Gβγ Mediates the Interplay between Tubulin Dimers and Microtubules in the Modulation of Gq Signaling

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Agonist stimulation causes tubulin association with the plasma membrane and activation of PLCβ1 through direct interaction with, and transactivation of, Goα. Here we demonstrate that Gβγ interaction with tubulin down-regulates this signaling pathway. Purified Gβγ, alone or with phosphatidylinositol 4,5-biphosphate (PIP2), inhibited carbachol-evoked membrane recruitment of tubulin and Goα transactivation by tubulin. Polymerization of microtubules elicited by Gβγ overrode tubulin translocation to the membrane in response to carbachol stimulation. Gβγ sequestration of tubulin reduced the inhibition of PLCβ1 observed at high tubulin concentration. Goα interacted preferentially with tubulin-GDP, whereas Goq was transactivated by tubulin-GTP. Prenylation of the γ polypeptide was required for Gβγ/tubulin interaction. Both microtubule formation and microtubule function were disrupted by tubulin-GTP interaction during carbachol stimulation of neuroblastoma SK-N-SH cells. In resting cells Gβγ localized predominantly at the cell membrane, whereas tubulin was found in well defined microtubules in the cytosol. Within 2 min of agonist exposure, a subset of tubulin translocated to the plasma membrane and colocalized with Gβ. Fifteen min post-carbachol addition, tubulin and Gβ/GGβ colocalized in vesicle-like structures in the cytosol. Gβ/GGβ colocalization increased after pretreatment of cells with the microtubule-depolymerizing agent, colchicine, and was inhibited by taxol. Taxol also inhibited carbachol-induced PIP2 hydrolysis. It is suggested that Gβγ/tubulin interaction mediates internalization of membrane-associated tubulin at the offset of PLCβ1 signaling. Newly cytosolic Gβγ/tubulin complexes might promote microtubule polymerization attenuating further tubulin association with the plasma membrane. Thus G protein-coupled receptors might evoke Goα and Gβγ to orchestrate regulation of phospholipase signaling by tubulin dimers and control of cell shape by microtubules.

Binding of agonists to G protein-coupled receptors causes receptors to interact with specific G protein α-βγ heterotrimers, which, in turn, triggers the replacement of GDP for GTP on the α subunit and the functional dissociation and activation of both α and βγ subunits. Goα and Gβγ are then able to activate effectors and initiate signal transduction.

Tubulin is a cytoskeletal protein that forms microtubules (1, 2) and regulates G protein-mediated signaling (3–5) through binding and hydrolysis of GTP. Transfer of GTP from tubulin to the α subunit of Goq, Gαq, and Gq (transactivation) leads to Go coupling to, and regulation of, adenyl cyclase and phospholipase Cβ1 (PLCβ1) (4–8).

Tubulin translocates to the plasma membrane to regulate these G proteins. Activation by Gq-coupled muscarinic receptors (3, 9) or glutamate receptors (mGlur1α) (10) causes microtubule depolymerization and association of tubulin with plasma membrane proteins in living cells. Upon binding to the plasma membrane tubulin transactivates Gαq, which, in turn, activates PLCβ1 (7). However, continued accumulation of tubulin at the membrane inhibits PLCβ1 as that tubulin binds to the PLCβ1 substrate, phosphatidylidylinositol 4,5 bisphosphate (PIP2) (9). Because the product of PLCβ1-directed PIP2 hydrolysis, inositol 1,4,5-trisphosphate (IP3), mobilizes stored calcium that evokes microtubule depolymerization (11), feedback inhibition of PLCβ1 may be caused by increased concentration of tubulin dimers in regions close to the plasma membrane (9).

Both G protein α and βγ subunits regulate the assembly of the microtubule cytoskeleton in vitro. Although Ga activates tubulin GTPase and increases microtubule dynamics (12), Gβγ promotes microtubule polymerization and stabilizes microtubules in vitro (13). Post-translational isoprenylation of Gγ appears important to this process, because the mutant β1γ(C68S), which is isoprenylation-deficient, and β1γ, which is farnesylated, rather than geranylgeranylated, do not support microtubule formation.

Although this Gβγ effect on in vitro microtubule assembly has been shown, the potential impact of a tubulin-Gβγ interaction in the cell and its effect on intracellular signaling has not been investigated. Interaction of Gβγ and PIP2 with the G protein receptor kinase 2 (GRK2) assists GRK2 membrane translocation and the subsequent phosphorylation of the activated β-adrenergic receptor (14). Gβγ might be similarly instrumental in translocation of tubulin to its membrane-associated signaling partners. Regulated interactions of tubulin with either Goα or Gβγ might differentially affect cellular signaling, because these subunits reciprocally regulate tubulin polymerization.

The abbreviations used are: PLCβ1, phospholipase Cβ1; AAGTP, P(4-azidoanilido)-P5’-GTP, tubulin-AAGTP, dimeric tubulin with AAGTP bound; GDPβS, guanyl-5’-y1 thiosphosphate; HPLC, high-performance liquid chromatography; Pipes, 1,4-piperazinediethanesulfonic acid; DTt, dithiothreitol; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; QNB, 1-quinuclidinyl-[phenyl-4-nitro]-1-propanesulfonate, muscarinic receptor antagonist; PIP2, phosphatidylidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-trisphosphate; GRK2, G protein receptor kinase 2; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; GTPyS, guanosine 5’-3-O-thio(triphosphate); FITC, fluorescein isothiocyanate; PI3K, phosphoinositide 3-kinase; MAP, mitogen-activated protein kinase; EMA, echinoderm MAP.

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Cytoskeletal Regulation of G Protein Signaling

ization. By promoting microtubule polymerization in response to a signal, G\textsubscript{\beta\gamma} might sequester cytosolic tubulin and inhibit tubulin involvement in the regulation of intracellular signaling.

This report investigates the interaction of tubulin with G\textsubscript{\beta\gamma} and the effect of this interaction on the interplay between agonist-evoked PLC\textsubscript{1} signaling and microtubule assembly. It is suggested that agonist stimulation recruits tubulin to the membrane, where it transactivates G\textsubscript{\alpha}. Subsequent to PLC\textsubscript{1} activation, tubulin sequesters with G\textsubscript{\beta\gamma}, and this complex internalizes to the cytosol. In vitro membrane association/microtubule polymerization experiments suggest that such complexes might seed microtubule polymerization. Data from transactivation, communoprecipitation and cross-linking experiments in vitro, as well as those from confocal immunofluorescence microscopy or communoprecipitation in cells, indicate a direct functional relationship between G protein-coupled receptor signaling and the dynamics of the microtubule cytoskeleton. This report provides first direct evidence for agonist-evoked and G\textsubscript{\beta\gamma}-mediated cross-regulation between G protein signaling and the dynamics of the microtubule cytoskeleton. Such a mechanism could be instrumental in the regulated reorganization of cytoskeletal elements or other cellular rearrangements leading to synapse formation, cell motility, and cell shape.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Sf9 cells were maintained in SF-900 II SFM media as described previously (7). SK-N-SH neuroblastoma cells were grown in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum according to standard procedures (7).

**Baculovirus-directed Protein Expression in Sf9 Cells**—Sf9 cells were simultaneously infected with baculoviruses bearing m1 muscarinic receptor, G\textsubscript{\textalpha}m1, and/or PLC\textsubscript{1}cDNA as described previously (7). The construction of these recombinant baculoviruses was described earlier (15–17). Membranes were prepared from cells collected 60 h post-infection. Membrane Preparation and Western Blotting—Sf9 or SK-N-SH cells were sonicated in ice-cold 20 mM Hepes, pH 7.4, 1 mM MgCl\textsubscript{2}, 100 mM NaCl, 1 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, and membrane pellets were prepared as described (7). Protein concentration was measured by the Bradford dye-binding assay (18) with bovine serum albumin (BSA) as a standard. Expression of receptors, G proteins, and PLC\textsubscript{1} was determined by immunoblotting. Membrane proteins transferred to polyvinylidene difluoride membranes (0.45 μm, Millipore Corp.) were probed with antiserum specific for the m1 muscarinic receptor (#71, from G. Luthin, University of Pennsylvania, PA), or PLC\textsubscript{1} (K-32-3, monoclonal, from S. G. Rhee, Bethesda, MD) at a dilution of 1:500. Goat anti-rabbit or anti-mouse IgGs were used as secondary antisera, respectively, followed by ECL detection of the corresponding protein bands. Expression levels were estimated by densitometry of the bands (Storm 840, Amersham Biosciences). They varied by no more than 10% for a given recombinant protein. Receptor binding studies using [\textsuperscript{3}H]QNB as a ligand were performed to monitor m1 muscarinic receptor expression (7). When coexpressed with G\textsubscript{\alpha}m1 and PLC\textsubscript{1} in the Sf9 cells, m1 muscarinic receptor density was estimated at 240 fmol/mg of membrane protein (7). SK-N-SH cells have a high density of m1 muscarinic receptors (500 fmol/mg of membrane protein) coupled to phosphoinositide turnover (19–21).

**Purification of Proteins**—Microtubule proteins were isolated as described previously (22). Microtubule-associated proteins were removed by phosphocellulose chromatography, and the remaining pure tubulin fraction was termed PC-tubulin (23). PC-tubulin was aliquoted and stored in liquid nitrogen until used.

Preparation of G\textsubscript{\beta\gamma} in tubulin was performed as described previously (5). Briefly, GTP was removed from PC-tubulin by charcoal pre-treatment followed by incubation of tubulin with 150 μM guanine nucleotide (GDP or [\textsuperscript{32}P]AATGTP) for 30 min on ice. Prior to use, these samples were passed through Bio-Gel P6DG desalting columns (Bio-Rad) twice to remove the unbound nucleotide. This procedure yields 0.3–0.4 μM G\textsubscript{\beta\gamma} (G\textsubscript{\beta1}G\textsubscript{\gamma1}, G\textsubscript{\beta1}G\textsubscript{\gamma2}, and G\textsubscript{\beta1}G\textsubscript{\gamma3} (68S)) and PC-tubulin in a GTP- or GDP-bound form, or devoid of nucleotide, as well as extracts from SK-N-SH neuroblastoma cells, were tested for tubulin/G\textsubscript{\beta\gamma} coimmunoprecipitation. Purified G\textsubscript{\beta\gamma} subunits and the different PC-tubulin species were tested at a protein ratio of 1:2. When tested, SK-N-SH membranes were extracted with 1% sodium cholate in buffer A for 1 h at 4 °C with constant stirring. Samples were centrifuged at 20,000 × g for 15 min, the membrane pellets were washed with buffer A and dissolved in SDS Laemmli sample buffer with 50 mM DTT as described (27). SDS-PAGE of the samples was performed, and the gels were either stained (Coomassie Blue) or subjected to Western blotting, followed by autoradiography (Kodak XAR-5 film) or phosphor image analysis (Storm 840, Amersham Biosciences).

**Immunoprecipitation**—Purified G\textsubscript{\beta\gamma} subunits (G\textsubscript{\beta1}G\textsubscript{\gamma2}, G\textsubscript{\beta1}G\textsubscript{\gamma1}, and G\textsubscript{\beta1}G\textsubscript{\gamma3} (68S)) and PC-tubulin in a GTP- or GDP-bound form, or devoid of nucleotide, as well as extracts from SK-N-SH neuroblastoma cells, were tested for tubulin/G\textsubscript{\beta\gamma} coimmunoprecipitation. Purified G\textsubscript{\beta\gamma} subunits and the different PC-tubulin species were tested at a protein ratio of 1:2. When tested, SK-N-SH membranes were extracted with 1% sodium cholate in buffer A for 1 h at 4 °C with constant stirring. Samples were centrifuged at 20,000 × g for 15 min at 4 °C, and the isolated membrane extracts (0.5 mg/ml membrane protein) were incubated with the indicated tubulin species (1 μM). Incubations were carried out in buffer A for 15 min at 24 °C and constant shaking. After precipitation with Pansorbin (Calbiochem), each sample was incubated overnight with appropriate specific anti-serum or preimmune serum (1:20 dilution) at 4 °C with constant shaking. Note, that dimeric tubulin has a very low GTPase activity, which is activated upon polymerization at temperatures higher than 24 °C. In addition, no tubulin (or possible aggregates), or G\textsubscript{\beta\gamma}, was precipitated with anti-beta tubulin antibody. Microtubule complexes were precipitated with Pansorbin, and each immunoprecipitate was subjected to SDS-PAGE, followed by immunoblotting and ECL detection of the protein bands. Polyclonal anti-tubulin (raised against the β-tubulin C-terminal region of 422–431 amino acids) (3) and anti-G\textsubscript{\beta1} (Santa Cruz Biotechnologies) antisera were utilized. No cross-reactivity was observed.

**Chemical Cross-linking**—The method of Tucker and Goldstein (28) was used. Briefly, PC-tubulin, in its GDP-bound form, and purified G\textsubscript{\beta1}G\textsubscript{\gamma2} subunits (at a protein ratio of 2:1) were incubated in buffer A for 30 min at 25 °C. The cross-linking agent EDC (Pierce) was added to the...
reaction mixture at the final concentration of 0.30 mM (concentration that limits nonspecific cross-linking), and the incubation was carried out for 2 h at 25 °C (28). The reaction was quenched by the addition of 2× SDS sample buffer. Proteins were separated by SDS-PAGE, followed by immunoblotting and ECL detection of the protein bands. Monoclonal anti-α-tubulin antibody (DM1A, Sigma) and polyclonal anti-Gβγ antisera (Santa Cruz Biotechnologies) were used for immunoblot detection.

PLCβ2, Assay—20 μg of SK-N-SH membrane protein was incubated with a [3H]IP3, substrate mixture (30 μM final concentration) as described (7). 10 μl of each GTPγS, tubulin-GDP, Gβγ, and carbachol was added at appropriate concentrations to a final volume of 120 μl as indicated. When tested, GTPγS was preincubated with the membranes for 30 min on ice, Gβγ subunits were also preincubated with tubulin for 30 min on ice before the experiment. The tubes were incubated for 15 min at 37 °C with constant shaking, as described previously (7). [3H]Insoluble triphosphate ([3H]IP3) production was measured as described (29).

Analysis of Phosphoinositide Hydrolysis in SK-N-SH Cells—SK-N-SH neuroblastoma cells were grown in 6-well plates in DMEM supplemented with 10% fetal bovine serum and 50 units/ml penicillin-streptomycin. 24 h before the experiment, insolvent-free DMEM supplemented with 2 μCi/well myo-[3H]inositol was added. The cells were washed three times with Locke’s buffer, containing 10 mM LiCl, and incubated for 1 h with or without 330 nM taxol in the same buffer. After triplicate wash with Locke’s buffer, 100 μM carbachol was added and the cells were incubated for 30 min at 37 °C. Carbachol effects were routinely controlled for by addition of 10 μM atropine. The reaction was stopped with ice cold 10% trichloroacetic acid, and the cells were scraped from wells with a rubber policeman and transferred to tubes. After sonication (as described above) and centrifugation at 20,000 × g for 15 min (4 °C), the supernatants were extracted with water-saturated ether and neutralized with 1 M NH4HCO3. Ion exchange chromatography (Dowex AG 1-X8 resin, formate form, Bio-Rad) of the samples was performed as described (29). Total [3H]inositol phosphates were quantified by liquid scintillation counting. The inositol phosphate content of SK-N-SH cells at the start of the experiment (0% increase) was 0.98 ± 0.31 × 10−7 dpm per 106 cells.

Microscopy—SK-N-SH neuroblastoma cells were plated onto glass coverslips in 12-well culture plates at a density of 1 × 104. Where indicated, cells were pretreated for 1 h with 330 nM taxol or atropine. After a PBS wash the cells were treated for the indicated times with 100 μM carbachol, 10 μM atropine, or both. After washing with PBS buffer the cells were immediately fixed in −20 °C methanol for 3 min and washed three times, 10 min each, in PBS, containing 0.1% Triton X-100. The cells were blocked for 40 min in PBS, containing 5% milk, and washed in PBS. Subsequently, the cells were incubated for 1 h with a polyclonal rabbit anti-Gβ-γ antisera (Santa Cruz Biotechnologies) at a dilution of 1:100. Following a PBS wash, a secondary FITC (fluorescein isothiocyanate-conjugated) goat anti-rabbit antisera (EY Labs) at an appropriate 1:100 dilution was added for 1 h at 4 °C. The cells were washed three times, 10 min each, in PBS, containing 0.1% Triton X-100. The cells were blocked for 20 min in PBS, containing 5% milk and 0.1% Triton X-100. After washing, the cells were incubated for 1 h in blocking buffer, containing 10 μg/ml rabbit IgG. Subsequently, a monoclonal α-tubulin antisera (DM1A, Sigma) was applied for 1 h at a dilution of 1:100. The cells were washed three times, 10 min each, in PBS, containing 0.1% Triton X-100. Following a PBS wash, a secondary Texas Red-conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories) was applied for 1 h at 4 °C. Image overlays were obtained using a laser scanning confocal microscope (Zeiss LSM 510). Images were acquired using a laser scanning confocal microscope (Zeiss LSM 510) equipped with a ×63 water immersion objective. A 488-nm beam from an argon-krypton laser was used for the excitation of FITC, whereas a 543-nm beam was used for Texas Red excitation. Emission from FITC was detected through a BP505 filter, whereas emission from Texas Red was detected through a LP560 filter. Areas of antibody colocalization were apparent in yellow. Differential interference contrast images of the cells were regularly acquired as well. Coverslips were examined at random.

Figure 1. Taxol-evoked stabilization of microtubules inhibits PLCβ signaling in SK-N-SH neuroblastoma cells. Myo-[3H]inositol-prelabeled SK-N-SH cells were treated for 1 h with 330 nM taxol as indicated. Carbachol (100 μM) was added (where indicated), the samples were incubated for 30 min at 37 °C, and the total inositol phosphate production was measured as described. Values are means ± S.D. of three independent experiments performed in triplicate. **, significantly different from control cells (p < 0.001); *, significantly different from carbachol-treated cells (p < 0.01), Student’s t test.

**RESULTS**

**Microtubule Stabilization Inhibits Agonist-induced PLCβ Activation**—To verify the physiological significance of tubulin regulation of PLCβ1 signaling in vivo, SK-N-SH neuroblastoma cells were treated with the microtubule-stabilizing agent taxol before stimulating the cells with the muscarinic receptor agonist, carbachol (Fig. 1). Because taxol increases the formation of microtubule polymers and inhibits microtubule dynamics, the pool of dimeric tubulin available for regulation of PLCβ1 is decreased. As seen in Fig. 1, the normal 6-fold increase in phosphoinositide hydrolysis elicited by carbachol (610 ± 67% (± S.D.)) was reduced by half in taxol-pretreated cells (350 ± 26% (± S.D.)).

**Agonist-evoked Membrane Association of Tubulin Is Not Assisted by Gβγ Subunits**—It has been previously shown that both Gα and Gβγ subunits interact with tubulin and these interactions reciprocally regulate microtubule polymerization in vitro (12, 13). Although we have shown that interaction of tubulin with certain Gα subunits, including Gαq, regulates intracellular signaling (4–8), the role of Gβγ in such membrane-located signaling events has not been evaluated. Because Gβγ subunits appear to regulate the translocation of signaling enzymes to their membrane-located signaling targets (30–32), we initially aimed to determine whether Gβγ also modifies the recruitment of tubulin to the plasma membrane in response to carbachol stimulation (3).

Membranes from SK-N-SH neuroblastoma cells were incubated with tubulin-[32P]AAGTP in the presence or absence of carbachol and/or purified Gβγ subunits (Fig. 2). As seen previously (7), association of tubulin with SK-N-SH membranes increased by 133 ± 11% (± S.D.) after carbachol stimulation. However, when Gβγ complexes were present in the medium, the carbachol-induced increase in tubulin association with the membrane was blocked. At the concentration used, Gβγ had no effect on the “basal” association of tubulin with the membrane.
Concomitant Binding of Gβγ and PIP2 to Tubulin Prevents Tubulin Association with the Plasma Membrane—Tubulin binds PIP2 (7), which appears to act as a membrane anchor (9). Coordinated binding of PIP2 and Gβγ to the pleckstrin homology domain of GRK2 synergistically enhances GRK2 membrane association in response to agonist stimulation (14). To test if PIP2 and Gβγ similarly affected tubulin, membranes from Sf9 cells containing recombinant m1 muscarinic receptors, Go_q, and PLCβ1, were incubated with tubulin-[32P]AAGTP ([32P]AAGTP (1 μM), with or without carbachol (100 μM), and/or Gβγ (20 μg/ml), and/or PIP2 (30 μM), for 5 min at 23 °C, as described under “Experimental Procedures.” Membranes were UV-irradiated, washed, and subjected to SDS-PAGE, immunoblotting with anti-Gβγ antisera, and phosphorimaging analysis. The phosphor image (A) of the Gβ immuno blot (B) of a representative of four identical experiments with similar results is shown. Gβγ inhibits tubulin association and the resulting transactivation of Go_q. When PIP2 was present, that inhibition is nearly complete.

Gβγ-bound tubulin dimers, because small amounts of Gβγ routinely associated with the membranes independent of carbachol stimulation. Carbachol stimulation tripled the amount of membrane-associated tubulin (199 ± 37% (±S.D.) increase above control), whereas the microtubule mass correspondingly decreased (254 ± 43% (±S.D.)). However, microtubule polymerization, but not membrane association of tubulin, was observed in the presence of carbachol and Gβγ,γ2 subunits. Thus, Gβγ,γ2 appeared to drive tubulin toward polymerization and away from association with the plasma membrane in response to agonist stimulation. This is consistent with the microtubule-polymerizing role of Gβγ seen with pure components (13). It is also consistent with the observation that taxol-induced microtubule polymerization inhibits membrane-located PLCβ1 signaling (Fig. 1).

Gβγ Reverses the Inhibition of PLCβ1 Caused by High Concentration of Tubulin Dimers—High, millimolar, concentrations of tubulin inhibit PLCβ1 presumably through receptor-independent association of tubulin with PIP2/PLCβ1 sites at the cell membrane (7, 9). Diminished access of PLCβ1 to its substrate, PIP2, and/or obstruction of Go_q coupling to PLCβ1, appear to be responsible for this inhibition (9). We wanted to determine whether Gβγ binding of tubulin dimers would also block this process. Tubulin-GDP was used in these experiments, because it did not transactivate Go_q, and, thus, did not activate PLCβ1 (3).

PLCβ activation was evaluated in the presence and absence of carbachol, GTPγS, tubulin-GDP, and Gβγ,γ2 subunits in Sf9 membranes containing recombinant m1 muscarinic receptors, Go_q, and PLCβ1 (Fig. 5). Both carbachol and GTPγS increased
Fig. 4. Gβ1γ2 inhibits agonist-evoked membrane recruitment of tubulin due to increased microtubule polymerization. Redistribution of tubulin between microtubules and membranes was studied during the course of carbachol stimulation of SK-N-SH membranes both in the presence and absence of purified Gβ1γ2. Membranes and microtubules were subjected to SDS-PAGE, immunoblotting with anti-tubulin antibody, and ECL detection of the protein bands. A representative of three identical experiments with similar results is shown. Numerical values are given under “Results.” Note that, although carbachol stimulation triggers tubulin association with membranes, the addition of Gβγ causes microtubule polymerization and an increase in the microtubule pellet even after carbachol exposure.

IP₃ generation. When applied together, they significantly increased the activation of PLCβ1 (90 ± 10% (± S.D.) above the control). (Note that coexpression of Go₁ and PLCβ1 in the SF9 cells increased significantly the basal PLCβ1 activity. This decreased the percent agonist stimulation of PLCβ1, because m₁ muscarinic receptors were moderately expressed (240 fmol/mg of membrane protein).) At the concentrations tested, tubulin-GDP or Gβ1γ2 did not affect the basal activity of PLCβ1. Gβ1γ2 also did not change carbachol activation of PLCβ1 regardless of the presence of GTPγS. This suggested that added Gβ1γ2 did not directly affect membrane PLCβ1 signaling. However, carbachol-activated PLCβ1 was inhibited by 64 ± 4% (± S.D.) by tubulin-GDP (note that tubulin with GTP-bound transactivates Go₁ (3)). When tubulin-GDP was preincubated with Gβ1γ2 subunits, this inhibition was reversed by 60 ± 13% (± S.D.). Thus, the binding of tubulin to Gβ1γ2 subunits appeared to prevent its membrane association and involvement in the regulation of PLCβ1. A mechanism for control of PLCβ1 inhibition by high tubulin concentrations was suggested by these experiments.

Gβ1γ2 Subunits Preferentially Interact with Tubulin-GDP—Go₁ interacts with, and is transactivated by, tubulin-GTP (3). Gβγ subunits decorate brain microtubules (13), which consist of tubulin-GDP (to promote microtubule polymerization, Gβγ must interact with tubulin at some time). We wanted to test directly whether Gβ1γ2 bound preferentially to tubulin-GDP. Purified Gβ1γ2 was incubated with tubulin-GTP, tubulin-GDP, or tubulin dimers, which had been stripped of nucleotide. Tubulin/Gβ1γ2 coimmunoprecipitation was tested with both anti-tubulin (Fig. 6A) and anti-Gβ1 antisera (Fig. 6B). In both cases, preferential coimmunoprecipitation of Gβ1γ2 subunits with tubulin-GDP was observed. Coimmunoprecipitation of Gβ1γ2 with tubulin-GDP was 157 ± 28% (± S.D.) higher that that with tubulin-GTP. This corresponded to the coimmunoprecipitation of tubulin-GDP with Gβ1γ2, which was 198 ± 32% (± S.D.) higher than that of tubulin-GTP. Similarly, when extracts from SK-N-SH neuroblastoma membranes were incubated with GTP- or GDP-ligated tubulin, preferential coimmunoprecipitation of Gβ1γ2 with tubulin-GDP was detected. Gβ1γ2/tubulin-GDP coimmunoprecipitation was 136 ± 25% (± S.D.) higher than that of Gβ1γ2/tubulin-GTP (Fig. 6C). Thus, although Go₁ interacted with, and was activated by, tubulin-GTP, Gβ1γ2 subunits interacted preferentially with the GDP-bound form of tubulin.

It has also been shown that Gβ1γ2 subunits in which the γ2 polypeptide is isoprenylated promote microtubule assembly (13). To evaluate if this lipid modification is important for Gβγ interaction with tubulin-GDP dimers, as it is for their microtubule binding, coimmunoprecipitation experiments of tubulin-GDP with Gβ1γ2, Gβ1γ1, and Gβ1γ3(68S) were performed (Fig. 6D). The mutant Gβ1γ2(68S) is unable to undergo post-translational modification and, thus, does not carry a geranylgeranyl moiety (33). Gβ1γ2, but not Gβ1γ3(68S) or Gβ1γ1 (which is farnesylated rather than geranylated), coimmunoprecipitated with the GDP-bound tubulin dimers. These results suggested that geranylgeranylation of the γ subunit is important for its interaction with the GDP-bound form of dimeric tubulin.

Chemical Cross-linking of Gβγ and Tubulin—The interaction between Gβ1γ2 and tubulin-GDP was verified by chemical cross-linking (Fig. 7). Tubulin-GDP was incubated with Gβ1γ2 or Gβ1γ1, followed by cross-linking of the proteins with the agent EDC, SDS-PAGE, and immunoblotting with anti-Gβγ or anti-tubulin antisera. A band consistent with the electrophoretic mobility of cross-linked Gβ1γ2 dimers (molecular mass of ~40–42 kDa), and a band, consistent with that of tubulin-Gβ1γ2 complexes (molecular mass of ~140 kDa), were detected.
with the anti-\(G\) antibody (Fig. 7, lane a). Immunoblotting with the anti-tubulin antibody revealed the presence of both tubulin monomers (at \(\sim 50\) kDa) and cross-linked tubulin dimers (at \(\sim 100\) kDa) (Fig. 7, lane c). A band at 140 kDa was also recognized, confirming the interaction between tubulin and \(G\). Under the chosen experimental conditions, (28) higher order molecular weight complexes were not detected by either antiserum. When \(G\) subunits were tested, labeling at 140 kDa was barely visible (Fig. 7, lanes b and d). These experiments confirmed tubulin-GDP interaction with \(G\) protein \(\beta\gamma\) subunits. It also indicated that the stoichiometry of this interaction was 1:1.

Agonist-regulated Spatiotemporal Pattern of Tubulin-\(G\) Colocalization in Neuroblastoma SK-N-SH Cells—Because tubulin and \(G\) interact with each other, they would be expected to colocalize at specific cellular locations. In addition, if tubulin/\(G\) interaction is signal-regulated, spatiotemporal colocalization should exist in the living cell. To test this, SK-N-SH cells were studied by confocal microscopy before and after carbachol stimulation (Fig. 8). Tubulin is shown to regulate PLC\(\beta\) signaling in this neuroblastoma cell line (3).

In unstimulated SK-N-SH cells, tubulin and \(G\) colocalized sporadically in the cytoplasm (Fig. 8, 0 min). \(G\), but not tubulin, was seen at the membrane of the resting cells. Two minutes after carbachol stimulation, microtubules depolymerized and tubulin translocated to the cell membrane (Fig. 8, 2 min). \(G\) and tubulin colocalized at the membrane. Fifteen minutes subsequent to carbachol exposure, tubulin and \(G\) were colocalized in vesicle-like structures in the cytosol (Fig. 8, 15 min). Diminished association of tubulin with the plasma membrane was seen at this time point. As previously reported (3, 7, 9), carbachol-induced redistribution of tubulin was inhibited by atropine.

These findings were corroborated by communoprecipitation studies of carbachol-treated SK-N-SH cells (Fig. 9). Two minutes after carbachol stimulation \(G\) and tubulin complexes were immunoprecipitated from detergent-extracted SK-N-SH membranes (increase by 61 \(\pm\) 10\% (\(\pm\) S.D.) compared with control untreated cells). Fifteen minutes later such complexes were observed in the cytosol (increase by 44 \(\pm\) 17\% (\(\pm\) S.D.) compared with control cells). Thus, both confocal microscopy and communoprecipitation experiments in cells indicated agonist-evoked interaction between tubulin and \(G\) at the plasma membrane as well as translocation of these complexes to the cell cytosol at the offset of signaling.

Microtubule Depolymerization Increases Membrane Recruitment of Tubulin and Its Colocalization with \(G\) —We used compounds that would either increase or decrease tubulin dimer concentrations to evaluate tubulin translocation and colocalization with \(G\) in response to agonist stimulation. As previously observed (9), colchicine depolymerized SK-N-SH cell microtubules and increased the amount of membrane-associated tubulin by 44 \(\pm\) 11\% (\(\pm\) S.D.) (Fig. 10B). These biochemical data correlated well with images showing colchicine-induced redistribution of tubulin toward the plasma membrane regardless of carbachol stimulation (Fig. 10, A and C, center panels). Tubulin/\(G\) colocalization in the cytosol was also readily observed. To the contrary, taxol stabilized the microtubule network and decreased membrane-associated tubulin by 27 \(\pm\) 17\% (\(\pm\) S.D.) (Fig. 10B). Images from these experiments showed taxol-bundled microtubules and no tubulin colocalization with
Cytoskeletal Regulation of G Protein Signaling

0 min

2 min

15 min

Fig. 8. Colocalization of tubulin and Gβ. SK-N-SH cells were treated for the indicated times with 100 μM carbachol as described under “Experimental Procedures.” The cells were fixed and incubated with anti-Gβ antiserum and monoclonal anti-α-tubulin antibody (DM1A). The anti-Gβ antiserum was recognized with a secondary FITC-conjugated antiserum (green), and the anti-tubulin antibody was recognized with a secondary Texas Red-conjugated antiserum (red). Microtubules are seen in samples prior to carbachol treatment and 15 min after the drug application but are not observed at the 2-min point. Areas of tubulin/Gβ colocalization appear in yellow. Carbachol-induced tubulin/Gβ colocalization is greatest at the cell membrane after 2 min and in vesicle-like structures in the cytosol after 15 min.

DISCUSSION

Gβγ heterodimers perform many diverse intracellular regulatory functions. Adenyl cyclases and PLCβ isozymes, ion channels, and kinases are directly regulated by G protein βγ subunits (for reviews see Refs 34 and 35). The membrane association of Gαi, Gαq, and Gαz requires Gβγ subunits (36–38). Gβγ subunits may also act as “molecular levers” for activated receptors to pry open the Gα guanine nucleotide binding pocket and thus, release the GDP (38). Gβγ is involved in the targeting of cytosolic GRKs, phosphoinositide 3-kinase (PI3K), as well as PLCβ1, to the membrane of the cell (30–32). Gβγ isoform selectivity for this process has been demonstrated (36, 39). Gβ1γ2, but not Gβiγ1 subunits, bind to microtubules and promote microtubule assembly (13) (Fig. 4). Gβγ subunits regulate the migration of the centrosome around the nucleus and, hence, the orientation of the mitotic spindle, in embryos of Caenorhabditis elegans (40). This report suggests that, in the cell, Gβγ interaction with tubulin is signal-regulated and responsible for both down-regulation of tubulin involvement in PLCβ1 signaling and remodeling of the microtubule network. Cross-regulation between intracellular signaling and cytoskeletal reorganization is proposed.

The ability of Gβ1γ2 subunits to affect tubulin regulation of PLCβ1 appeared related to their preferential interaction with tubulin-GDP. Both immunoprecipitation and chemical cross-linking experiments showed the specificity of this interaction (Figs. 6 and 7). This was in contrast to the Gα subunits Gα11, Gαq, and Gαi, which interacted preferentially with tubulin-GTP (3–5, 7–9, 25, 41). Such preferred interactions suggest a mechanism by which receptor activation evokes membrane association of tubulin-GTP leading to Gαi transactivation and initiation of PLCβ1 signaling. This is followed by internalization of tubulin-GDP/Gβγ complexes at the offset of signaling. They also explain the accelerated microtubule dynamics caused by Gβγ.
FIG. 10. Microtubule stabilization prevents agonist-evoked translocation of tubulin and colocalization with membrane Gβ. SK-N-SH cells were pretreated for 1 h with or without 330 nM of the microtubule-depolymerizing agent, colchicine, or the microtubule-stabilizing agent, taxol. A, representative images of control and drug-treated cells, as indicated. After the treatment the cells were fixed and incubated with DM1A anti-α-tubulin antibody and secondary Texas Red-conjugated antibody. Cells were examined by confocal microscopy as described under “Experimental Procedures.” Note the extensive microtubule network of control cells, depolymerized tubulin along the cell membrane in colchicine pretreated cells and microtubule bundles in taxol-pretreated cells. B, membrane fractions were prepared from cells similar to those shown in A and membrane-associated tubulin was detected by immunoblotting. A representative experiment of three with similar results is shown. Colchicine
Cytoskeletal Regulation of G Protein Signaling

by purified Go1 (12) and the increased microtubule polymerization promoted by Gβγ2 (13) (Fig. 4). Chemical cross-linking also indicated that the ratio of tubulin/Gβγ binding was significant (1:1 (Fig. 7). EMAP, an echinoderm MAP, which has significant sequence homology with Gβ (42), binds to both microtubules and tubulin dimers (43). The ratio of dimeric tubulin/EMAP binding is 1:1.

A simple antagonist relationship between Go and Gβγ in microtubule-dependent processes is unlikely to exist. Go has been observed to associate with microtubules in developing neurites from PC12 pheochromocytoma cells (59) and a dominant-negative Go, which binds to microtubules but prevents transactivation of Go by tubulin, prevents COS1 cells from sending out cellular processes (60). It is noteworthy in this regard that developing cellular extensions, such as neuronal growth cones, contain microtubules that are more dynamic. Thus, a syncopated interaction between Go and Gβγ may exist in which dynamic microtubules in a developing neurite give way to Gβγ-stabilizing microtubules in a more mature structure (61).

In summary, we have demonstrated that purified Gβγ subunits do not translocate tubulin to the plasma membrane for

pretreatment increased membrane-associated tubulin, whereas taxol pretreatment did not. C, cells pretreated with the indicated agents were subsequently treated for 2 min with either 100 μM carbachol or vehicle. Cells were fixed and incubated with anti-Gβ antiserum and DM1A anti-α-tubulin antibody. The secondary antibody for anti-Gβ was FITC-conjugated (green), whereas that for anti-α-tubulin was Texas Red-conjugated (red). Areas of tubulin/Gβ colocalization appear in yellow. An arrow denotes such an area in the control cells at 2 min post-carbachol. Carbachol stimulation does not evoke tubulin/Gβγ colocalization at the plasma membrane in taxol-pretreated cells.

2 J. S. Popova and M. M. Rasenick, manuscript in preparation.
the regulation of PLCβ1 signaling but instead promote microtubule assembly. Because Gβγ associates preferentially with tubulin-GDP, this may be relevant for regions of the cell where microtubules depolymerize in response to agonist-evoked elevation in calcium. In these areas, receptor-independent inhibition of PLCβ1 by high tubulin concentrations may be reversed by tubulin interaction with local Gβγ subunits and subsequent “diversion” of that tubulin into microtubules. Furthermore, during the course of agonist stimulation, membrane Gβγ subunits could assist sequestration of membrane-associated tubulin and its subsequent internalization. Taken together, this may constitute a novel mechanism for agonist-mediated cross-regulation between intracellular signaling and the reorganization of the microtubule cytoskeleton. Such consequence of events could prove important to the regulation of neuronal plasticity and development, cell motility, and changes in cell shape.

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