Cellular Signalling 22 (2010) 600–609

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Resistance of the dopamine D4 receptor to agonist-induced internalization and degradation

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ARTICLE INFO

Article history:
Received 23 October 2009
Received in revised form 12 November 2009
Accepted 16 November 2009
Available online 20 November 2009

Keywords:
GPCR
Dopamine
D4 receptor
β-arrestin
Internalization
Phosphorylation

ABSTRACT

Dopamine receptors are G-protein-coupled receptors involved in the control of motivation, learning, and fine-tuning of motor movement, as well as modulation of neuroendocrine signalling. Stimulation of G-protein-coupled receptors normally results in attenuation of signalling through desensitization, followed by internalization and down-regulation of the receptor. These processes allow the cell to regain homeostasis after exposure to extracellular stimuli and offer protection against excessive signalling.

Here, we have investigated the agonist-mediated attenuation properties of the dopamine D4 receptor.

We found that several hallmarks of signal attenuation such as receptor phosphorylation, internalization and degradation showed a blunted response to agonist treatment. Moreover, we did not observe recruitment of β-arrestins upon D4 receptor stimulation. We also provide evidence for the constitutive phosphorylation of two serine residues in the third intracellular loop of the D4 receptor.

These data demonstrate that, when expressed in CHO, HeLa and HEK293 cells, the human D4 receptor shows resistance to agonist-mediated internalization and down-regulation. Data from neuronal cell lines, which have been reported to show low endogenous D4 receptor expression, such as the hippocampal cell line HT22 and primary rat hippocampal cells, further support these observations.

1. Introduction

Dopamine receptors belong to the superfamily of G-protein-coupled receptors (GPCRs) and are involved in the regulation of locomotion, reward, and cognitive processes. Dysregulation of dopaminergic neurotransmission is associated with multiple neurological and psychiatric conditions, such as attention deficit hyperactivity disorder (ADHD), mood disorders and schizophrenia. Dopamine receptors are divided in two subfamilies: the D1-like subfamily (D1 and D5 receptors) and the D2-like subfamily (D2, D3 and D4 receptors) that signal through Gs and Gi/o, respectively. The D2-like subfamily forms a target for antipsychotic medication and among them the D4 is of particular interest as it displays high affinity for the antipsychotic clozapine. The dopamine D4 receptor also shows an interesting polymorphism in its third intracellular loop and genetic studies have found an association between this highly polymorphic repeat sequence and ADHD [1].

Attenuation of GPCR signalling after activation is necessary to prevent overstimulation and to regain responsiveness towards future extracellular stimuli. Three main processes have been described to contribute to attenuation of signalling: desensitization, internalization and down-regulation whereby most information comes from studies with the prototypic β2-adrenergic receptor.

As a first step, desensitization usually involves phosphorylation of GPCRs by GPCR kinases (GRK) or second messenger-dependent kinases, such as protein kinase A on the C-terminal tail and the third intracellular loop or sometimes also on the first or second intracellular loop (reviewed by Tobin, [2]). Phosphorylation of GPCRs on serine and threonine residues can create a binding site for β-arrestins. Binding of β-arrestin1 or 2 to the receptor uncouples them from G-proteins, thus leading to an attenuation of GPCR signalling. In earlier studies it was shown that at least two phosphorylated residues in close proximity are involved in β-arrestin binding. Later it was published that the conformational change induced upon activation of most GPCRs, often exposes β-arrestin-interacting residues, which allows phosphorylation-independent binding of β-arrestin (reviewed in [3]).

Abbreviations: GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; ER, endoplasmic reticulum; HEK293, human embryonic kidney cell line; CHO, Chinese hamster ovary cell line.

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doi:10.1016/j.cellsig.2009.11.013

Subsequently, internalization can occur as a result of recruitment of an endocytic protein complex by β-arrestins, containing among others clathrin and dynamin, to the receptor. Finally, after endocytosis the receptor can either be degraded (down-regulation) or recycled to the plasma membrane (resensitization) [4,5].

Upon agonist stimulation, D1-like receptors undergo multiple levels of regulation which are not that different from the prototypic β2-adrenergic receptor. Agonist-occupied D1-like receptors undergo internalization, via a pathway involving protein kinase A, GRKs, β-arrestin, clathrin and dynamin [6,7]. Interestingly, both D1-like receptors contain distinct specific phosphorylation sites that may underlie the differences of internalization rates of both receptor subtypes. The observation that the D5 receptor undergoes slower agonist-induced internalization than the D1 receptor, could be due to the higher constitutive activity of the D5 receptor, hereby favouring a constitutive desensitization of the D5 receptor [8]. Most studies on regulation of D2-like receptors report that continuous agonist application results in phosphorylation of the receptor, for example by GRK2, leading to G-protein uncoupling, subsequent β-arrestin recruitment and internalization. Internalization of the receptor seems to be dependent on a classical dynamin-dependent mechanism of endocytosis in early endosomes [9–12]. Also heterologous desensitization of D2 receptors by protein kinase C promotes internalization through β-arrestin- and dynamin-dependent pathways [13].

Concerning the D3 receptor subtype, some differences in desensitization and trafficking compared to the D2 receptor, have been reported [10]; only subtle agonist-mediated receptor phosphorylation, β-arrestin translocation to the plasma membrane and receptor internalization was observed.

Studies on the desensitization, internalization and subsequent degradation properties of the D4 receptor are very rare; a resistance for desensitization has been mentioned in the review by Oak et al. [14] and a role for the SH3 domains in the third intracellular loop in this process has been suggested [15].

Endocytotic trafficking of the D4 receptor, upon agonist stimulation, is not governed by common processes, such as robustly increased phosphorylation of the receptor, recruitment of β-arrestin1 or -2 or significant internalization. Overall we observed a blunted or negligible response of the D4 receptor to agonist-mediated attenuation mechanisms. Therefore, we can conclude that the different D2 receptor subtypes vary substantially in their regulation processes in the cell.

2. Materials and methods

2.1. Reagents

Minimal essential medium, fetal bovine serum, neomycin (G418), Neurobasal medium, B27 medium, Lipofectamine 2000 were purchased from Invitrogen. [^{3}H]spiperone was obtained from Amersham Biosciences. Brefeldin A, butaclamol(+), dopamine, haloperidol, lactacystin, poly-L-lysine were purchased from Sigma. Laminin was obtained from Roche.

Antibodies used were mouse monoclonal anti-HA (clone 16B12; Covance Research Products, Berkeley, CA), mouse monoclonal anti-FLAG M1 (Sigma), and mouse monoclonal anti-FLAG M2 antibody (Sigma). Horseradish peroxidase-conjugated sheep anti-mouse antibodies were purchased from Amersham Biosciences and horseradish peroxidase-conjugated anti-FLAG M2 mouse monoclonal antibody from Sigma.

2.2. Plasmids, cell culture and transfection

The dopamine D4 receptor contains a polymorphism, namely a 'variable number of tandem repeats', in the third intracellular loop. This number of repeats can vary from two to eleven and is denoted as D4.2 to D4.11 receptor. Most common are D4.2, D4.4 and D4.7 receptors, and the discussed results are obtained with the D4.2 or D4.4 receptors, dependent on the preferred tag. We have not seen a difference between both receptor variants concerning internalization and degradation. The plasmids pHA D4.2 receptor, pFLAG D4.4 receptor and pFLAG D2 receptor have been described before [16,17]. β-arrestin1-GFP was a kind gift from Prof. Dr. S. Wilson (SmithKline Beecham Pharmaceuticals, Harlow, Essex, UK). β-arrestin2-GFP, FLAG β2-adrenergic receptor, HA β2-adrenergic receptor and FLAG β-arrestin2 were kind gifts from Prof. Dr. R. Lefkowitz (Duke University, Durham, NC).

CHO-K1, HeLaT5 and HEK293(T) cells were purchased from American Type Culture Collection (Manassas, VA). The HT22 cell line was a kind gift from Prof. Dr. D. Schubert (Salk Institute, LaJolla, California). Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a controlled environment (37 °C, 98% humidity, 5% CO_2). Generation of CHO-K1 cells stably expressing the FLAG D4.4 receptor and pharmacological characterization has been described before [16,17].

HEK293T cells were transfected using the Polyethylenimine (PEI) method. Cells were grown in 10 cm dishes until subconfluency. The medium was refreshed with 9 ml DMEM, supplemented with 2% fetal calf serum before transfection. A mixture of 475 μl serum-free medium and 25 μl (1 μg/μl) PEI was added to a solution of 500 μl serum-free medium containing 10 μg DNA. Upon mixing thoroughly and incubation for 10 min at room temperature, the DNA/PEI mixture was added dropwise to the cells. 6 h later, the medium was refreshed with DMEM, supplemented with 10% fetal calf serum. For the immunofluorescence microscopy experiments, cells were grown on coverslips in 6-well plates and transfected using the calcium phosphate method. The medium on cells was refreshed with 1.8 ml DMEM, supplemented with 10% fetal calf serum, 1 h before transfection. A 100 μl mixture containing 78 μl TE (1 mM Tris–HCl pH 7.2, 0.1 mM EDTA pH 8), 20 μl $CaCl_2$/HEPES (1.25 M $CaCl_2$, 0.125 M HEPES; final pH 7.05) and 2 μg plasmid DNA, was added dropwise to 100 μl of BS/HEPES (0.275 M NaCl, 0.01 M KCl, 1.4 mM $Na_2HPO_4 \cdot 12H_2O$, 11 mM dextrose, 25 mM HEPES; final pH 7.05). Upon pipeting gently up and down, the total mix was added dropwise to the cells. The medium containing the transfection mix was refreshed with DMEM, supplemented with 10% fetal calf serum, after 6 h.

2.3. Primary hippocampal culture and transfection

Primary hippocampal cultures were prepared from embryonic day 19 (E19) rat brains and cultured according to procedures described previously [18,19]. Briefly, cells were grown on coverslips coated with poly-L-lysine (30 μg/ml) and laminin (2 μg/ml). Hippocampal neurons were grown in Neurobasal medium supplemented with B27, 0.5 mM glutamine, 12.5 mM glutamate, and penicillin/streptomycin mixture. At 14 day in vitro (DIV) hippocampal neurons were transfected with 0.9 μg FLAG D4.4 receptor or FLAG D2 receptor (a kind gift from Hubert Van Tol) with Lipofectamine 2000 according to manufacturer's protocol, and cultured for the next 6 days.

2.4. Pharmacological studies

D4 receptor binding using [^{3}H]spiperone was performed as described previously [16, 17, 20]. Drug treatments were done by addition of quinpirole and butaclamol(+) to the medium at a final concentration of 10 μM for 16 h.

2.5. Co-immunoprecipitation assay and Western blot analysis

As described before [21], cells were lysed in 400 μl RIPA buffer without EDTA or EGTA (150 mM NaCl; 5 mg/ml sodium deoxycholate; 50 mM Tris–HCl pH 7.5; 1% Nonidet P-40; 0.1% SDS) supplemented with freshly added protease inhibitors (2.5 μg/ml aprotinin; 1 mM PEFA-block; 10 μg/ml leupeptin; 10 mM β-glycerolphospate; 10 mM NaF).