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Selective interactions of spinophilin with the C-terminal domains of the δ- and μ-opioid receptors and G proteins differentially modulate opioid receptor signaling

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ABSTRACT

Previous studies have shown that the intracellular domains of opioid receptors serve as platforms for the formation of a multi-component signaling complex consisting of various interacting partners (Leontiadis et al., 2009, Cell Signal. 21, 1218–1228; Georganta et al., 2010, Neuropharmacology, 59(3), 139–148). In the present study we demonstrate that spinophilin a dendritic-spine enriched scaffold protein associates with δ- and μ-opioid receptors (δ-OR, μ-OR) constitutively in HEK293 an interaction that is altered upon agonist administration and enhanced upon forskolin treatment for both μ-OR and δ-OR. Spinophilin association with the opioid receptors is mediated via the third intracellular loop and a conserved region of the C-terminal tails. The portion of spinophilin responsible for interaction with the δ-OR and μ-OR is narrowed to a region encompassing amino acids 151–444. Spinophilin, RGS4, Gα and Gβγ subunits of G proteins form a multi-protein complex using specific regions of spinophilin and a conserved amino acid stretch of the C-terminal tails of both δ-μ-ORs. Expression of spinophilin in HEK293 cells potentiated DPDPE-mediated adenylyl-cyclase inhibition of δ-OR leaving unaffected the levels of cAMP accumulation mediated by the μ-OR. Moreover, measurements of extracellular signal regulated kinase (ERK1,2) phosphorylation indicated that the presence of spinophilin attenuated agonist-driven ERK1,2 phosphorylation mediated upon activation of the δ-OR but not the μ-OR. Collectively, these findings suggest that spinophilin associates with both δ- and μ-OR and G protein subunits in HEK293 cells participating in a multimeric signaling complex that displays a differential regulatory role in opioid receptor signaling.

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1. Introduction

Opioid receptors can physically interact with a variety of accessory proteins, confirming that signal transduction of these receptors is not restricted to heterotrimeric G protein activation. Such interactions can alter the effectiveness of opioid agonist-driven cell signaling, trafficking, targeting, fine tuning and cellular localization of these receptors by providing a scaffold that links the receptors to the cytoskeletal network [1]. Previous observations employing pull down assays utilizing the C-terminal domain of the μ- and δ-opioid receptor (μ-OR, δ-OR) expressed as a glutathione-S-transferase (GST) fusion peptides, demonstrated the ability of RGS4 to interact directly with these receptors to modulate [D-Ala^2,N-MePhe^4,Gly^5-ol]-enkephalin (DAMGO)-mediated adenylyl cyclase inhibition and accelerate [D-Ser^2, Leu^5, Thr^6]-enkephalin (DSLET)-mediated internalization of the δ-OR in HEK293 cells [2,3]. These interactions were part of a signaling complex consisting of the μ or δ-opioid receptors, Gα, Gβγ and RGS4. Moreover it was shown that the δ-CT serves as a platform for the formation of a multi-component signaling complex (signalosome), consisting of STAT5B, c-Src and selective G protein members [4]. Consequently, we hypothesized that additional proteins by interacting directly with opioid receptors might promote or inhibit receptor signaling and thus shed light to our understanding regarding the intracellular events that are affected by agonists that target opioid receptors.

Spinophilin (also known as Neurabin II) is a ubiquitous multidomain-scaffold protein that modulates synaptic transmission by interacting with the filamentous actin cytoskeleton that supports the dendritic spines [5–7]. Spinophilin has multiple domains including an actin-binding domain, a protein phosphatase 1 (PP1)-binding and a regulatory domain, a PSD-95/Discs large/ZO-1 homology (PDZ) domain and the

Abbreviations: GPCR, G protein-coupled receptors; RGS, Regulators of G protein Signaling; ΔNRGS4, RGS4 lacking its N-terminal domain; 6xHis, hexahistidine; aa, amino acids; GEFs, Guanine nucleotide exchange factors; AC, adenylyl cyclase; DAMGO, [D-Ala^2,N-MePhe^4,Gly^5-ol]-enkephalin; δ-CT, δ-opioid receptor carboxyl-terminal tail; δ-OR, δ-opioid receptor; μ-OR, μ-opioid receptor; μ-CT, μ-opioid receptor carboxyl-terminal tail; δi3-L, GST fusion of the third intracellular loop of the δ-opioid receptor; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; DSLET, [D-Ser^2]-Leucine enkephalin-Thr; IPTG, isopropyl β-D-1-thiogalactopyranoside; IBMX, 1-Methyl-3-isobutylxanthine; ERK, extracellular signal-regulated protein kinase; GST, glutathione-S-transferase; HA, hemaglutinin; MAPK, mitogen activated protein kinase; NMS, normal mouse serum; NRS, normal rabbit serum; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PVDF membrane, polyvinylidene difluoride membrane; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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coiled-coil domain [5,8,9]. Previous studies have demonstrated that the sequence region encompassing the amino acids 151–444 (RBD domain) of spinophilin interacts with GPCRs specifically *via* their third intracellular loops as demonstrated for the Gi/Go-coupled D2 dopamine receptor and all three α2AR subtypes [10,11]. More recently, interaction of spinophilin with the α1AR, CCKA, CCKB and muscarinic M3 receptors have also been reported [6]. Apart from its interaction with the GPCRs spinophilin has previously been demonstrated to interact or to be co-localized with a number of other regulatory proteins, such as enzymes, RGS proteins, arrestins, ion channels, cell adhesion molecules, to modulate cell signaling [5,12,13]. The nature and the role of these scaffolding protein complexes mediated by spinophilin are still unclear. Observations in knockout mice have shown that spinophilin is involved in the acute and chronic actions of opiates, whereas it has been reported that at the cellular level spinophilin affects μ-OR functional responses by promoting endocytosis and by inhibiting μ-OR signaling in mice striatal extracts [14].

Based on these observations in the present study we investigate the nature of spinophilin interaction with the δ-OR and μ-OR in living cells. Moreover, using GST fusion peptides encompassing specific regions of the δ-OR and μ-OR we demonstrate that spinophilin interacts directly within the third intracellular loop and the proximal conserved region of the C-termini of these receptors forming the predicted helix VIII. The structural determinant of spinophilin association with these receptors relies within the spinophilin's receptor binding region. We further explored whether the multiple protein-interacting domains of spinophilin allow the formation of a multi-protein complex consisting of RSG4, Gα and Gβγ subunits of G proteins, known to interact within the same conserved juxtamembrane region of the C-termini of the δ- and μ-ORs. Finally we demonstrate that spinophilin modulates δ-OR signaling in a different manner than that of μ-OR in HEK293 cells, suggesting that spinophilin displays a differential role in the signaling of these opioid receptors.

2. Material and methods

2.1. Constructs and reagents

The c-DNAs of the rat Myc-spinophilin and the wild type rat 6xHis-spinophilin were kindly provided by Drs R. Colbran Nashville, TN, USA and P. B. Allen, New Haven, CT, USA, respectively. GtαGDP and Gβγ purified from bovine retina were a gift of Dr H. Hamm, Vanderbilt University, Nashville, TN, USA and Hemagglutinin (HA)-tagged human RGS4 (HA-RGS4) was kindly provided by Dr G. Milligan University of Glasgow, Glasgow, Scotland. [^{3}H]-adenine (23 Ci/mmol) was from Amersham Pharmacia (Vienna, Austria). Opioid ligands DSLET and DPDPE were obtained from Tocris (Cookson MI, USA). Protease inhibitors, monoclonal anti-flag antibody and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine 2000 and anti-mouse secondary antibody were purchased from Invitrogen (Carlsbad, CA, USA). TurboFect *in vitro* transfection reagent was supplied by Fermentas Life Sciences. Anti-Goα (OCI) and anti-Giα$_2$ (SG3) antibodies were kindly provided by Dr G. Milligan University of Glasgow, Glasgow, Scotland. Monoclonal anti-His antibody was obtained from BD Pharmingen. Protein A and A/G plus sepharose beads, polyclonal Neurabin II and monoclonal anti-GST, anti-pERK1,2 and anti-Giα$_{1,3}$ were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal anti-ERK1,2 and monoclonal anti-myc antibodies were from Cell Signaling Technology Inc. (Danvers, MA, USA) and anti-EE was from QED Bioscience Inc. (San Diego, CA, USA).

2.2. Preparation of the GST fusion constructs

GST fusion peptides encompassing the third intracellular loop of the δ-OR and the C termini of the μ- and δ-OR were generated from cDNA clones of the rat μ-OR and mouse δ-OR as described by [2], whereas the truncated C-termini of the μ- and δ-ORs were carried out as described by [3]. For the production of the spinophilin GST fusion peptide encompassing the receptor-interacting domain (RBD), located between amino acids 151 and 446, the following primers were designed for PCR: RBD (151–446), (forward) 5′-AAATTGGA TCCTTATGTTCGAACGGAGCGTCCCC-3′ and (reverse) 5′-AAAAAGCG GCCGCTACTCGGGGCTGGGTCCTCTTC-3′. For the generation of the PP1-binding and PDZ domains (PP1/PDZ) located between amino acids 447–586 the following primers were designed: PP1/PDZ, (forward) 5′-AAAAAGAATTCTCGGAAGATCCATTTCAGCACC-3′ and (reverse) 5′-AAAATCTCGAGTAAGGCCGCTCCCGGCCAAT-3′. Finally, the primers for the GST spinophilin encompassing the coiled-coil domain (664–817) were: (forward) 5′-AAAATGGATCCTACCTGAAAAGCTGGTGCACAAG-3′ and (reverse) 5′-AAAAACTCGAGCCAGTAGAATTGGAATTCCTCAGTGT-3′. The PCR products were engineered into the pGEX-5x3vector (GE Healthcare). All positive constructs were verified by nucleotide sequence analysis.

2.3. Protein expression

DNA from positive clones was transformed in *E. coli* (BL21) cells for protein expression (Invitrogen). 10 ml of LB medium with ampicillin (100 μg/ml) was cultured overnight from a single colony with subsequent inoculation in 500 ml LB with the same ampicillin concentration. Induction of the GST fusion proteins was done using 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at $OD_{600} = 0.6$ and cultures were shaken at 29 °C for 3 h. Cells were pelleted, lysed with ice cold PBS buffer pH 7.6 containing 1% Triton X-100, 20 μg/ml leupeptin, 20 μg/ml antipain, 0.2 mM PMSF and protease inhibitor cocktail, sonicated and incubated under rotation at 4 °C for 1 h. Soluble protein was obtained after centrifugation at 50,000 rpm for 45 min at 4 °C; glycerol was added and lysates were stored at −20 °C.

2.4. Purification of 6xHis-spinophilin

Preparation of recombinant histidine tagged (6xHis-spinophilin) was purified using a pRSET plasmid vector containing the cDNA of rat spinophilin in-frame with a six histidines tag sequence (kindly provided by Dr R. Colbran, University of Vanderbilt, Nashville, TN, USA) after expression in Escherichia coli (BL21-SI). Positive clones were selected in the presence of ampicillin (100 μg/ml) and the protein was expressed after induction with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 37 °C for 2.5 h. Purification of 6xHis-spinophilin was performed as described by Hsieh-Wilson et al., [9]. Briefly, cells were resuspended in ice cold 50 mM Tris–HCl, 300 mM NaCl, and 10 mM imidazole, pH 8.0, containing protease inhibitors and lysed by sonication. Lysozyme was added at a final concentration of 0.2 mg/ml. After centrifugation at 25,000 rpm for 45 min, soluble proteins were purified from the supernatant by affinity chromatography using Ni-NTA agarose. Protein was eluted using a gradient of 10–250 mM imidazole and fractions of high purity were selected for the assays.

2.5. GST pull down assays

Approximately 1 μM of the GST-fusion proteins in PBS containing protease inhibitor cocktail, 0.2 mM PMSF, 20 μg/ml leupeptin and 20 μg/ml antipain, were immobilized on glutathione sepharose 4B beads in PBS. The mixture was washed three times with PBS, and the immobilized fusion proteins were incubated with cell membranes, or purified recombinant 6xHis-spinophilin, 6xHisRGS4, or heterotrimeric Gαtβγ and Gβγ complexes for 15 min to 1 h at 4 °C. The GST-slurry with the bound proteins was subsequently washed extensively with PBS containing 0.1% Igepal and bound proteins were eluted with 30 μl of Laemmli loading buffer, boiled and resolved

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