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Cytoplasmic tail of D1 dopaminergic receptor differentially regulates desensitization and phosphorylation by G protein-coupled receptor kinase 2 and 3

Keyvan Sedaghat, Mario Tiberi *

Ottawa Hospital Research Institute, Neuroscience Program and Departments of Medicine/Cellular and Molecular Medicine/Psychiatry, University of Ottawa, Ottawa, ON, Canada

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ABSTRACT

Herein, we investigate the differential D1 dopaminergic receptor (D1R) regulation by G protein-coupled receptor kinase (GRK) 2 and 3 using two truncated receptors lacking the distal (Δ425) and distal–central (Δ379) cytoplasmic tail (CT) regions. We first show the association between D1R and GRKs in co-transfected cells and rat striatum. Our studies further indicate that deletion of distal CT region of D1R does not alter the association between receptor and GRK2. Meanwhile, removal of both distal and central CT regions culminates in a drastic increase in the basal association between Δ379 and GRK2 relative to D1R and Δ425. Interestingly, CT truncations have no effect on the basal and DA-induced association of receptors with GRK3. Furthermore, we demonstrate that desensitization of D1R is considerably more robust in cells expressing GRK3. Notably, the robust GRK3-induced D1R desensitization is not attenuated by CT deletions. However, GRK2-induced Δ425 desensitization is not detectable whereas we unexpectedly find that Δ379 desensitization is similar to GRK2-induced D1R desensitization. GRK2 and GRK3-dependent desensitization of wild type D1R is not linked to differences in the extent of DA-induced receptor phosphorylation. Moreover, our studies show that GRK2-induced D1R phosphorylation is only modulated by deletion of distal CT region while distal and central CT regions control GRK3-induced D1R phosphorylation. Intriguingly, dopamine-induced Δ379 phosphorylation by GRK3 was significantly lower than receptor phosphorylation in cells harboring Δ379 alone or Δ379 and GRK2. Overall, our study suggests an intricate interplay between CT regions of D1R in differentially regulating receptor responsiveness by GRK2 and GRK3.

1. Introduction

Physiological roles of dopamine (DA) in the central nervous system (CNS) and periphery are mediated through activation of six distinct cell-surface proteins. These structurally related seven-transmembrane (7TM) proteins belong to the large family of G protein-coupled receptors (GPCRs). DA receptors are grouped into D1-like (D1R and D5R) and D2-like ($D2R_{short}$ and $D2R_{long}$, D3R and D4R) subtypes. This classification is based on their primary amino acid sequence identity, pharmacological properties and ability to activate or inhibit adenylyl cyclase (AC) activity, respectively [1]. Dysfunction of dopaminergic systems and receptors has been implicated in the etiology and phenotypic expression of numerous diseases [1,2]. Notably, studies suggest that D1R represents an important therapeutic target [3,4].

Studies of the rhodopsin and β2-adrenergic receptor (β2AR), two prototypical 7TM receptors, have suggested a model whereby phosphorylation of GPCRs reduces their ability to respond to stimuli [5]. In this canonical model, the regulation of 7TM receptor responsiveness by Ser and Thr phosphorylation is mostly linked to the actions of G protein-coupled receptor kinases (GRK1–7) and second messenger-dependent kinases [5,6]. GRKs have been shown to play an important role in the stimulus-induced desensitization and internalization of 7TM receptors, two processes regulating the degree and duration of receptor responses [5,6]. Thus, GRK-induced receptor phosphorylation plays a pivotal role in the signal transduction efficacy of agonist-bound GPCRs [7]. Given the large number of genes coding for GPCRs and low degree of primary sequence identity within their intracellular domains harboring putative phosphorylation sites, it remains unclear whether the mechanisms implicated in GRK-mediated phosphorylation of rhodopsin and β2AR can be generalized to all 7TM receptors [8].

The molecular complexity underlying agonist-driven phosphorylation and desensitization of GPCRs is also highlighted by studies

Abbreviations: 7TM, seven-transmembrane; AC, adenylyl cyclase; Bmax, maximal binding capacity; BSA, bovine serum albumin; β2-AR, β2-adrenergic receptor; CHO, Chinese Hamster Ovary; CT, cytoplasmic tail; D1R, dopamine D1 receptor; D2R, dopamine D2 receptor; DA, dopamine; DMEM, Dulbecco's modified Eagle's medium; DSP, dithiobis [succinimidylpropionate]; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HA, hemaglutinin; HEK293, human embryonic kidney 293; IB, immunoblotting; IP, immunoprecipitation; MEM, minimum essential medium; PBS, phosphate-buffered saline; RIPA, radioimmunoprepitation assay; SDS, sodium dodecyl sulfate; WT, wild type.

* Corresponding author. University of Ottawa, Neurosciences, 451 Smyth Road, Roger-Guindon Hall, Ottawa, ON, K1H 8M5, Canada. Tel.: +1 613 562 5800x4848; fax: +1 613 562 5403.
E-mail address: mtiberi@uottawa.ca (M. Tiberi).

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investigating D1R phosphorylation and desensitization [9–13]. Indeed, D1R can serve as a substrate for GRK2, GRK3 and GRK5 [10]. Interestingly, overexpression of Gβγ subunit-dependent GRK2 and GRK3 (two closely related isoforms) promotes a differential quenching of receptor responsiveness (GRK3>GRK2) in cells exhibiting similar levels of agonist-induced D1R phosphorylation [10]. The formation of a signaling complex between D1R and GRK2/3 in vivo has yet to be tested. Meanwhile, studies using GRK2 and GRK3 knockout mice suggest that these two kinases may differentially regulate D1R function in vivo [14]. It is unclear whether regulation of D1R by GRKs reported in heterologous cells and in vivo requires a direct interaction and signaling complex formation between these proteins. Likewise, the molecular basis underlying GRK isoform-specific regulation of D1R responsiveness remains to be explored in a more mechanistic fashion. This is of physiological importance as neuronal GRK expression patterns correlate with dopaminergic pathways containing D1Rs [15,16]. Most importantly, changes in the expression levels and subcellular distribution of GRK isoforms along with aberrant D1R phosphorylation and responsiveness have been implicated in the etiology of numerous pathologies [16–20]. Interestingly, a recent study has shown that striatal-targeted GRK overexpression alleviates L-DOPA-induced dyskinesia in animal models of Parkinson's disease [21]. Overall, these studies highlight the clinical relevance of targeting GRK-mediated D1R phosphorylation and desensitization in diseases linked to impaired D1R activity.

Previously, we demonstrated that removal of Ser and Thr residues located downstream of the putative α-helical domain or H8 region of the cytoplasmic tail (CT) of rat D1R completely abolished basal and DA-induced phosphorylation of D1R [13]. In fact, this truncated D1R displayed DA-mediated desensitization albeit at a reduced level relative to wild type counterpart suggesting that D1R desensitization is regulated by receptor phosphorylation-independent and dependent mechanisms [13]. Meanwhile, the mechanisms underlying GRK isoform-specific D1R desensitization mediated through receptor phosphorylation likely involve distinct determinants of CT of D1R, but this issue remains to be investigated in more detail. Indeed, mutations of Thr446, Thr439 and Ser431 located in the distal region of CT of D1R had no effect on agonist-mediated receptor phosphorylation and desensitization while inhibiting DA-induced D1R internalization [11]. In striking contrast, we have shown that deletion of CT region harboring these residues mediated a loss of DA-induced phosphorylation and desensitization [13]. Moreover, studies from our lab and others suggest that the removal of these Ser and Thr residues did not prohibit DA-induced D1R internalization [13,22]. Different cells used in previous studies potentially explained these discrepancies. Thus, repertoire of GRK isoforms and their relative expression levels are likely imparting GRK isoform-specific desensitization and phosphorylation patterns to GPCRs.

In the present study, we show that D1R forms a signaling complex with GRK2 and GRK3 in human embryonic kidney 293 (HEK293) cells and rat striatum. We also demonstrate that truncation of the distal (Tyr426-Thr446) and central (Ser380-Asp425) regions of CT of D1R distinctly modulate GRK2 but not GRK3-mediated desensitization using a co-transfection approach in HEK293 cells. Additionally, results indicate that truncation of these two CT regions does not inhibit DA-induced D1R interaction with GRK2 and GRK3 but relieves the molecular constraints restricting GRK2 interaction with D1R under basal state. We also investigated the effect of truncations on D1R phosphorylation mediated by GRK2 and GRK3 and identified distinct molecular determinants controlling GRK isoform-specific regulation of basal and DA-induced phosphorylation. Notably, our data imply a disconnect between the extent in GRK-mediated phosphorylation and desensitization of GPCRs. Collectively, our data strongly suggest that distinct GRK2 and GRK3 phosphorylation and desensitization mechanisms operate at the level of D1R signaling complex.

2. Material and methods

2.1. Materials

SlowFade Gold antifade (Molecular Probes®) and cell culture reagents were obtained from Invitrogen (Burlington, Ontario, Canada). Radioligands, protein A-sepharose beads, horseradish peroxidase (HRP)-conjugated goat antibodies, HRP-conjugated streptavidin and enhanced chemiluminescent (ECL) reagents were purchased from GE Healthcare (Baie d'Urfé, Québec, Canada). The rat monoclonal anti-D1R antibody (D-187), drugs and general chemicals were from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada) unless stated otherwise. Dithiobis [succinimidylpropionate] (DSP) was obtained from MJS Biolynx Inc. (Brockville, Ontario, Canada). Sodium dodecyl sulfate (SDS) and Dowex AG 50W-4X resin were purchased from Bio-Rad Laboratories Inc. (Mississauga, ON, Canada). Alumina N Super I was from MP Biomedicals Canada (Montréal, Québec, Canada). The rat monoclonal anti-hemaglutinin epitope (HA) affinity matrix was purchased from Roche Diagnostics (Laval, Québec, Canada). Biotinylated anti-HA antibody was from Covance Research Products (Richmond, CA, USA). Alexa 488-conjugated goat anti-mouse and Texas red-conjugated goat anti-rabbit antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). The rabbit polyclonal anti-GRK2/GRK3 antibody [15] was a generous gift from Dr. Robert Lefkowitz (Duke University, NC, USA). Protein A/G-agarose beads, preimmune rabbit IgG, mouse monoclonal anti-GRK2 (SC-13143; C-9) and rabbit polyclonal anti-GRK3 (SC-563; C-14) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.2. Expression constructs

HA-tagged wild type (WT) and truncated forms (Δ425 and Δ379, CT deleted at Asp425 and Gly379, respectively) of rat D1R (Fig. 1A) along with the untagged rat GRK2 and GRK3 were subcloned into the mammalian expression vector pCMV5 as previously described [10,15,23].

2.3. Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells transformed with the human adenovirus type 5 (Ad5) (CRL-1573; American Type Culture Collection, Manassas, VA, USA) were cultured at 37 °C and 5% CO_2 in minimal essential medium (MEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and gentamicin (10 μg/ml) referred to as complete MEM. Cells were seeded into 100-mm dishes (2.5×10^6 cells/dish) and transiently transfected with a total of 5 μg of DNA/dish using a modified calcium phosphate precipitation method as described previously [24]. When less than 5 μg of plasmid DNA constructs for HArD1R (WT, Δ425 and Δ379), rGRK2 and rGRK3 was used in transfections, empty pCMV5 vector was added to adjust the total amount of DNA to 5 μg.

2.4. Whole cell phosphorylation assay

Following an overnight transfection, culture medium was replaced with fresh complete MEM until the next day. Cells were then reseeded in six-well dishes ($1–1.5\times10^6$ cells/well; for phosphorylation) and 100-mm dishes (for receptor binding assays and GRK2/3 immunoblotting). The next day, cells were labeled in 20 mM HEPES-buffered phosphate free Dulbecco's modified Eagle's medium (DMEM) (pH 7.4) containing gentamicin (10 μg/ml) and 0.15 mCi/ml [^{32}P]-orthophosphate for 90 min at 37 °C. At the end of the labeling period, cells were incubated in the presence of 0.1 mM ascorbic acid alone or with 10 μM DA for 10 min at 37 °C. Cells were then placed on ice and washed three times with ice-cold phosphate-buffered saline (PBS) and solubilized for 1 h at 4 °C on a rotating wheel in 800 μl of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1%