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Activation of β- and α2-adrenergic receptors stimulate tubulin polymerization and promote the association of Gβγ with microtubules in cultured NIH3T3 cells

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ABSTRACT

Microtubules (MTs) constitute a crucial part of the cytoskeleton and are essential for cell division and differentiation, cell motility, intracellular transport, and cell morphology. Precise regulation of MT assembly and dynamics is essential for the performance of these functions. Although much progress has been made in identifying and characterizing the cellular factors that regulate MT assembly and dynamics, signaling events in this process is not well understood. Gβγ, an important component of the G protein-coupled receptor (GPCR) signaling pathway, has been shown to promote MT assembly *in vitro* and in cultured NIH3T3 and PC12 cells. Using the MT depolymerizing agent nocodazole, it has been demonstrated that the association of Gβγ with polymerized tubulin is critical for MT assembly. More recently, Gβγ has been shown to play a key role in NGF-induced neuronal differentiation of PC12 cells through its interaction with tubulin/MTs and modulation of MT assembly. Although NGF is known to exert its effect through tyrosine kinase receptor TrkA, the result suggests a possible involvement of GPCRs in this process. The present study was undertaken to determine whether agonist activation of GPCR utilizes Gβγ to promote MT assembly. We used isoproterenol and UK 14,304, agonists for two different GPCRs (β- and α2-adrenergic receptors, respectively) known to activate Gs and Gi respectively, with an opposing effect on production of cAMP. The results demonstrate that the agonist activation of β- and α2-adrenergic receptors promotes the association of Gβγ with MTs and stimulates MT assembly in NIH3T3 cells. Interestingly, the effects of these two agonists were more prominent when the cellular level of MT assembly was low (30% or less). In contrast to MT assembly, actin polymerization was not affected by isoproterenol or UK 14, 304 indicating that the effects of these agonists are limited to MTs. Thus, it appears that, upon cellular demand, GPCRs may utilize Gβγ to promote MT assembly. Stimulation of MT assembly appears to be a novel function of G protein-mediated signaling.

1. Introduction

The major component of microtubules (MTs) is the heterodimeric protein tubulin. Numerous proteins are known to interact with tubulin and regulate MT assembly either by promoting the polymerization of tubulin or by stabilizing MTs. These proteins are collectively known as microtubule-associated proteins (MAPs). Members of this group of proteins, such as MAP2 and tau, are known to promote MT assembly and stabilize MTs *in vivo* and *in vitro* [1,2]. Several other proteins, including Op18/stathmin, katanin, and some kinesin-related motor proteins, act on MTs by destabilizing the polymer [3–5]. Although these studies identified and characterized the cellular factors that regulate MT assembly and dynamics, the precise spatial and temporal control of the process has not been clearly understood.

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Heterotrimeric G proteins play an important role in transferring signals from cell surface receptors (G protein-coupled receptors, GPCRs) to intracellular effector molecules. G protein heterotrimer, consisting of guanine nucleotide binding α plus βγ subunits, is inactive in cellular signaling. Agonist binding to GPCRs triggers structural rearrangements in the receptors such that the intracellular domain then catalyzes a nucleotide exchange in the α subunit of heterotrimeric G proteins. Subsequently, activated Gα changes its association with Gβγ in a manner that permits both subunits to participate in the regulation of intracellular effector molecules [6–8]. Although G proteins are likely to be membrane-bound when coupled to receptors, recent results from various laboratories suggest that G proteins associate with MTs, and regulate assembly/dynamics of MTs [reviewed in 9, 10]. It has been shown that α subunits of G proteins (Gsα, Gi1α and Gqα) inhibit MT assembly and promote MT dynamics *in vitro* [11]. In contrast, Gβγ promotes MT assembly [12]. Also, reconstituted Gαβγ heterotrimers were found to be inactive in the modulating MT assembly [13], suggesting that receptor-mediated activation of G proteins may involve modulation of MT assembly by G proteins.

To investigate the potential link between Gβγ and MT assembly *in vivo*, cultured NIH3T3 and PC12 cells were used. The role of Gβγ in MT assembly was demonstrated using nocodazole, a MT depolymerizing drug [14]. We found that Gβγ was preferentially bound to MTs in NIH3T3 and PC12 cells and treatment with nocodazole indicated that the dissociation of Gβγ from MTs is an early step in the depolymerization process [14]. When MTs were allowed to recover after the removal of nocodazole, the tubulin-Gβγ interaction was restored in reassembled MTs suggesting that the association of Gβγ with MTs is important for MT assembly and/or stability [14]. More recently, using biochemical and immunofluorescence analysis, it has been demonstrated that Gβγ-MT interactions and modulation of MT assembly is critical for nerve-growth-factor (NGF)-induced neuronal differentiation of PC12 cells [15]. Although, NGF is known to induce neurite outgrowth from PC12 cells by activating the receptor tyrosine kinase, TrkA [16], our study clearly suggests the possible involvement of GPCR since Gβγ is a component of the GPCR pathway.

GPCRs mediate physiological responses to extracellular signaling molecules, such as hormones or neurotransmitters. The GPCR family of proteins is highly diverse; more than 1000 genes encoding GPCRs are found in the human genome [17]. GPCRs consist of seven transmembrane domains, connected by three extracellular loops and three intracellular loops. The extracellular region is responsible for agonist binding (neurotransmitters, hormones, and odorants, among others), and the intracellular region is responsible for interacting with heterotrimeric G proteins [6–8]. G-protein heterotrimers are typically classified into four classes: Gs, Gi, GQ and G12-13. Typical effectors of Gα/Gβγ signaling include adenylyl cyclase, phospholipases, ion channels, and several kinases and transcription factors [6–8,18–20]. While activation of the β-adrenergic receptor leads to activation of Gαs and in turn, stimulates adenylyl cyclase and subsequent production of cAMP, activation of the α2-adrenergic receptor mediates a decrease in adenylyl cyclase activity and cAMP production through the inhibitory G protein Gαi [7].

The present study was undertaken to determine whether agonist activation of GPCR utilizes Gβγ to promote MT assembly. We found that the activation of β- and α2-adrenergic receptors by isoproterenol and UK 14, 304 respectively alters the association of Gβγ with tubulin/MTs and stimulates MT assembly in NIH3T3 cells. This finding may provide a novel mechanism for hormone- or neurotransmitter-mediated alterations of the MT cytoskeleton.

2. Material and methods

2.1. Cell culture and agonist treatments

NIH3T3 cells (ATCC, Manassas, VA) were grown in 75-cm^2 culture flasks at 37 °C in DMEM (with 4.5 g/L glucose and L-glutamine), supplemented with 10% bovine calf serum, 0.1 mM sodium pyruvate, and penicillin-streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin) in 10% CO_2. For agonist treatment, cells were grown on 150-mm plates to 80% confluence over 1–2 days and were then treated with 50 μM isoproterenol or 10 μM UK 14,304 (Sigma Chemicals. St. Louis, MO), as indicated in the figures. Stock solutions of 50 mM isoproterenol in deionized water, and 10 mM 14,304 in DMSO were prepared, and diluted in culture media to final concentrations of 50 μM isoproterenol and 10 μM UK14,304 respectively. Initial experiments were performed by varying the agonist concentration and the incubation time. A maximal effect was observed when NIH3T3 cells were incubated in the presence of 50 μM isoproterenol or 10 μM UK 14,304 for 30–60 min, and therefore was used for subsequent experiments. The similar concentrations (10–50 μM) of isoproterenol/UK 14,304 were used in earlier studies showing their effects on cAMP production [7].

2.2. Extraction of microtubules (MT) and soluble tubulin (ST) fractions

NIH3T3 cells were grown on 150-mm plates to 80% confluence over 1–2 days as previously described and subjected to isoproterenol or UK 14,304 as indicated in the figures. The plates were used in duplicate for each condition. Microtubules (MT) and soluble tubulin (ST) were prepared by extracting soluble, and polymerized tubulin fractions in an MT stabilizing buffer (MS) as previously described [14,15]. Briefly, cells were rinsed in PBS and incubated with 0.5–1 ml MS buffer (0.1 M PIPES, pH 6.9, 2 M glycerol, 5 mM MgCl2, 2 mM EGTA, 0.5% Triton X-100) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), 1 mM DTT, and 10 μM GTP for ~10 min at room temperature until the cells began to lyse. The cells were removed with a cell scraper and centrifuged for 10 min. The supernatant constitutes the ST fraction and the cell pellets represent the MT fraction. The pellets were resuspended in PEM buffer (100 mm PIPES, pH 6.9, 2 mM EGTA, 1 mM MgCl2) containing 1 mM DTT, 10 μM GTP, and protease inhibitor cocktail followed by incubation in ice for 30 min, sonicated for 20 s 3 times and centrifuged for 10 min. Supernatant represents the MT fraction. This procedure yielded highly reproducible results in technically replicated samples used for each condition in a given experiment.

2.3. Preparation of cytosolic fractions

NIH3T3 cells were grown on 100-mm plates to 80% confluence over 1–2 days. Cells were then treated with or without isoproterenol as indicated in the figure. Incubations were terminated by discarding the media and adding 1 mL ice-cold HEPES-sucrose buffer (15 mM HEPES, 0.25 M sucrose, 1 mM DTT, pH 7.5) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) for 10 min. Subsequently, cells were removed mechanically, homogenized, and centrifuged at 10,000 × g for 10 min. Supernatants represent cytosolic fractions.

2.4. Immunoprecipitation

For immunoprecipitation, 100 μL aliquots of cytosolic fractions (~0.5–1 mg/ml) were incubated with and without polyclonal rabbit anti-Gγ2 (Santa Cruz Biotechnology) or non-specific IgG (anti-

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