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PDZK1/NHERF3 Differentially Regulates Corticotropin-releasing Factor Receptor 1 and Serotonin 2A Receptor Signaling and Endocytosis

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ABSTRACT

The corticotropin-releasing factor receptor 1 (CRFR1) and serotonin 2A receptor (5-HT_{2A}R) are linked to cellular mechanisms underlying stress anxiety and depression. Both receptors are members of the G protein-coupled receptor (GPCR) superfamily and encode class I PSD-95/DiscsLarge/Zona Occludens 1 (PDZ) binding motifs (-S/T-x-V/I/L) at the end of their carboxyl-terminal tails. We have identified PDZK1, also referred to as Na(+)/H(+) exchange regulatory cofactor 3 (NHERF3) as both a CRFR1- and 5-HT_{2A}R-interacting protein. We have examined whether PDZK1 plays a role in regulating both CRFR1 and 5-HT_{2A}R activity. We find that while PDZK1 interactions with CRFR1 are PDZ binding motif-dependent, PDZK1 associates with 5-HT_{2A}R in a PDZ binding motif-independent manner and CRFR1 expression, but not 5-HT_{2A}R expression, redistributes PDZK1 to the plasma membrane in PDZ binding motif-dependent manner. PDZK1, negatively regulates 5-HT_{2A}R endocytosis and has no effect upon 5-HT_{2A}R-mediated ERK1/2 phosphorylation. In contrast, PDZK1 overexpression does not affect CRFR1 endocytosis, but selectively increases CRFR1-stimulated ERK1/2 phosphorylation. Similar to what has been previously reported for PSD-95 and SAP97, PDZK1 positively influences 5-HT_{2A}R-stimulated inositol phosphate formation, but does not contribute to the regulation of CRFR1-mediated cAMP signaling. Taken together, these results indicate that PDZK1 differentially regulates the signaling and trafficking of CRFR1 and 5-HT_{2A}R via PDZ-dependent and -independent mechanisms, respectively.

1. Introduction

Corticotropin-releasing factor (CRF) is both a peptide hormone and neurotransmitter, and serotonin (5-HT) is a monoamine neurotransmitter. CRF and 5-HT have both been implicated in the etiology of neuropsychiatric disorders due to their critical role in the regulation of diverse physiologic and behavioral functions that are associated with stress, anxiety and depression [1–3]. CRF regulates the physiological response to stress and its involvement in the regulation of stress, anxiety and depression provides a functional link between endocrine signaling and neurobehavioral responses [1,4]. 5-HT is involved in the regulation of feeding behaviors, sleep, cognitive function and mood [2]. Most importantly, the serotonergic system is regulated by the hypothalamic-pituitary-adrenal (HPA) axis and serotonergic dysfunction is well-established in mood disorders. Because altered endocrine regulation of the HPA axis activity and lower 5-HT function [5,6] are considered the most convincing biological correlates of mood disorders, alterations in both systems are commonly associated with mental health diseases [7].

CRF and 5-HT exert their biological actions by activating a distinct subset of G protein-coupled receptors (GPCRs). CRF binds to the CRF receptors 1 and 2 (CRFR1, 2) and 5-HT binds to the 5-HT receptors 1-7 (5-HT_{1-7}R), all of which are GPCRs, except for the 5-HT_3R, which is a ligand-gated ion channel [8–10]. 5-HT_{2A}R and the CRFR1 represent excellent molecular targets for the treatment of anxiety, eating, and obsessive-compulsive disorders, as well as depression and schizophrenia [11]. CRFR1 antagonists have been shown to have anxiolytic- and antidepressant-like effects [12,13]. Moreover, in depressed individuals an increased expression of 5-HT_{2A}R in the brain has been identified [11]. Antidepressants and atypical antipsychotics also down-regulate

Abbreviations: ANOVA, analysis of variance; β_1AR, β_1-adrenergic receptor; β_2AR, β_2-adrenergic receptor; CRF, corticotropin-releasing factor; CRFR1, CRF receptor 1; CRFR2, CRF receptor 2; ERK1/2, extracellular signal-regulated kinase; GKAP, guanylate kinase-associated protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HA, hemagglutinin; (hIPR), human prostacyclin receptor; HEK 293, human embryonic kidney 293; KLHL17, kelch-like family member 17; MAPK, mitogen-activated protein kinase; PDZK1/NHERF3, Na(+)/H(+) exchange regulatory cofactor; NMDAR, N-methyl-D-aspartate receptor; PDZ, PSD95/Disc Large/Zona Occludens; 5-HT_{2A}R, 5-HT_{2A} receptor; 5-HT_{2C}R, 5-HT_{2C} receptor; SAP97, synapse-associated protein 97; serotonin, 5-HT; SHANK, SH3 and multiple ankyrin repeat domains protein; SSTR, somatostatin receptor; SYNGAP1, Synaptic Ras GTPase-activating protein 1.

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cortical 5-HT_{2A}R expression in the brain, linking the 5-HT_{2A}R to the symptoms of depression [11,14]. Thus, the CRFR1 and the 5-HT_{2A}R and their associated interacting proteins constitute enormous potential for the development of pharmacological targets to treat mood disorders.

Once a GPCR is activated by its agonist, the physiological result is the engagement of downstream subcellular signaling cascades as well as intracellular trafficking pathways that function to regulate the availability of the receptor for subsequent agonist activation [15–19]. The CRFR1 couples to and activates several different G proteins. However, CRFR1 preferentially couples to $G\alpha_s$ which activates adenylyl cyclase resulting in the production of cAMP and the subsequent stimulation of protein kinase A [20–24]. In contrast, the 5-HT_{2A}R couples to $G\alpha_{q/11}$, leading to the activation of phospholipase C, formation of inositol 1,4,5 trisphosphate and diacylglycerol, the release of intracellular Ca^{2+} stores and the subsequent activation of protein kinase C. In addition, several other signaling pathways are activated by CRFR1 and 5-HT_{2A}R including the extracellular-regulated protein kinase 1/2 (ERK1/2) pathway that can be activated by both G protein-dependent and-independent mechanisms [25].

Many GPCRs exert cell specific functions that involve, in part, their interaction with PSD-95/Disc Large/Zona Occludens-1 (PDZ) domain-containing proteins, that have been identified to be involved in a variety of regulatory aspects of GPCR function such as signaling, trafficking, receptor distribution and stability [19,26]. PDZ proteins are cytoplasmic adapter proteins that interact with GPCRs through specific consensus sequences most typically located at the distal carboxyl-termini of the receptor [19]. Such consensus sequences are called PDZ binding motifs. Both the CRFR1 and 5-HT_{2A}R encode class I PDZ binding motifs (-S/T-x-V/I/L) at their C-termini, which enables their interactions with PDZ proteins [27,28]. A large number of GPCR/PDZ protein interactions have been identified over the last decade, and this number is growing [19,29]. Previous work from our laboratory comprise a number of examples, including the negative regulation of CRFR1 endocytosis by SAP97 and the requirement of SAP97 for CRFR1-mediated ERK1/2 phosphorylation [27]. Similarly, SAP97 was reported to be involved in the regulation of 5-HT_{2A}R endocytosis and ERK1/2 activation [28]. Interestingly, the activation of CRFR1 enhances subsequent 5-HT_{2A}R-mediated inositol phosphate signaling, via a PDZ-motif-dependent mechanism [30]. CRFR1-mediated heterologous sensitization of 5-HT_{2A}R signaling represents a novel functional crosstalk between GPCRs that is mediated by PDZ protein interactions that requires receptor endocytosis and recycling.

PDZK1, alternatively called Na(+)/H(+) exchange regulatory cofactor 3 (NHERF3) is one of the PDZ proteins, we identified in our previous proteomic screen for PDZ proteins that interact with both the CRFR1 and 5-HT_{2A}R C-tails [27]. PDZK1/NHERF3 is predominantly expressed in epithelial and endothelial cells, in the liver, kidney and small intestine, but it is also localized at neuronal synapses [31–34]. PDZK1/NHERF3 interacts with several ion transporters, receptors and GPCRs including the human prostacyclin receptor and somatostatin receptors [35,36]. In the present study we have identified PDZK1 as a protein that interacts with both CRFR1 and 5-HT_{2A}R. We find that PDZK1 mediates CRFR1 and 5-HT_{2A}R endocytosis, G protein signaling and ERK1/2 activation. Thus, PDZK1 interactions with CRFR1 and 5-HT_{2A}R clearly differentiate the regulation of these two functionally important GPCRs. Thus, PDZK1 differentially regulates the signaling and trafficking of CRFR1 and 5-HT_{2A}R in a manner that is not consistent with the heterologous sensitization of 5-HT_{2A}R signaling.

2. Materials and Methods

2.1. Materials

Eagle's minimal essential medium (MEM), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, trypsin/0.25% EDTA, Lipofectamine 2000, Zenon Alexa Fluor 647 mouse IgG1 and Zenon Alexa Fluor 647 rabbit IgG1 labeling kits as well as Alexa Fluor 647 donkey anti-mouse IgG, Alexa Fluor 633 goat anti-rabbit IgG and anti-GFP rabbit serum antibodies were purchased from Invitrogen (Burlington, ON, Canada). Rabbit anti-Actin antibody and goat anti-rabbit IgG (H + L) HRP conjugate were purchased from Santa Cruz (Dallas, TX). Rabbit anti-p44/42 MAPK and rabbit anti-phospho-p44/42 MAPK antibodies were obtained from Cell Signaling (Boston, MA). QuantiFast SYBR Green RT-PCR and RNeasy Mini Kits were purchased from Qiagen (Toronto, ON, Canada).QuikChange™ site-directed mutagenesis was obtained from Stratagene (LaJolla, CA). Clarity Western ECL Substrate was from Bio-Rad (Mississauga, ON, Canada). Rat anti-HA peroxidase high affinity antibody was from Roche (Mississauga, ON, Canada). CRF was purchased from R&D Systems (Minneapolis, MN). Protein G Sepharose was obtained from GE Healthcare (Mississauga, ON, Canada). *myo*-[^{3}H]-Inositol was obtained from Perkin Elmer (Akron, OH). NheI and KpnI restriction endonucleases were from Thermo Scientific (Waltham, MA). AEBSF, leupeptin and aprotinin were obtained from BioShop Canada Inc. (Burlington, ON, Canada). PDZK1 siRNA (PDZK1 GenBank accession no. AF012281) and control siRNA were obtained from Ambion (Burlington, ON, Canada). Rabbit anti-FLAG antibody, mouse anti-HA antibody, serotonin (5-HT), ANTI-FLAG M2 affinity gel and all other biochemical reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.2. Plasmid Constructs

FLAG-tagged 5-HT_{2A}R, 5-HT_{2A}R-ΔSCV and HA-tagged CRFR1,CRFR1-ΔTAV constructs were described previously [24,30]. 3xHA-tagged CRFR2 was kindly provided by Aditi Bhargava (Center for Neurobiology of Digestive Diseases, University of California San Francisco). 3xHA-CRFR2 + TAV was generated using QuikChange™ site-directed mutagenesis (Stratagene, LaJolla, CA), thereby deleting an alanine at position 410 (^{408}TAAV^{411}) to introduce a C-terminal TAV motif. The exchange protein activated by cAMP (EPAC) biosensor was the gift of Drs. Ali Salahpour (University of Toronto) and Marc Caron (Duke University) [37]. Mouse myc-PDZK1 was generously provided by Dr. Randy Hall (Emory University School of Medicine, Atlanta). The mouse PDZK1 cDNA was cloned into the eukaryotic expression vector pEYFP-N1 and C-terminally fused to the enhanced yellow fluorescent protein (eYFP).Mouse PDZK1 was amplified by PCR using a forward primer that adds a NheI restriction site upstream of the PDZK1 start codon and a reverse primer that introduced the restriction site KpnI downstream of the PDZK1 coding sequence thereby deleting the stop codon. The PDZK1 coding sequence, restriction digested with NheI/KpnI, was then ligated in-frame into peYFP-N1 restriction enzyme digested with NheI/KpnI. The constructs were verified by DNA sequencing.

2.3. Cell Culture and Transfection

Human embryonic kidney (HEK 293) cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Cells were seeded on 10 cm dishes at 70-80% confluency 24 h prior to transfection. Transfections were performed using a modified calcium phosphate method, as described previously [38]. Cells were transfected with 1 μg for the CRFR1/2 and PDZK1 constructs, 2 μg for the 5-HT_{2A}R constructs and 3 μg for EPAC, respectively. Empty pcDNA3.1 vector was used to equalize the total amount of plasmid cDNA used for each transfection. 18 h post-transfection, cells were washed with phosphate buffered saline (PBS) and suspended with trypsin, 0.25% EDTA. All experiments were conducted 48 hours after transfection, with the exception of inositol phosphate (IP) formation assays which were performed 72 hours after transfection.