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RalA employs GRK2 and β-arrestins for the filamin A-mediated regulation of trafficking and signaling of dopamine D_2 and D_3 receptor

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ABSTRACT

Filamin A (FLNA) is known to act as platform for the signaling and intracellular trafficking of various GPCRs including dopamine D_2 and D_3 receptors (D_2R, D_3R). To understand molecular mechanisms involved in the FLNA-mediated regulation of D_2R and D_3R, comparative studies were conducted on the signaling and intracellular trafficking of the D_2R and D_3R in FLNA-knockdown cells, with a specific focus on the roles of the proteins that interact with FLNA and the D_2R and D_3R. Lowering the level of cellular FLNA caused an elevation in RalA activity and resulted in selective interference with the normal intracellular trafficking and signaling of the D_2R and D_3R, through GRK2 and β-arrestins, respectively. Knockdown of FLNA or coexpression of active RalA interfered with the recycling of the internalized D_2R and resulted in the development of receptor tolerance. Active RalA was found to interact with GRK2 to sequester it from D_2R. Knockdown of FLNA or coexpression of active RalA prevented D_3R from coupling with G protein. The selective involvement of GRK2- and β-arrestins in the RalA-mediated cellular processes of the D_2R and D_3R was achieved via their different modes of interactions with the receptor and their distinct functional roles in receptor regulation. Our results show that FLNA is a multifunctional protein that acts as a platform on which D_2R and D_3R can interact with various proteins, through which selective regulation of these receptors occurs in combination with GRK2 and β-arrestins.

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1. Introduction

Filamin stabilizes cortical three-dimensional actin filament networks [1] and links them to cellular membranes by binding to transmembrane receptors or ion channels [2]. Filamin has essential scaffolding functions and integrates multiple cellular behaviors. Among the three isoforms of filamins, filamin A (FLNA) and B are ubiquitously expressed, whereas the expression of filamin C is largely restricted to skeletal and cardiac muscle [3].

FLNA interacts with receptors, including G protein-coupled receptors (GPCRs), and it has been proposed that FLNA provides a platform through which receptors signal or undergo appropriate intracellular trafficking. For example, FLNA is required for the trafficking of androgen receptors from the cytoplasm to the nucleus [4], internalization and desensitization of the μ opioid receptor [5], signaling via the calcium sensing receptor [6], constitutive inhibition of the insulin receptor [7], and for actin-dependent clustering of HIV receptors via interactions with CD4 and its coreceptors (CCR5 or CXCR4) [8].

Studies using M2 and A7 cells have suggested that FLNA plays an important role in the functional regulation of the dopamine D_2 and D_3 receptors (D_2R and D_3R). For example, it has been reported that FLNA is required for correct expression of the D_2R on the plasma membrane [9], and for signaling through both D_2R and D_3R [10–12]. However, the molecular mechanism underlying the FLNA-mediated regulation of D_2R and D_3R has not been understood.

Previous studies have shown that FLNA interacts with caveolin1 and RalA [13,14], while other studies have shown that FLNA interacts with β-arrestin2 [12,15]. These results imply that FLNA interacts with multiple proteins and contributes to the proper function of GPCRs.

Among the dopamine receptor subtypes that have been characterized, D_2R and D_3R are the major targets for the most commonly used neuroleptics. Disturbances in motor and endocrine functions are the most serious problems caused by the neuroleptics targeted to the D_2R [16]. Hence, the development of specific ligands or the ability to manipulate specific signaling pathways of the D_3R has been suggested as a means to separate the desired therapeutic activities from the unwanted side effects of neuroleptics. In our preliminary studies, we observed that FLNA exerted differential effects on the signaling and intracellular trafficking of the D_2R and D_3R. To understand the molecular mechanisms involved in the differential regulation of D_2R and D_3R signaling, we analyzed the functional interactions among the proteins that make up the

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signaling and regulatory complexes with FLNA and receptor proteins such as β-arrestins and RalA.

2. Materials and methods

2.1. Materials

Human embryonic kidney (HEK-293) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cell culture reagents were obtained from either Cellgro (Herndon, VA, USA) or Invitrogen Life Technologies, Inc. (Carlsbad, CA, USA). [^{3}H]-Sulpiride (84 Ci/mmol) and [^{3}H]-spiperone (85.5 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Dopamine (DA), (−) quinpirole, forskolin, antibodies to actin, GFP, FLAG, and anti-M2 FLAG antibody conjugated agarose beads were obtained from Sigma/Aldrich Chemical Co. (St. Louis, MO, USA). The anti-FLNA antibody was obtained from Research Diagnostics, Inc. (Flanders, NJ, USA). Antibodies to GRK2, phospho-ERK1/2, ERK2, and HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to β-arrestin were kindly provided by Dr. Lefkowitz (Duke University, NC, USA).

2.2. Cell culture and transfection

HEK-293 cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2. The cells were transfected using either the calcium phosphate precipitation method or polyethyleneimine (PEI) which was purchased from Polysciences, Inc. (Warrington, PA, USA).

2.3. Plasmid constructs

The human D_2R and D_3R in pCMV5 or pRC/CMV, which are either untagged or are tagged with a FLAG epitope at the N-terminus, have been described elsewhere [17,18]. Chimeric receptors between D_2R and D_3R in which the 2nd and 3rd intracellular loops were exchanged have been described previously [19,20]. In D_2R-(IL2/3-D_3R), the 2nd and 3rd intracellular loops of the D_2R were exchanged with those of the D_3R. In D_3R-(IL2/3-D_2R) the 2nd and 3rd intracellular loops of the D_3R were exchanged with those of the D_2R. In D_2R-Arr3, β-arrestin2 was fused to the *C*-terminus of the D_2R [21]. GFP-tagged FLNA was provided by Dr. Fumihiko Nakamura (Brigham and Women's Hospital, Boston, MA, USA). RNA interference plasmids for β-arrestin 1 and β-arrestin 2 were published previously [22]. Small hairpin RNAs of FLNA and GRK2, and the yellow fluorescence protein (YFP)-Gαo were reported previously [23–25]. Ral constructs were provided by Dr. Larry Feig (Tufts University, Boston, MA, USA) and Dr. Hans Bos (University Medical Center Utrecht, Utrecht, Netherlands).

2.4. Determination of the ratio of D_2R and D_3R expressed on the cell surface and in total subcellular regions

Surface expression of D_2R or D_3R were determined by radioligand binding assay, and the ratio of intracellular-to-total receptor levels was calculated as previously described [17]. Total receptor level was assessed using [^{3}H]-spiperone (final concentration: 2 nM), which labels D_2R and D_3R expressed both on the cell surface and in intracellular regions. The levels of the receptors expressed on the cell surface were determined using [^{3}H]-sulpiride (final concentration: 2.2 nM for D_2R, 7.2 nM for D_3R). Nonspecific binding was determined in the presence of 10 μM haloperidol.

To establish stable cell lines that express D_2R and D_3R in FLNA knockdown cells, D_2R or D_3R in pRC/CMV were transfected into the cells that express scrambled shRNA (Con-KD) or FLNA shRNA in pLKO.1 (FLNA-KD), and positive clones were selected in the presence of puromycin and G418. To adjust the expression levels of D_2R and D_3R on the cell surface at similar levels, multiple double cell lines were selected and the cell lines which express similar levels of receptors were chosen for the functional studies. In case of transient transfection, about three times of D_3R cDNAs were transfected into FLNA-KD cells compared with Con-KD cells. Receptor expression levels on cell surface were determined by [^{3}H]-sulpiride binding.

2.5. 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 25 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, 10% glycerol, 1% NP-40) three times for 5 min each. Immunoprecipitates and cell lysates were analyzed by SDS-PAGE, transferred to nitrocellulose membrane filters, and immunoblotted with corresponding antibodies.

2.6. Measurement of GTP-bound RalA

Activation of Ral was measured using a GST-pull down assay with an activation-specific probe, the Ral-binding domain (397–518 amino acid residues) of RalBP (RBD) [26,27]. For this, RBD was bacterially expressed as a fusion protein with glutathione-S-transferase (GST-RBD). Lysates obtained from the cells transfected with GFP-tagged RalA were added to a column containing GST-RBD which was pre-coupled to glutathione-agarose beads and incubated for 45 min with continuous shaking at 4 °C. Beads were washed four times with GST-binding buffer and then treated with Laemmli sample buffer to elute bound proteins.

2.7. Immunocytochemistry

Cells were plated onto cover slips, and fixed with 4% paraformaldehyde in PBS with 0.2% Triton X-100 for 20 min at room temperature. Cells were then incubated with PBS containing 3% FBS and 1% BSA for 1 h, then incubated with FLAG antibodies at 1:1000 for 1 h at room temperature. After three washes, cells were incubated with an Alexa-594-conjugated anti-rabbit antibody (Invitrogen, Carlsbad, CA, USA) at a 1:500 dilutions. After three washes with wash buffer, the cells were mounted onto slides using Vectashield (Vector Laboratories, Burlingame, CA, USA) and imaged using a laser scanning confocal microscope (TCS SP5/AOBS/Tandem, Germany).

2.8. Luciferase reporter gene assay

Cellular cAMP was measured by an indirect method [27,28]. Cells were transfected with either a reporter plasmid containing the firefly luciferase gene under the transcriptional control of multiple cAMP responsive elements (CRE) or a pRL-TK control plasmid (Promega, Madison, WI, USA). Transfected cells were seeded onto 24-well plates, and treated with 2 μM forskolin and quinpirole (10^{-12}–10^{-8} M) for 4 h. The cells were then harvested and the relative luciferase expression was measured. To determine the development of D_2R tolerance, cells were pre-treated with 10 μM DA for 30 min and washed three times with 1 ml of ice-cold serum-free media for 5 min per wash. Data were normalized by expressing cAMP levels as a percentage of the forskolin-stimulated cAMP for each experiment. Dose-response curves were fitted with GraphPad Prism (GraphPad Software, San Diego, CA).

2.9. Measurement of ERK activation

ERK assays were conducted as previously described [29]. HEK-293 cells (Con-KD and FLNA-KD) which stably express human D_2R or D_3R were used. Cells were cultured in 25 mm-diameter well plates, and

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