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Research Report

Chronic morphine acts via a protein kinase Cγ–G_β–adenylyl cyclase complex to augment phosphorylation of G_β and $G_{\beta\gamma}$ stimulatory adenylyl cyclase signaling

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Abstract

Chronic morphine augments protein kinase C (PKC) phosphorylation of G_β, which enhances the potency of $G_{\beta\gamma}$ to stimulate adenylyl cyclase II (ACII) activity. The present study demonstrates an in vivo association between phosphorylated G_β and a specific PKC isoform, PKCγ. We investigated the association of G_β and PKCγ by assessing the ability of anti-PKCγ antibodies to co-immunoprecipitate G_β from ^{32}P-radiolabeled Chinese Hamster Ovary cells stably transfected with a μ-opioid receptor (MOR-CHO). PKCγ immunoprecipitate (IP) obtained from MOR-CHO membranes contained radiolabeled signals of ≈33 and 36–38 kDa that were subsequently identified as G_β(s). Chronic morphine significantly increased (≈75%) the magnitude of ^{32}P incorporated into G_β present in PKCγ IP. This suggests that G_β is an in vivo substrate for PKCγ, which mediates the chronic morphine-induced increment in G_β phosphorylation. In order to evaluate AC as a putative effector for phosphorylated $G_{\beta\gamma}$, its presence in IP obtained using anti-AC antibodies was evaluated. Autoradiographic analyses of AC IP also revealed the presence of phosphorylated G_β(s), the magnitude of which was significantly enhanced (≈60%) following chronic morphine treatment. This indicates that phosphorylated $G_{\beta\gamma}$ associates and presumably interacts in vivo with AC, indicating that it is a target for the enhanced phosphorylated $G_{\beta\gamma}$ that is generated following chronic morphine treatment. This would contribute to the previously observed shift from predominantly $G_{i\alpha}$ inhibitory to $G_{\beta\gamma}$ stimulatory AC signaling following chronic morphine. The PKCγ–G_β–AC complex identified in this study provides an organizational framework for understanding the well-documented participation of PKCγ in opioid tolerance-producing mechanisms.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Second messengers and phosphorylation

Keywords: Protein kinase C γ; Phosphorylation; MOR-CHO; Opioid; Tolerance; G protein

1. Introduction

The $G_{\beta\gamma}$ subunit that is generated in parallel with G_α following activation of heterotrimeric G proteins is now recognized as a major signaling entity that targets wide-ranging effectors [8,9,11,15,16,18,33]. In vivo, G_β can exist as a phosphorylated protein. Histidine-phosphorylated G_β has been demonstrated in rod outer segment membranes of bovine retina [38], cell membranes obtained from liver, brain, and placental tissue [26], and human leukemia (HL-60) cells [39]. More recently, threonine-phosphorylated G_β has been demonstrated in spinal tissue [3]. Importantly, the phosphorylation state of G_β exhibits plasticity. Specifically, in both the guinea pig longitudinal muscle myenteric plexus tissue as well as spinal cord, the magnitude of G_β phos-

Abbreviations: AC, adenylyl cyclase; IP, immunoprecipitate; MOR-CHO, Chinese Hamster Ovary cells stably transfected with μ-opioid receptor; PKCγ, protein kinase C γ; BC1, anti-G_β common polyclonal antibody; BBC-4, anti-adenylyl cyclase monoclonal antibody

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phorylation is significantly augmented following chronic systemic morphine treatment [3,6].

The phosphorylation state of G_β influences multiple determinants of its signaling activity. In vitro phosphorylation of G_β by protein kinase A (PKA) and/or protein kinase C (PKC) augments its $G_{s\alpha}$-dependent stimulation of ACII activity [3]. Additionally, G_β phosphorylation decreases its association with G protein receptor kinase 2/3 [6]. The ensuing increased availability of $G_{\beta\gamma}$ subunits to interact with effectors such as the type II family of AC isoforms would further contribute to increase $G_{\beta\gamma}$ stimulatory AC signaling. Participation of (histidine) phosphorylated G_β in an enzymatic transfer of a phosphate to GDP at G_α subunits [10,38] would also serve to enhance $G_{\beta\gamma}$ signaling by preventing G_α–$G_{\beta\gamma}$ re-association, thereby maintaining the pool of free $G_{\beta\gamma}$ subunits available for signaling. Collectively, these observations underscore the relevance of the phosphorylation state of G_β to modulating $G_{\beta\gamma}$ signaling activity.

In vitro phosphorylation of purified G_β by PKC is isoform-specific. PKCγ achieves a high stoichiometry of G_β phosphorylation comparable to that produced by PKA or PKC catalytic subunits [3]. In contrast, phosphorylation of G_β via PKCα, PKCζ, or PKCβ is negligible [3,41]. This suggests a critical role for PKCγ in regulating G_β phosphorylation in vivo.

Hypothesized in vivo interactions between PKCγ and G_β were investigated by assessing their co-immunoprecipitation with either anti-G_β or anti-PKCγ antibodies. This study reveals that G_β and PKCγ can be co-immunoprecipitated by either antibody. This indicates the formation of stable protein complexes suggesting their interaction in vivo. Moreover, chronic morphine augments the magnitude and phosphorylation state of the co-immunoprecipitated G_β. As expected, G_β co-immunoprecipitates with AC. Notably, chronic systemic morphine also enhances the phosphorylation state of the G_β that co-immunoprecipitates with AC. This suggests that AC is a putative effector for phosphorylated $G_{\beta\gamma}$. The relevance of these changes to the previously reported chronic morphine-induced emergence of opioid receptor-coupled $G_{\beta\gamma}$ stimulatory AC signaling is discussed.

2. Materials and methods

2.1. Cell culture

Chinese Hamster Ovary cells stably transfected with rat μ-opioid receptors (MOR-CHO) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) high glucose with L-glutamine (Mediatech, Herndon, VA) supplemented with 10% fetal calf serum (Nova-Tech Inc., Grand Island, NE), 100 units/ml penicillin/streptomycin and 100 μg/ml geneticin (Mediatech). Cells were plated (3.8×10^6 cells/150 mm dishes) and grown at 37 °C in a humidified atmosphere of 90% air/10% CO_2. Two days later, at 90–95% confluency, cells were treated with or without morphine (1 μM) for 48 h. Morphine was replenished in fresh media and media were changed for the untreated cells after every 24 h.

2.2. ^{32}Pi labeling of MOR-CHO cells and Immunoprecipitation

On the day of harvest, cells were incubated for 2 h in phosphate- and serum-free DMEM at 37 °C under normal culture conditions. Later, MOR-CHO cells were washed once with 10 ml phosphate- and serum-free media and incubated with 10 ml of the same media containing [^{32}P]orthophosphate (150 μCi/ml; PerkinElmer, Boston, MA) for additional 2 h at 37 °C under 90% air/10% CO_2. Subsequently, cells were washed thoroughly (twice, 15 ml each) with ice-cold phosphate buffered saline (pH 7.3) and harvested directly in 20 mM HEPES, pH 7.4, containing 10% sucrose, 5 mM EDTA, 1 mM EGTA, 2 mM Dithiothreitol [DTT], 10 mM Na-pyrophosphate, 10 mM NaF, 0.2 mM Na-orthovanadate; protease inhibitors 1 mM Benzamidine, 0.2 mg/ml bacitracin, 2 mg/l Aprotinin, 3.2 mg/l each of Trypsin Inhibitor from soybean and Leupeptin, 20 mg/l each of *N*-tosyl-L-phenylalanine chloromethyl ketone, N^α-*p*-tosyl-L-lysine chloromethyl ketone and phenylmethylsulfonyl fluoride, complete cocktail inhibitor tablet/50 ml and phosphatase inhibitors 0.5 μM Okadaic acid and 25 nM Calyculin A. Cells were homogenized and centrifuged at $1000 \times g$, 4 °C for 10 min. Supernatants obtained from the low-speed spin were subjected to a high-speed spin at $30{,}000 \times g$ for 40 min at 4 °C.

Membrane fractions obtained were re-suspended in HEPES buffer (pH 7.4) containing 1 mM each of EDTA, EGTA and DTT, 10 mM Na-pyrophosphate and the same protease and phosphatase inhibitors as mentioned above. Membranes were either stored at −80 °C in aliquots or processed further. For immunoprecipitation, membranes were solubilized in the same buffer containing 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% Na-deoxycholate, 0.1% Na-dodecyl sulfate and 10% glycerol, agitated 60 min at 4 °C and centrifuged ($14{,}000 \times g$ for 20 min at 4 °C). Clear supernatants were used for Protein Assay [2] and immunoprecipitation.

PKCγ was immunoprecipitated using a mouse monoclonal antibody (6 μl/600 μg protein, Sigma Chemical Co.) generated against rat PKCγ amino acid residues 684–697. G_β was immunoprecipitated from cell membranes using a mouse monoclonal antibody generated against amino acid residues 130–145 (3 μl/300 μg protein; BD Biosciences). G_β from spinal cord was immunoprecipitated using a polyclonal anti-G_β common antibody (BC1) raised against the carboxyl terminal 11 amino acids of G_β [20]. This antibody was generously supplied by Dr. J. Hildebrandt (Medical University of South Carolina, Charleston, SC). AC was immunoprecipitated using BBC-4 monoclonal antibodies generated against AC purified from bovine