [3H]SCH 23390 with a Kd of 552.9 ± 27 pm (n = 6). Incubation of membranes with increasing concentrations of D1DR TM6 peptide resulted in a dose-dependent reduction of [3H]SCH 23390 binding (Fig. 1). No effect on binding was observed when membranes were incubated with a peptide derived from TM5 of the D2 dopamine receptor (D2DR) or a peptide based on a portion of the D2DR third intracellular loop domain. The IC50 of peptide inhibition of [3H]SCH 23390 binding to D1DR was 1.7 ± 0.2 mm (n = 6). The Ki was determined to be 460 ± 39 μM.

The saturation isotherms for [3H]SCH 23390 in the presence of increasing concentrations of the D1DR TM6 peptide revealed a dose-dependent blockade of antagonist binding in an irreversible manner. The unoccluded receptors remained homogenous, with no change in the saturable nature of antagonist binding.

To determine whether the D1DR TM6 peptide had the ability to bind the ligand directly, an assay was performed in which the concentration of ligand present in the filtrate was measured after collecting the membranes on a filter. These results showed there was <10% depletion of the concentration of free ligand by the addition of the TM peptide (data not shown). Furthermore, attenuation of D1DR binding was still observed in membranes that were washed extensively after peptide treatment (data not shown). This indicates that the D1DR TM6 peptide does not affect binding because of an interaction between free peptide and ligand and also confers that the peptide-receptor interaction is likely to be reversible.

Effect of D1DR TM6 Peptide on Antagonist Binding to Other GPCRs—In Sf9 cell membranes expressing the D2DR, [3H]Spirpene binding was unaffected by pretreatment with 1 mM D1DR TM6 peptide (control, Bmax = 1.8 ± 0.2 pmol/mg; KD = 323 ± 24 pm; peptide-treated, Bmax = 1.7 ± 0.3 pmol/mg; KD = 337 ± 43 pm). D1DR TM6 peptide also had no effect on
[3H]carboxamidotryptamine maleate binding in 5-HT1D serotonin receptor-expressing Sf9 cell membranes (control, \( B_{\text{max}} \) 1.3 ± 0.2 pmol/mg; \( K_D \) 2.73 ± 0.13 nM; peptide-treated, \( B_{\text{max}} \) 1.3 ± 0.1 pmol/mg; \( K_D \) 2.74 ± 0.38 nM).

Effect of D1DR TM6 Peptide on Agonist Interaction with the D1DR—In membranes prepared from vehicle-treated cells, analysis of agonist competition curves indicated two agonist-detected affinity states (25% high affinity state, 75% low affinity state). The high and low affinity values were determined to be 10 ± 1.4 and 1100 ± 151 nM, respectively. For membranes in which the D1DR had been exposed to the D1DR TM6 peptide, two agonist-detected affinity states were also observed with inhibition constants that were similar to those of untreated cells (Fig. 2). However, total specific binding was significantly reduced, as would be predicted from the saturation binding experiments.

D1DR TM6 Peptide Effect on GTP\(_{\gamma}\)S Binding—Treatment of membranes from D1DR-expressing Sf9 cells with dopamine resulted in a dose-dependent increase in the binding of [\(^{35}\)S]GTP\(_{\gamma}\)S (Fig. 3A). This agonist-induced binding was attenuated when membranes were incubated with D1DR TM6 peptide (data not shown). Furthermore, D1DR TM6 peptide treatment of the D1DR-expressing membranes in the absence of dopamine resulted in a suppression of basal [\(^{35}\)S]GTP\(_{\gamma}\)S binding to a level approaching that of membranes from wild-type baculovirus-infected Sf9 cells (Fig. 3B).

D1DR TM6 Peptide Effect on Adenylyl Cyclase Activity—D1DR in Sf9 cells mediated a dose-dependent stimulation of adenylyl cyclase activity by dopamine (Fig. 4A). Dopa-}

\[ ^{35} \text{S} \text{GTP}_{\gamma} \text{S binding to D1DR-expressing Sf9 cell membranes.} \]

\[ \text{A, dopamine-induced [}^{35}\text{S}]\text{GTP}_{\gamma}\text{S binding. Binding is expressed as a percentage of the maximum dopamine-induced increase in specific [}^{35}\text{S}]\text{GTP}_{\gamma}\text{S binding. The result shown is from one of three independent experiments.} \]

\[ \text{B, attenuation of basal [}^{35}\text{S}]\text{GTP}_{\gamma}\text{S binding by D1DR TM6 peptide. Binding is expressed as a percentage of specific [}^{35}\text{S}]\text{GTP}_{\gamma}\text{S binding in the absence of dopamine. The data shown are from one of three independent experiments.} \]
specific for the c-myc epitope, with no cross-reactivity to Sf9 proteins (23, 24), and is ideal for the immunodetection of the c-myc-D1DR in these cells, as we have previously reported (1, 3, 25). The immunoblot analysis of D1DR-expressing Sf9 cells revealed the presence of a ~48-kDa protein, representing a receptor monomer, and a ~100-kDa protein, representing a receptor dimer and higher species (Fig. 5, lane 1). We have shown previously that Sf9 cell-expressed GPCRs are not heavily glycosylated and do not co-migrate with G protein when subjected to SDS-polyacrylamide gel electrophoresis (1–5). Thus, we conclude that the higher molecular weight species of specific immunoreactivity represent higher order oligomers of the D1DR, possibly tetramers.

Exposure of membranes to the D1DR TM6 peptide resulted in no significant change in both the monomer and the dimer species of the D1DR and no alteration of the overall proportion of receptor dimers to monomers (Fig. 5, lane 2). The effect of other peptides derived from TM domains of other receptors and membrane proteins was also examined for effects on the D1DR. A peptide based on the TM6 of the β2AR (Fig. 5, lane 3) and other α-helical peptides (data not shown) did not disrupt the D1DR dimer.

**DISCUSSION**

In the present study, we have shown that incubation of the dopamine D1DR with a peptide derived from TM6 of the same receptor disrupted D1DR function in an irreversible, dose-dependent manner. D1DR TM6 peptide treatment resulted in inhibition of ligand binding to the D1DR and attenuation of the D1DR-linked functions of G protein activation and adenylyl cyclase stimulation. These effects on the D1DR were specific for the D1DR TM6 peptide and were not seen with comparable TM peptides from other GPCRs or with other unrelated peptides. D1DR TM6 peptide treatment did not affect ligand binding of the D2DR and the 5-HT1D serotonin receptor. Peptide treatment was not accompanied by any change in the oligomeric state of the D1DR, because no disruption of receptor dimers or higher order oligomers was observed even at peptide concentrations that completely prevented antagonist binding and dopamine-stimulated adenylyl cyclase activation.

This report is the first to demonstrate that a peptide based on a TM of a GPCR can inhibit ligand binding and agonist-induced G protein activation by the receptor from which it is derived. We provide evidence that the D1DR TM6 peptide has properties of a D1DR inverse agonist, because peptide treatment suppressed the effect of D1DR on GTPγS binding and adenylyl cyclase activity to levels significantly below those observed in the absence of agonist. Furthermore, the observations of inverse agonism under agonist-absent conditions and of minimal ligand depletion by the D1DR TM6 strongly suggested that the peptide interacted with the receptor and did not mediate its effects through binding ligand independently of receptor.

These results differ from what has been reported for the β2AR, for which a peptide based on TM6 of the β2AR has been shown to dissociate β2AR dimers to monomers and to disrupt agonist-stimulated adenylyl cyclase activity without affecting ligand binding (6). It was postulated that β2AR dimers were critical for receptor-mediated adenylyl cyclase activity and that the dissociation of β2AR dimers by β2AR TM6 peptide resulted in the loss of cAMP production. The reason for the discrepancy with our results in not clear. Hebert et al. (6) proposed that a motif in TM6 of the β2AR that is analogous to one reported for the membrane protein glycoporphin A (26–28) was the basis for β2AR dimerization. However, in the D1DR, there is no similar motif in any of the putative transmembrane domains. Thus, the D1DR may undergo dimerization by some other mechanism, independent of what has been proposed for the β2AR in which TM6 was postulated to participate in the critical dimer interface.

The ability of a single peptide derived from a TM region to disrupt function of the D1DR attests to the critical intermolecular interactions that must be present within the receptor to maintain the three-dimensional conformation necessary for agonist interaction and signal transduction. The D1DR and other
GPCRs are predicted to have a tightly packed TM core consisting of the seven TMs aligned as α-helices arranged sequentially in a counterclockwise arrangement (29). These TM regions contain highly conserved amino acid residues that are important in maintaining the three-dimensional structure of the receptor and the pocket for agonist binding (20). There is now an abundance of evidence demonstrating that the precise positioning of individual residues within the helical bundle to allow contact points between neighboring helices is critical for the creation of the agonist binding site and other necessary conformations of the receptor (30–33). Mutagenesis studies of GPCRs have suggested that the proper folding of GPCR monomers involves intramolecular interactions between TM1 and TM7 (34). Furthermore, the necessity of amino acids residing on different TM domains to come together to form the ligand-binding crevice deep within the TM core has been elegantly demonstrated (35). Nevertheless, the assignment of specific helical positions relative to one another and the location of residues that mediate the interhelical interactions are not fully known at present. From our studies, it would appear that for the D1DR, the D1DR TM6 peptide is able to disturb the receptor conformation sufficiently to prevent its proper interaction with agonist or antagonist ligands and to interfere with basal intrinsic activity of the receptor.

We propose that the mechanism of the antagonism exerted by the TM-based peptide is the interaction and binding of the peptide to the TMs of the receptor protein, resulting in competition and interference with the intramolecular interactions of the receptor. This type of interference may result in disruption of the three-dimensional structure of the receptor. There have been reports showing that polypeptide fragments containing the TM segments of bacteriorhodopsin, when expressed together, are able to reassemble into functional proteins (36) suggesting that the TM domains of a GPCR are able to “find” the specific interacting amino acids from neighboring domains. Therefore, it is possible that a peptide mimicking a TM segment of the D1DR is able to interact specifically with the TMs of the D1DR. The exact manner in which the TM peptide interacts with the receptor protein is not known. Several possibilities exist, and these include contact with an external face of a TM or the direct insertion into the hydrophobic interior core of the receptor. It has been suggested that GPCRs may even “open up” their hydrophobic core during their interconversion between monomer and dimer (37), and this process may provide the means for a TM peptide to embed itself within a receptor core. Despite the speculative nature of these ideas, they may provide the basis for novel strategies in the design of receptor-selective antagonists.

Our results also indicate that the interference from the TM6 peptide was insufficient to disrupt the D1DR dimer. In a dimerized receptor protein, each monomer may contribute multiple residues or receptor domains toward common structural elements, such as the ligand binding pocket or the dimer interface. One recent model proposed postulates TM domain swapping between receptor monomers resulting in dimerization (21). If such a model is true, it is unlikely that interfering with a single transmembrane domain would result in disruption of dimers into monomers. Our results show the TM peptide interferes with receptor function probably by mimicking the natural dynamics of this domain of the receptor and that the dimerization or oligomerization process likely involves a complex interaction of multiple receptor domains because it is not affected by interference with a single TM domain.

This study represents the first demonstration that a peptide derived from the TM of a GPCR disrupts ligand binding in the receptor from which it was derived. We also have shown that the D1DR peptide disrupts intrinsic D1DR activity despite the lack of oligomer dissociation. TM peptides derived from GPCRs may represent a novel category of antagonists with the possible advantage over traditional receptor blockers in being highly selective to a particular class or subtype of receptor.

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