Roles of Dopamine D₂ Receptor Subregions in Interactions with β-Arrestin2

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Abstract

β-Arrestins are one of the protein families that interact with G protein-coupled receptors (GPCRs). The roles of β-arrestins are multifaceted, as they mediate different processes including receptor desensitization, endocytosis, and G protein-independent signaling. Thus, determining the GPCR regions involved in the interactions with β-arrestins would be a preliminary step in understanding the molecular mechanisms involved in the selective direction of each function. In the current study, we determined the roles of the N-terminus, intracellular loops, and C-terminal tail of a representative GPCR in the interaction with β-arrestin2. For this, we employed dopamine D₂ and D₃ receptors (D₂R and D₃R, respectively), since they display distinct agonist-induced interactions with β-arrestins. Our results showed that the second and third intracellular loops of D₂R are involved in the agonist-induced translocation of β-arrestins toward plasma membranes. In contrast, the N- and C-termini of D₂R exerted negative effects on the basal interaction with β-arrestins.

Key Words: β-Arrestin, G protein-coupled receptors, Dopamine D₂ receptor, Dopamine D₃ receptor

INTRODUCTION

G protein-coupled receptors (GPCRs) are classified into rhodopsin-like, secretin-like, and metabotropic glutamate-like families based on the size and shape of their N-terminal region (Bockaert and Pin, 1999). Rhodopsin-like GPCRs are further classified into subfamilies, which bind to different ligands including catecholamines, peptides, and glycoprotein hormones. Dopamine D₂ and D₃ receptors (D₂R and D₃R, respectively) belong to the catecholamine subfamily and are important targets for the treatment of various diseases associated with motor, emotional, and endocrine dysfunction (Thomas et al., 2008; Cho et al., 2010b).

GPCRs including D₂R and D₃R contain relatively well-conserved transmembrane regions, but the sizes and amino acid sequences of the N-termini and intracellular loops are variable (Cho et al., 2010b). According to the crystal structure of D₃R (Chien et al., 2010), the three-dimensional structure around the TM domains was clearly defined, and the ionic interactions among the well-conserved amino acid residues of the TM regions and adjacent submolecular domains were well predicted. However, the structural features of the other regions, especially those of the N-terminal region could not be determined.

Upon stimulation by agonists, GPCRs activate G proteins and are phosphorylated by G protein-coupled receptor kinases (GRKs). In response to agonistic stimulation, some GPCRs undergo GRK2/3-mediated phosphorylation and interact with β-arrestins, which results in desensitization of the GPCRs, and this was exemplified by β₂ adrenergic receptor (β₂AR) (Lohse et al., 1990; Lohse et al., 1992). When exposed to agonists for extended periods, GPCRs are endocytosed in a complex with β-arrestin (Ferguson et al., 1996; Goodman et al., 1996), and may or may not dissociate from the co-internalized β-arrestins after entering the cells. Based on the postendocytic behaviors of GPCRs with β-arrestins, GPCRs are classified as either class A or class B (Oakley et al., 2000).

Recent studies have suggested that β-arrestins mediate G protein-independent atypical signaling, and evidence for this was mainly demonstrated by ERK activation (Ahn et al., 2003; Ahn et al., 2004). It remains unclear how β-arrestins mediate various cellular processes such as desensitization, endocytosis, and atypical signaling. As an initial step toward understanding the multifaceted roles of β-arrestins in these processes, especially those of the N-terminal region could not be determined.
es, we determined the roles of distinct GPCR subregions in the interactions with β-arrestins.

MATERIALS AND METHODS

Materials
Human embryonic kidney 293 (HEK-293) cells were purchased from American Type Culture Collection (Manassas, VA, USA). HEK-293 cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA USA), 100 units/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere containing 5% CO2. The cells were transfected using polyethyleneimine (PEI) (Warrington, PA, USA). [3H]-spiperone was purchased from PerkinElmer Life Sciences (Boston, MA, USA). Dopamine (DA), antibodies to Flag, and anti-M2 Flag antibody-conjugated agarose beads were obtained from Sigma/Aldrich Chemical Co. (St. Louis, MO, USA). Antibodies to β-arrestin were kindly provided by Dr. Lefkowitz (Duke University, NC, USA).

Plasmid constructs
The human genes encoding for D2R and D3R in the pCMV5 vector, either untagged or tagged with Flag epitopes at the N-termini, were described elsewhere (Kim et al., 2001; Cho et al., 2007). D2R and D3R chimeric receptors, in which the second and third intracellular loops or N/C-termini were exchanged, were described previously (Robinson and Caron, 1997; Zheng et al., 2011), or prepared by site-directed mutagenesis. Specifically, in D2R-(IL2/3-D3R), the second and third intracellular loops of D2R were exchanged with those of D3R. In D3R-(IL2/3-D2R), the second and third intracellular loops of D3R were exchanged with those of D2R. D2R mutants lacking either the N-terminus or C-terminus were prepared by site-directed mutagenesis.

Immunoprecipitation
Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) on a rotation wheel for 1 h at 4°C. The supernatant was mixed with 35 µL of 50% slurry of anti-Flag antibody-conjugated agarose beads for 2-3 h on the rotation wheel. The beads were washed with washing buffer (50 mM Tris, pH 7.4, 137 mM NaCl, 1% glycerol, 1% NP-40) three times for 10 min each.

Confocal microscopy
For β-arrestin translocation assays, HEK-293 cells were transfected with β-arrestin2-GFP and corresponding GPCRs (Kim et al., 2001). One day after transfection, cells were seeded onto 35-mm dishes containing a centered, 1-cm well and allowed to recover for 1 day. Cells were then incubated with 2 mL MEM containing 20 mM HEPES (pH 7.4) and viewed on a Zeiss laser scanning confocal microscope.

RESULTS

D2R but not D3R mediates agonist-induced β-arrestin translocation
Upon binding to agonists, GPCRs undergo conformational changes resulting in signaling and a series of regulatory

Fig. 1. Comparison between D2R and D3R in agonist-induced β-arrestin translocation. (A) Visualization of β-arrestin2-GFP translocation in response to agonistic stimulation of D2R or D3R in HEK-293 cells. Cells stably expressing D2R or D3R (2.7 and 3.5 pmol/mg protein, respectively) were transfected with 2 µg β-arrestin2-GFP per 100 mm culture dish. Cells were stimulated with 10 µM DA for 5 min. Receptor expression levels were determined by [3H]-spiperone binding in saturating concentrations (3 nM). (B) Schematic diagram of D2R and D3R chimeric receptors. Detailed information about these constructs is described in previous studies (Robinson and Caron, 1996; Robinson and Caron, 1997).
Interactions between GPCRs and β-Arrestins

For example, Gbγ helps recruit GPCR kinase2/3 (GRK2/3) from the cytosol to the plasma membrane to phosphorylate activated receptors (Pitcher et al., 1992). Phosphorylated receptors exhibit a higher affinity to β-arrestin, causing translocation of β-arrestins from the cytosol to the extracellular membrane (Barak et al., 1997). Translocated β-arrestins bind to agonist-occupied GPCRs to which they exert multiple effects such as desensitization and receptor endocytosis. Thus, agonist-induced translocation of β-arrestins is a biomarker to predict the uncoupling of the receptors from G proteins and GPCR endocytosis.

As shown in Fig. 1A, agonist stimulation of D2R but not D3R resulted in β-arrestin2 translocation, suggesting that these two receptors could be used to determine the particular regions involved in β-arrestin2 translocation. The second and third intracellular loops are the main regions involved in GPCR interactions with various proteins including G proteins (Robinson and Caron, 1996). To test whether the second or third loops are involved in the agonist-induced β-arrestin2 translocation, six different D2R and D3R chimeric receptors were prepared (Fig. 1B). For each chimeric receptor, either the second or third intracellular loop or both loops were replaced with those of the other receptor.

**The second and third intracellular loops are both involved in agonist-induced translocation of β-arrestin2**

Fig. 2. Evaluation of β-arrestin2-GFP translocation after agonist activation of D2R, D3R, or D2R and D3R chimeric receptors. HEK-293 cells expressing D2R (2.3 pmol/mg of protein), D3R (3.7 pmol/mg of protein), or D2R and D3R chimeric receptors (between 2.0 and 3.5 pmol/mg protein) were transfected with 1 μg of β-arrestin2-GFP. Cells were stimulated with 10 μM DA for 5 min and viewed on a Zeiss laser scanning confocal microscope.

![Image](image_url)
when the second or third intracellular loop was replaced with that of D2R, while D3R could not, as clearly, gain the ability to translocate β-arrestin2 when both loops were replaced with those of D2R. Thus, it could be concluded that the second and third intracellular loops of D2R play central roles in the translocation of β-arrestin2, though other subregions also possess certain roles.

**Constitutive interactions are controlled by the N- or C-terminus**

The roles of the N-terminus of D2R or D3R in the interaction with β-arrestins have not been reported. The N-terminus of D2R consists of 37 amino acids and contains three N-linked glycosylation sites (N-X-S/T) (Fig. 3A). Since N-linked glycosylation is important in the surface expression of certain GPCRs, we prepared three constructs in which approximately 10 consecutive amino acids were deleted. For example, Δ(2-10) represents a deletion mutant of D2R in which the amino acids 2 through 10 were deleted. Surface expression of Δ(2-34) was too seriously impaired (data not shown), and thus could not be used for functional studies. As shown in Fig. 3B, agonist stimulation of D2R resulted in an increase in the interaction between D2R and β-arrestin2. However, deletion of 21 amino acid residues located within the N-terminus of D2R, Δ(2-22), resulted in a marked increase in the basal interaction with β-arrestin2.

When the carboxyl tails of D2R and D3R are compared, it becomes apparent that D2R contains four extra residues near the seventh transmembrane domain. Other than this difference, the only difference found between the two C-termini was one amino acid residue that precedes the cysteine residue, which is the last amino acid residue of each receptor (Fig. 4A). Replacement of the N-terminus of D2R with that of D3R (D2R-D3C) did not affect the pattern of the interaction with β-arrestin2, that is, the interaction was increased in response to treatment with the agonist (Fig. 4B). Conversely, deletion of the C-terminus of D2R (CTX-D2R) inhibited the receptor expression levels, but also markedly increased the basal interaction with β-arrestin2 (Fig. 4C).

**DISCUSSION**

Like other GPCRs, D2R and D3R contain the characteristic topology of heptahelical receptors: N-terminus, seven transmembrane (TM) domains, three extracellular and intracellular loops, and C-terminal tail. Although the TM regions are relatively well-conserved among GPCRs, most GPCRs including D2R and D3R differ in their amino acid sequences and in the lengths of their N-terminal region, intracellular loops, and C-terminal tail (Cho et al., 2010b).

The roles of the intracellular loops and C-terminal tails of some GPCRs have been extensively characterized in terms of G-protein coupling and intracellular trafficking (Ostrowski et al., 1992; Cho et al., 2010b). However, both D2R and D3R have relatively short C-terminal tails, consisting of fourteen and ten amino acid residues, respectively; thus, it was difficult to study functional roles of the C-termini of D2R and D3R. It is unlikely that the C-terminal tail of D2R plays a major role in determining the interaction with β-arrestin2, as the results presented herein show that the replacement of the C-terminus of D2R with that of D3R did not affect the interaction with β-arrestin2.

The roles of the N-terminal regions of rhodopsin family GPCRs have been difficult to study owing to technical limitations such as challenges in getting three-dimensional information from their crystal structures (Chien et al., 2010). Additionally, it is unclear how the N-terminus of D2R, which is located extracellularly, affects the interaction between D2R and β-arrestin2, which presumably occurs through intracellular regions of D2R. It could be speculated that shortening the N-terminus alters the overall conformation of D2R, such that potential strains that prevent the constitutive interaction between D2R and β-arrestin2 are relieved.

In a previous study, it was reported that the second and
cells were transfected with Flag-D2R or Flag-CTX-D2R in pCMV5 together with immunoblotted with antibodies against b-arrestin2. (A) Alignment of the amino acid sequences within the C-terminal tail of D2R on the interaction with b-arrestin2. Though further studies are needed to understand completely the molecular details involved this process, the results presented herein may provide clues for elucidating the functional roles of the N- and C-termini of D2R in the interaction with b-arrestin2.

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