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Palmitoylation on the carboxyl terminus tail is required for the selective regulation of dopamine D_2 versus D_3 receptors

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ABSTRACT

Dopamine D_2 receptor (D_2R) and D_3 receptor (D_3R) possess highly conserved amino acid sequences but this study showed that D_3R was more extensively palmitoylated than D_2R. Based on this finding, the molecular basis of this selective palmitoylation of D_3R was determined and the roles of palmitoylation in the regulation of D_3R functions were investigated. D_3R was palmitoylated on the cysteine residue on its carboxyl terminus tail, the last amino acid residue of D_3R, and an exchange of the carboxyl terminus tail between D_2R and D_3R (D_2R-D_3C and D_3R-D_2C) resulted in the switching of the palmitoylation phenotype. When the consensus site for palmitoylation was mutated or the palmitoylation of D_3R was inhibited by treatment with 2-bromopalmitate (2BP), a palmitoylation blocker, cell-surface expression, PKC-mediated endocytosis, agonist affinity, and agonist-induced tolerance of D_3R were all inhibited. However, these changes were not observed when D_3R palmitoylation was inhibited by replacing its carboxyl tail with that of D_2R (D_3R-D_2C) or when the palmitoylation of D_2R-D_3C was inhibited by treatment with 2BP. Overall, this study shows that D_3R is palmitoylated more extensively than D_2R even though the carboxyl terminus tails of D_2R and D_3R are highly homologous, and thus provides a new clue regarding the consensus sequence for palmitoylation. This study also shows that palmitoylation controls various functionalities of D_3R only when the receptor is in the intact D_3R configuration.

1. Introduction

G protein-coupled receptors (GPCRs) undergo four major types of posttranslational modification: glycosylation, phosphorylation, ubiquitination, and palmitoylation. During palmitoylation, a 16C saturated fatty acid, palmitic acid, is inserted into one or more cysteine residues located in the carboxyl-terminal or the intracellular loops of GPCRs [1]. Several GPCRs have been reported to be palmitoylated, including rhodopsin [2], β_2-adrenoceptor [3], α_2-adrenoceptor [4,5], dopamine D_1 receptor [6], dopamine D_2 receptor in insect cells [7,8], and metabotropic glutamate mGlu4 receptor [9].

The most common outcome of lipid modification is the enhancement of the hydrophobicity of proteins, which leads to alterations in the properties and localization of proteins. However, abolishment of palmitoylation has been shown to be capable of affecting various functional aspects of GPCRs. For example, palmitate binding is required for proper processing and localization of various receptors on the plasma membrane [10,11], and for downregulation but not sequestration of α_2-adrenoceptor [5]. Furthermore, palmitoylation is required for receptor-G protein coupling in certain GPCRs such as β_2-adrenoceptor [3] and endothelin receptor type B [12], but not in α_2-adrenoceptor [4,5] and M2 muscarinic receptor [13]. The palmitoylation of dopamine D_3 receptor has not been reported yet.

Dopamine D_2 and D_3 receptors (D_2R, D_3R) show high homology in amino acid composition (46% overall amino acid homology and 78% identity in the transmembrane domain) [14] and share most of their signaling pathways [15]. Nevertheless, D_2R and D_3R undergo intracellular trafficking to markedly distinct degrees: D_2R undergoes robust GRK/β-arrestin-dependent endocytosis, whereas only a negligible fraction of D_3R is internalized through this pathway [16,17]. Conversely, D_3R undergoes more extensive PKC-mediated endocytosis as compared to D_2R [17].

Rhodopsin, one of the most extensively studied GPCRs, has been shown to be palmitoylated at its 322nd and 323rd cysteine residues [2]. One of these cysteine residues is conserved in an equivalent position in the carboxyl terminus tail of most members of the GPCR family [3]. Both D_2R and D_3R contain the conserved cysteine residue on their carboxyl termini, and their last 10 amino acid residues are identical except for the penultimate residue (Fig. 2A, histidine in D_2R and serine in D_3R). Unexpectedly, our preliminary studies showed that D_3R is

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more extensively palmitoylated than D_2R. These findings prompted us to conduct comparative studies between D_2R and D_3R, such as determination of their palmitoylation site and the functional roles of palmitoylation.

2. Materials and methods

2.1. Cell culture and transfection

HEK-293 cells were acquired from American Type Culture Collection (Manassas, VA, USA) and cell culture reagents were purchased from Invitrogen-Life Technologies, Inc. (Carlsbad, CA, USA). HEK-293 cells were cultured in MEM supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2. Transfections were performed using polyethylenimine, which was purchased from Polysciences (Warrington, PA, USA).

2.2. Materials

The following reagents were acquired from commercial sources: dopamine (DA), quinpirole, haloperidol, phorbol myristate acetate (PMA), forskolin, antibodies against hemagglutinin (HA), hydroxylamine, mouse anti-FLAG antibodies conjugated to agarose beads, and 2-bromopalmitate (2BP), (Sigma-Aldrich Chemical Co., St. Louis, MO, USA); [^{3}H]-sulpiride (84 Ci/mmol), [^{3}H]-spiperone (85.5 Ci/mmol), and [^{3}H]-CGP-12,177 (41.7 Ci/mmol) (PerkinElmer Life Sciences; Boston, MA, USA); tunicamycin, rabbit anti-FLAG and anti-GRK2 antibodies (Santa Cruz Biotechnology; Dallas, TX, USA); an Alexa Fluor 594-conjugated anti-rabbit antibody, Molecular Probes (Invitrogen); PNGase F (New England Biolabs; Ipswich, MA, USA); and N-ethylmaleimide, Sulfo-NHS-SS-Biotin, and Avidin beads (Hudson, NH, USA).

2.3. Plasmid constructs

Wild-type and FLAG-tagged human β_2-adrenergic receptor (β_2AR), human D_2R (short form), and human D_3R in pCMV5 were described previously [16,18]. D_3R-(IL2-D_2R), D_3R-(IL3-D_2R), D_2R-(IL2/3-D_3R), and D_3R-(IL2/3-D_2R), in which the second and/or the third intracellular loop was switched between D_3R and D_2R were described previously [19]. D_2R-D_3C and D_3R-D_2C, in which the carboxyl terminal tails of D_2R and D_3R were replaced with those of D_3R and D_2R, respectively, were created using site-directed mutagenesis, as were C341G-β_2AR, C400G-D_3R, H413S-D_2R, H413A-D_2R, S399 A-D_3R, S399H-D_3R, C414G-D_2R, C404G-D_3R-D_2C, and C410G-D_2R-D_3C.

2.4. Immunoprecipitation

After 48 h of transfection, cells were disrupted in lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100, and 10% glycerol) on a rotation wheel for 4 h at 4 °C. The supernatants were mixed with 35 μL of a 50% slurry of anti-FLAG-agarose beads with incubation for 8 h on the rotation wheel. The beads were washed with lysis buffer (3×, 5 min each), and the resulting immunoprecipitates were analyzed by SDS-PAGE with electrotransfer and probed with antibodies against target proteins.

2.5. Detection of palmitoylated proteins

In the IP-ABE assay, immunoprecipitation is used to isolate target proteins, and this procedure is followed by the ABE chemical reaction [20]. The multistep IP-ABE assay was performed as follows: After transfection for 36 h with plasmids encoding the proteins under study, cells were treated with lysis buffer containing 20 μM N-ethylmaleimide (NEM) for 3 h at 4 °C. This step is used to block free cysteine residues that are not palmitoylated. The supernatants were mixed with 35 μL of a 50% slurry of anti-FLAG-agarose beads with incubation for 4 h on the rotation wheel at 4 °C. Next, immunoprecipitates were treated with 1 M hydroxylamine (HAM) in lysis buffer for 1 h at room temperature, to selectively cleave the cysteine residues that were palmitoylated. The HAM-treated immunoprecipitates were incubated with 20 μM HPDP-biotin for 1 h at room temperature. HPDP-biotin binds with high affinity to free thiol groups of cysteine residues, and this step selectively biotinylates the cysteine residues that were palmitoylated. Immunoprecipitated protein samples were eluted from the FLAG beads by treatment with SDS sample buffer containing no reducing agents. Here, a protein is assumed to be palmitoylated if it was detected by blotting with streptavidin-HRP in the HAM (+) sample and yielded no specific signal in the HAM (−) sample. Total receptor levels were determined by immunoblotting the immunoprecipitates with antibodies that recognize the target proteins.

2.6. Determination of the ratio of D_2R and D_3R expressed on the cell surface and in the total subcellular fraction

Surface expression of D_2R or D_3R was determined by a radioligand-binding assay and a cell surface biotinylation assay. In case of the radioligand-binding approach, the ratio of intracellular-to-total receptor levels was calculated as previously described [16]. The total receptor level was assessed using [^{3}H]-spiperone (final concentration 3 nM), which labels D_2R and D_3R, expressed both on the cell surface and in the 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 or 7.2 nM for D_3R). Nonspecific binding was determined in the presence of 10 μM haloperidol.

In the cell surface biotinylation assay, HEK-293 cells expressing corresponding receptors were treated with 0.5 mg/mL Sulfo-NHS-`-biotin for 40 min at 4 °C, followed by treatment with 0.1 M glycine for 10 min. After we suspended them in RIPA buffer, cell lysates were centrifuged for 10 min, and 100 μL of the supernatant was mixed with 50 μL of the 50% slurry of immobilized avidin beads, with incubation overnight at 4 °C. Immunoprecipitates were treated with 50 μL of sample buffer at 65 °C, and eluents were analyzed by immunoblotting.

2.7. Immunocytochemistry and confocal microscopy

One day after the transfection, cells were seeded onto glass coverslips coated with poly-L-lysine; the next day, the cells were fixed with 4% paraformaldehyde (15 min, room temperature). Then, the cells were permeabilized with 0.1% Triton X-100 in PBS at room temperature. Next, the cells were incubated with PBS containing 3% fetal bovine serum and 1% bovine serum albumin for 1 h and then incubated with anti-FLAG antibodies (1:1000 dilution) for 1 h at room temperature. After 3 washes, cells were incubated with Alexa 647-conjugated anti-rabbit antibodies (Invitrogen, Carlsbad, CA, USA) at 1:500 dilution. After 3 washes with washing buffer, the cells were mounted on a Vectashield (Vector Laboratories, Burlingame, CA, USA) and viewed under a laser scanning confocal microscope (TCS SP5/ABOS/Tandem, Germany). Ten cells were randomly selected and examined for the subcellular distribution of D_2R. The same sets of cells were used for determination of [^{3}H]-sulpiride and [^{3}H]-spiperone binding.

2.8. Detection of ubiquitinated receptors

FLAG-D_3R and HA-tagged ubiquitin (HA-Ub) were transfected into HEK-293 cells, and the cells were solubilized in lysis buffer for 4 h at 4 °C. The supernatants were mixed with 35 μL of a 50% slurry of anti-FLAG-agarose beads with incubation for 8 h on the rotation wheel at 4 °C. The immunoprecipitates were analyzed by SDS-PAGE with immunoblotting by means of antibodies against HA and FLAG tags.