Clathrin-mediated Endocytosis of \( m_3 \) Muscarinic Receptors

ROLES FOR \( G_\beta_\gamma \) AND TUBULIN*

Receptors as well as some G protein subunits internalize after agonist stimulation. It is not clear whether \( G_{ai} \) or \( G_\beta_\gamma \) undergo such regulated translocation. Recent studies demonstrate that \( m_3 \) muscarinic receptor activation in SK-N-SH neuroblastoma cells causes recruitment of tubulin to the plasma membrane. This consequently transactivates \( G_{ai} \) and activates phospholipase \( C_\beta_1 \). Interaction of tubulin-GDP with \( G_\beta_\gamma \) at the offset of phospholipase \( C_\beta_1 \) signaling appears involved in localization of tubulin and \( G_\beta_\gamma \) to vesicle-like structures in the cytosol (Popova, J. S., and Rasenick, M. M. (2003) \textit{J. Biol. Chem.} 278, 34299–34308). The relationship of this internalization to the clathrin-mediated endocytosis of the activated \( m_3 \) muscarinic receptors or \( G_{ai} \) involvement in this process has not been clarified. To test this, SK-N-SH cells were treated with carbachol, and localization of \( G_{ai} \), \( G_\beta_\gamma \), tubulin, clathrin, and \( m_3 \) receptors were analyzed by both cellular imaging and biochemical techniques. Upon agonist stimulation both tubulin and clathrin translocated to the plasma membrane and co-localized with receptors, \( G_{ai} \), and \( G_\beta_\gamma \). Fifteen minutes later receptors, \( G_\beta_\gamma \) and tubulin, but not \( G_{ai} \), internalized with the clathrin-coated vesicles. Immunoprecipitation of \( m_3 \) receptors with \( G_\beta_\gamma \) tubulin, and clathrin from the cytosol of carbachol-treated cells was readily observed. These data suggested that \( G_\beta_\gamma \) subunits might organize the formation of a multiprotein complex linking \( m_3 \) receptors to tubulin since they interacted with both proteins. Such protein assemblies might explain the dynamin-dependent \( \beta\)-arrestin-independent endocytosis of \( m_3 \) muscarinic receptors since tubulin interaction with dynamin might guide or insert the complex into clathrin-coated pits. This novel mechanism of internalization might prove important for other \( \beta\) arrestin-independent endocytic pathways. It also suggests cross-regulation between G protein-mediated signaling and the dynamics of the microtubule cytoskeleton.

Tubulin is a structural protein that builds the microtubule network of the cell. Tubulin also regulates adenylyl cyclase and phospholipase \( C_\beta_1 \) (PLC\( _{\beta_1} \))

* This study was supported by National Institutes of Health Grants MH 39595 and AG 15482 to M. M. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Physiology and Biophysics, College of Medicine, University of Illinois at Chicago, 835 S. Wolcott Ave. M/C 901, Chicago, IL 60612-7342. Tel.: 312-996-6641; Fax: 312-996-1414; E-mail: jsp@uic.edu

1 The abbreviations used are: PLC, phospholipase C; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; HA, hemagglutinin; DIC, differential interference contrast; GRK, G protein-coupled receptor kinase.
ric receptors, Gβγ, and tubulin internalize at the offset of PLCβ2 signaling through a clathrin-mediated endocytic mechanism. It is suggested that coupling of m3 receptors to tubulin through Gβγ might guide receptor sequestration, since tubulin interacts with dynamin and, thus, might facilitate the insertion of the complexes in the clathrin-coated pits. This may represent a novel mechanism for receptor internalization that is alternative to the β-arrestin-mediated pathway. It is finally hypothesized that, if excluded from the early endosomes, tubulin-Gβγ complex might activate a 1±103 cells-mediated reverse the prototubule depolymerization caused by PLCβ2, evoked Ca2+ increase. This suggests a regulated interconnection between G protein-mediated intracellular signaling and the remodeling of the cytoskeleton.

EXPERIMENTAL PROCEDURES

Expression Constructs—cDNAs encoding HA-tagged wild type and dominant negative K44E dynamin I were originally obtained from Dr. Richard Vallee (Columbia University) (35). They were subsequently cloned into pcDNA3.1zeo using Xhol and XbaI and kindly provided by Dr. Mark von Zastrow (University of California, San Francisco) (36).

Cell Culture and Transfection—SK-N-SH neuroblastoma cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum according to standard procedures (4). Where indicated, the cells were seeded in 12-well plates and transfected with 1 μg/well cDNA of wild type or dominant negative dynamin I K44E using GenePORTER transfection reagent (GTS, Inc.) according to the manufacturer’s instructions. Assays were performed 48 h after the start of transfection. Protein expression was verified by immunoblotting.

Membrane Preparation and Western Blotting—SK-N-SH cells were sonicated in ice-cold 20 mM Hepes, pH 7.4, 1 mM MgCl2, 100 mM NaCl, 1 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride (30 mM sodium deoxycholate and 1% Triton X-100 (1 h, 4°C, with constant shaking) before and after stimulation with 0.1 mM carbachol as described. Samples (10 μg of protein or 0.1 mg/ml membrane extract) were preclarified with normal rabbit, goat, or mouse IgG (depending on the experimental protocol) and protein A/G PLUS-agarose (Santa Cruz) according to the manufacturer’s instruction. Isolated supernatants were incubated for 4 h with 8 μl of polyclonal goat anti-clathrin or rabbit anti-Gαq antisera (Santa Cruz) or mouse tubulin antibody (DM1A, Sigma) at 4°C with constant shaking and then were precipitated with protein A/G PLUS-agarose as instructed by the manufacturer, and each immunoprecipitate was washed 4 times with radioimmuno precipitation assay buffer. The pellets were resuspended in 1× sample buffer and subjected to SDS/PAGE followed by immunoblotting and ECL detection of the protein bands. Monoclonal anti-tubulin antibody (DM1A, Sigma) (dilution of 1:1000) and polyclonal anti-Gαq, m3 muscarinic receptors, and clathrin were tested in radioimmuno precipitation assay buffer-stripped SK-N-SH membranes or crude cytosolic fractions adjusted to 1% sodium deoxycholate and 1% Triton X-100 (1 h, 4°C, with constant shaking) before and after stimulation with 0.1 mM carbachol as described. Samples (10 μg of protein or 0.1 mg/ml membrane extract) were preclarified with normal rabbit, goat, or mouse IgG (depending on the experimental protocol) and protein A/G PLUS-agarose (Santa Cruz) according to the manufacturer’s instruction. Isolated supernatants were incubated for 4 h with 8 μl of polyclonal goat anti-clathrin or rabbit anti-Gαq antisera (Santa Cruz) or mouse tubulin antibody (DM1A, Sigma) at 4°C with constant shaking and then were precipitated with protein A/G PLUS-agarose as instructed by the manufacturer, and each immunoprecipitate was washed 4 times with radioimmuno precipitation assay buffer. The pellets were resuspended in 1× sample buffer and subjected to SDS/PAGE followed by immunoblotting and ECL detection of the protein bands. Monoclonal anti-tubulin antibody (DM1A, Sigma) (dilution of 1:1000) and polyclonal anti-Gαq, m3 muscarinic receptor, and anti-m3 muscarinic receptor (dilution of 1:200) antisera were used to detect proteins of interest as described. No cross-reactivity was observed between the antisera used.

Materials—Carbachol and atropine sulfate were from Sigma. FITC- and TRITC-conjugated rabbit anti-goat antisera were from EY Laboratories Inc., Rhodamine-conjugated goat anti-rabbit antisera from Roche Applied Science and FITC-conjugated anti-goat-antisera from Vector Laboratories were also utilized. Texas Red-conjugated goat anti-mouse antisera was from Jackson Laboratories. All other reagents were of analytical grade.

RESULTS

Internalization of Membrane-associated Tubulin with Gβγ, but Not Gαq, after Carbachol Stimulation of SK-N-SH Neuroblastoma Cells—Dimeric tubulin rapidly associates with the plasma membrane in response to carbachol stimulation (4, 37). Cellular imaging demonstrates that 15 min after carbachol addition tubulin colocalizes with Gβγ in vesicle-like structures in the cytosol (8). To analyze biochemically the sequence of these events, temporal patterns of colocalization of tubulin, Gαq, and Gβγ at the membrane and in the cytosol were studied in intact SK-N-SH neuroblastoma cells (Fig. 1). Cells were incubated with carbachol for different time periods, and the content of tubulin, Gαq, and Gβγ in membrane and cytosolic fractions was analyzed by immunoblotting.

As previously observed (4), tubulin translocated to the plasma membrane in response to m3 muscarinic receptor activation (Fig. 1A). Two minutes after carbachol addition membrane-associated tubulin increased by 243% (n = 4) and gradually decreased subsequent to this time point. Ten minutes...
indicated times with 100 μM carbachol stimulation of SK-N-SH cells. Cells were incubated for the
10-min time point, Gβq decreased by 33% (n = 4) compared with the initial quantity of this protein. This
corresponded to a Gβ increase in the cytosol. Neither tubulin nor Gβ translocation occurred when the cells were pretreated
with 10 μM atropine.

In contrast, membrane or cytosolic distribution of Gaq did not significantly change during the course of carbachol stimulation
(Fig. 1C). This observation was in line with previous findings showing a lack of Gaq translocation to the cytosol
after receptor stimulation in neurons or in transiently transfected HEK 293 cells (39, 40). Thus, although tubulin was recruited
to the plasma membrane to transactivate Gaq in response to carbachol stimulation (4, 37), it translocated back
to the cytosol with Gβγ subunits but not Gaq.

Confocal microscopy was used to verify these findings. The intracellular localization of tubulin, Gaq, and Gβγ was studied
during the course of carbachol stimulation of intact SK-N-SH cells (Fig. 2). In unstimulated cells, Gaq and Gβγ colocalized at focal spots of the plasma membrane, presumably where Gaq/Gβ heterotrimers were located (Fig. 2A). However, significant
Gaq/Gβ colocalization was also observed in the perinuclear region of the cells. Two minutes after carbachol application, the areas of Gaq/Gβ colocalization at the plasma membrane appeared somewhat merged and significantly broader. At the
15-min time point, Gaq and Gβ colocalized predominantly in the perinuclear region. Gβ was also seen in vesicle-like structures
in the cytosol. However, Gaq was not detected at these intracellular locations.

Next, tubulin colocalization with Gaq or Gβγ was evaluated (Fig. 2, B and C). In unstimulated cells tubulin colocalized with
Gaq in the perinuclear area of the cells as well as along microtubules (Fig. 2B). This was concordant with previous observations
demonstrating Gaq, Gaq, and Gaq decoration of microtubules in cell-free system and in PC12 cells (6, 41). Gβγ sparsely
colocalized with tubulin in untreated SK-N-SH cells. However, 2 min after carbachol addition, tubulin associated with the
plasma membrane and colocalized with both Gaq and Gβγ (Fig. 2, B and C). Fifteen minutes post-carbachol, significant colo-
calization of tubulin and Gβγ, but not Gaq, was observed in vesicles in the cytosol. Gaq was again seen along microtubules.
Magnified images of SK-N-SH cells are shown to underline the differences between tubulin/Gβγ and tubulin/Gaq colocalization
during the course of carbachol stimulation (Fig. 2D). These findings confirmed the biochemical observations showing a cor-
relation between tubulin and Gβγ, but not Gaq, translocation to the cytosol (Fig. 1). It appeared that the pool of cytosolic Gaq
was different from that at the plasma membrane, since membrane Gaq did not appear to internalize in response to agonist
stimulation (Refs. 39 and 40 and herein). Thus, it appeared that tubulin engaged in revolving interactions with Gaq and
Gβγ during the course of PLCβ signaling.

**Tubulin and Gβγ Internalize through Clathrin-mediated Endocytic Mechanism**—Both biochemical and cellular imaging
approaches were utilized to identify the nature of endocytic vesicles containing tubulin and Gβγ. The Gaq-coupled m3 musca-
ricin receptors of neuroblastoma cells internalize through clathrin-mediated endocytosis (42). We tested whether tubulin
and/or Gβγ utilized this endocytic pathway.

Clathrin presence at the membrane and in the cytosol was investigated during the course of carbachol stimulation of in-
tact SK-N-SH cells (Fig. 3). A striking similarity with the pattern of translocation of tubulin and Gβγ was observed. Similar-
ly to tubulin, clathrin association with the membrane increased within 2 min of carbachol addition (220%, n = 4). Moreover, clathrin translocated back to the cytosol after 10 min

![Graph A](image1.png)

**Graph A**

**Graph B**

**Graph C**

![Graph B](image2.png)

**Graph C**

**Fig. 1.** Redistribution of tubulin, Gβγ, and Gaq during carbachol stimulation of SK-N-SH cells. Cells were incubated for the
indicated times with 100 μM carbachol, as described. Membrane and cytosol fractions were subjected to SDS/PAGE (50 μg of membrane
protein in each lane) and immunoblotting with anti-α-tubulin (A), anti-Gβγ (B), or anti-Gaq (C) antisera, as described under “Experimental
Procedures.” Values are the means ± S.E. of four independent experiments with similar results. A representative experiment is shown on
the top of each graph. When 10 μM atropine was applied before carbachol, no redistribution of any protein was detected. While tubulin associ-
ated with the membrane after 2 min of carbachol exposure of the cells, it decreased somewhat in the cytosol. Gβγ release in the cytosol followed
tubulin increase in this fraction (seen at 10 and 15 min of carbachol addition), whereas Gaq remained unchanged both at the membrane and
in the cytosol.

![Diagram A](image3.png)

**Diagram A**

![Diagram B](image4.png)

**Diagram B**

![Diagram C](image5.png)

**Diagram C**

![Diagram D](image6.png)

**Diagram D**

**Fig. 2.** Confocal microscopy of SK-N-SH cells showing tubulin/Gβγ and tubulin/Gaq redistribution. (A) Redistribution of tubulin,
Gβγ, and Gaq is shown in untreated cells at indicated time points. (B) Redistribution of tubulin/Gβγ and tubulin/Gaq at indicated time
points in carbachol-stimulated cells. (C) Redistribution of tubulin/Gβγ and tubulin/Gaq at indicated time points in carbachol-stimulated
cells with 10 μM atropine. (D) Magnification of tubulin/Gβγ and tubulin/Gaq colocalization during carbachol stimulation.
Fig. 2. Colocalization patterns of tubulin, Gaq, and Gβ during carbachol stimulation of SK-N-SH cells. Cells were treated with 100 μM carbachol for the periods indicated before fixation and immunostaining, as described under “Experimental Procedures.” When 10 μM atropine was applied before carbachol, the images were identical to control cells. Confocal micrographs of untreated (0 min) and carbachol-treated (2 and 15 min) cells are shown. Confocal images of 1-μm thick sections at the same level within the cell are compared. For each experimental condition, three independent experiments with similar results were performed. Images shown are representative of ~60 cells examined at each time point. A, colocalization of Gaq and Gβ. Gaq appears in green, and Gβ is in red. Areas of Gaq/Gβ colocalization appear in yellow. In unstimulated cells Gaq and Gβ colocalized in the perinuclear region and at highly localized sites of the cell membrane. Their membrane colocalization increased after the addition of agonist (2 min). Gβ, but not Gaq, was seen in vesicles in the cytosol (15 min). B, colocalization of tubulin and Gβ. Gβ appears in green, and tubulin is in red. Areas of tubulin-Gβ colocalization are seen in yellow. Carbachol-induced tubulin-Gβ colocalization at the cell membrane was greatest after 2 min. The two proteins colocalized in vesicles in the cytosol at 15 min of agonist stimulation. C, colocalization of tubulin and Gaq. Gaq appears in green, and tubulin is in red. Areas of tubulin-Gaq colocalization are seen in yellow. Tubulin and Gaq colocalized along microtubules. At 2 min of agonist exposure, depolymerization of microtubules and tubulin-Gaq colocalization at the cell membrane were observed. D, magnified images of control and carbachol-treated SK-N-SH cells labeled for Gβ and Gaq. Tubulin colocalization with both proteins at the plasma membrane at 2 min of agonist stimulation is apparent. At 15 min Gβ is at the plasma membrane and colocalized with tubulin in vesicle-like structures in the cytosol, whereas Gaq is at the membrane and along the microtubules, as seen in the unstimulated cells.
of agonist stimulation, as did tubulin and Gβ (Fig. 1). These results suggested agonist-dependent internalization of tubulin and Gβ with the clathrin-coated vesicles.

Clathrin colocalization with tubulin and Gβ in SK-N-SH cells was studied by confocal microscopy (Fig. 4). Gaq was used as negative control, since it did not internalize (43). In untreated cells clathrin did not colocalize with tubulin, Gβ, or Goq. However, 2 min after carbachol addition, clathrin translocated to the plasma membrane and colocalized with Gaq and Gβ (Fig. 4, B and C) as well as membrane-associated tubulin (Fig. 4A). Although areas of colocalization of clathrin and Gaq were broad and not well defined, colocalization with tubulin and Gβ was found in pit-like regions of the plasma membrane.

Vesicles containing clathrin and Gβ as well as clathrin and tubulin were also observed in proximity to the cellular cortex. Fifteen minutes after carbachol stimulation, both tubulin and Gβ were seen in clathrin-coated vesicles throughout the cytosol (Fig. 4, A and B). Gaq was not present in these cellular structures (Fig. 4C). All carbachol-mediated processes were inhibited by atropine (10 μM). Thus, internalization of membrane-associated tubulin and Gβ appeared to proceed through clathrin-mediated endocytosis.

To test if clathrin, tubulin, and Gβγ were internalized together, coimmunoprecipitation of clathrin with tubulin, Gβ, or Goq from the cytosol of SK-N-SH cells was tested before and 15 min after carbachol stimulation (Fig. 5). Tubulin, but not Gβ, coimmunoprecipitated with clathrin from the cytosol of untreated cells. Fifteen minutes after agonist addition tubulin/clathrin coimmunoprecipitation increased by 258% (n = 4). Although Gβ also coimmunoprecipitated with cytosolic clathrin after carbachol stimulation, Goq did not either before or after agonist addition. This experiment supported the view that carbachol-evoked involvement of tubulin in Goq-mediated signaling was followed by tubulin internalization with Gβγ through a clathrin-mediated endocytic mechanism.

Complexes of m3 Muscarinic Receptor, Gβγ, and Tubulin Internalize with the Clathrin-coated Vesicles—Gβγ subunits might mediate tubulin internalization through their interaction with both tubulin (5, 8) and the m3 muscarinic receptors (22). Although tubulin does not appear to bind to Goq-coupled muscarinic receptors (37), Gβγ interacts with the third intracellular loop of the m3 type (22). Thus, Gβγ might “bridge” tubulin internalization with these activated receptors. Under such a scenario, complexes of tubulin-Gβγ-m3 muscarinic receptors should coimmunoprecipitate with clathrin-coated vesicles from the cytosol of agonist-stimulated cells.

Cytosolic fractions of SK-N-SH cells were tested for coimmunoprecipitation of m3 muscarinic receptors with Gβ, tubulin, and clathrin before and 15 min after carbachol stimulation (Fig. 6). As seen for the m1 muscarinic receptor (37), tubulin did not coimmunoprecipitate with the m3 receptors of unstimulated cells. However, 15 min after carbachol addition an increase in coimmunoprecipitation of m3 receptors with Gβ, clathrin, and tubulin from the cytosol was observed. This observation was consistent with the notion that m3 muscarinic receptor-Gβγ-tubulin complexes might internalize through clathrin-mediated endocytosis after carbachol stimulation of the SK-N-SH cells.

This hypothesis was tested by confocal microscopy. Because m3 muscarinic receptors interact with Gβγ (22) and are shown to internalize with clathrin-coated vesicles in neuroblastoma cells (42), their potential agonist-evoked colocalization with tubulin was investigated (Fig. 7). The m3 receptors did not colocalize with tubulin in untreated SK-N-SH cells. However, 2 min after carbachol addition they colocalized with membrane-associated tubulin. At 15 min of agonist exposure colocalization of m3 receptors and tubulin was seen in vesicle-like formations in the cytosol and along microtubules. These results supported the view that the internalization of agonist-activated m3 muscarinic receptors involves membrane-associated tubulin.

Dominant-negative Dynamin Inhibits Tubulin Translocation during Carbachol Stimulation—Because internalization of m3 muscarinic receptors depends on dynamin (11) and tubulin interacts with this essential endocytic protein (33) we tested whether a dominant-negative dynamin I construct, K44E, would affect tubulin internalization after agonist exposure. Dynamin K44E binds GTP poorly (35) and, thus, does not pinch vesicles from the plasma membrane and inhibits agonist-evoked internalization. As seen in Fig. 8, wild type dynamin I coimmunoprecipitated with tubulin at the plasma membrane at 2 min of carbachol stimulation, and both proteins translocated to vesicle-like structures in the cytosol after 15 min of agonist exposure. To the contrary, tubulin did not colocalize with dynamin K44E at the plasma membrane or in vesicles in the cytosol during the course of carbachol stimulation. Thus, similarly to the activated m3 muscarinic receptors (11), the agonist-evoked translocation of tubulin was a dynamin-dependent process.

DISCUSSION

Internalization of receptors and other cell surface components in neuronal cells occurs via clathrin-mediated endocytosis, although other less characterized pathways are also reportedly involved. Receptors internalized in clathrin-coated vesicles are subsequently delivered to early endosomes, where they are sorted to recycle back to the plasma membrane or be transported to late endosomes/lysosomes for degradation. Other proteins involved in signal transduction, like GRKs and β-arrestins, are also found in the clathrin-coated vesicles (9, 44). GRKs phosphorylate activated receptors, which allows for the binding of β-arrestins. The latter interact with both the heavy chain of clathrin and the AP2 complex and, thus, sequester activated receptors to the clathrin-coated pits for internalization (9, 10).

Similarly to GRKs and β-arrestins, tubulin associates with the plasma membrane in response to agonist stimulation (4, 37). However, membrane-associated tubulin performs a differ-
ent function, which is to transactivate \( \text{G}_{\alpha_q} \) and, thus, initiate PLC_\beta_1 signaling. Although the fate of membrane-associated tubulin after termination of PLC_\beta_1 signaling has not been

**FIG. 5.** Clathrin colocalization with tubulin, G\( \beta \), and Go_q during carbachol stimulation of SK-N-SH cells. Cells were treated with 100 \( \mu \)M carbachol for the periods indicated before fixation and immunostaining, as described under “Experimental Procedures.” When 10 \( \mu \)M atropine was applied before carbachol, the images were identical to control cells. Confocal micrographs of untreated (0 min) and cells treated with carbachol (2 and 15 min) are shown. Confocal images of 1-\( \mu \)m-thick sections at the same level within the cell are shown. Each experimental condition was tested in three independent experiments with similar results. The images shown are representative of ~60 cells examined at each time point. A, colocalization of tubulin and clathrin. Clathrin appears in green, and tubulin is in red. Areas of clathrin/tubulin colocalization appear in yellow; colocalization increased at the cell membrane after 2 min of agonist stimulation. At 15 min clathrin and tubulin colocalized predominantly in vesicles in the cytosol. B, colocalization of clathrin and G\( \beta \). Clathrin appears in green, and G\( \beta \) is in red. Areas of clathrin-G\( \beta \) colocalization are seen in yellow. Carbachol-induced clathrin-G\( \beta \) colocalization was greatest in vesicles in the cytosol 15 min after agonist exposure of the cells. C, lack of colocalization of clathrin and Go_q. Go_q appears in green, and clathrin is in red. Areas of clathrin-Go_q colocalization are seen in yellow. After carbachol stimulation Go_q was observed at the cell membrane but not in clathrin vesicles in the cytosol.

**FIG. 6.** m_3 muscarinic receptor coimmunoprecipitation (IP) with tubulin, G\( \beta \), and clathrin from the cytosol increases after carbachol stimulation of SK-N-SH cells. Cytosolic fractions of SK-N-SH cells were tested before and 15 min after stimulation with 100 \( \mu \)M carbachol, as described under “Experimental Procedures.” It is suggested that protein complexes containing these proteins are immunoprecipitated from the cytosol after agonist exposure. The figure is representative of four independent experiments with similar results.
clarified, a decrease in tubulin at the plasma membrane beginning 5 min after \( m_1 \) or \( m_3 \) muscarinic receptor stimulation has been observed (4, 37).

Here we demonstrate that tubulin associated with the plasma membrane in response to agonist stimulation of SK-N-SH neuroblastoma cells internalized through clathrin-mediated endocytic mechanism. It also appeared that this tubulin participated in the clathrin-mediated internalization of the \( m_3 \) muscarinic receptors, perhaps through their mutual interaction with \( G_\beta\gamma \).
Gβγ subunits are multifunctional complexes that are involved in variety of signal transduction mechanisms. Although Gβγ assist agonist-evoked membrane translocation of GRKs (26), they do not support the membrane association of tubulin in response to agonist stimulation (8). To the contrary Gβγ appears involved in dissociation of tubulin-GDP from the plasma membrane at the offset of PLCγ1 signaling. This hypothesis was tested in the present study.

Both biochemical experiments and cellular imaging indicated plasma membrane colocalization and subsequent cointernalization of tubulin and Gβγ after carbachol stimulation of SK-N-SH neuroblastoma cells (Fig. 1). The patterns of tubulin and Gβγ translocation to the cytosol were identical to that of clathrin (Figs. 1 and 3), and both proteins were found in the clathrin-coated vesicles (Fig. 4). Thus, it appeared that tubulin-Gβγ complexes might internalize through a clathrin-mediated endocytic mechanism. This was supported by the observation that coimmunoprecipitation of tubulin, Gαq, and clathrin from the cytosol of carbachol-stimulated cells increased after 15 min of agonist exposure (Fig. 5). This endocytic mechanism did not involve Gαq, which did not appear to dissociate from the plasma membrane (Refs. 39 and 40; Fig. 1) and was not found in the clathrin-coated vesicles (Fig. 4). Gαq-clathrin coimmunoprecipitation from the cytosol was also not observed (Fig. 5).

These findings are supported by previous observations showing association of tubulin and clathrin with the plasma membrane in response to receptor activation (37, 45). Gβγ involvement in clathrin-mediated endocytosis has also been reported since Gβγ capture by overexpressed Gα strongly inhibits this process (46). Thus, Gβγ binding of tubulin at the membrane (8) might engage tubulin in the clathrin-mediated endocytic pathway at the offset of PLCγ1 signaling.

Activated m1 and m3 receptors sequester through a clathrin-mediated endocytotic mechanism (42), and muscarinic receptors are present in coated vesicles from bovine brain (47–49). Amino acids 286–292 in the 3rd intracellular loop of m1 muscarinic receptors are involved in receptor internalization (50). A similar region in the closely related m3 receptor (residues 289–330) binds Gβγ (22). M3 receptor constructs lacking Gβγ binding motifs sequester significantly less than wild type receptors, although they retain ligand recognition properties and the ability to increase intracellular calcium (22). In addition, although agonist-induced phosphorylation and uncoupling of m3 muscarinic receptors from Gαq/11 in SH-SY5Y neuroblastoma cells is significantly attenuated by expression of kinase-dead mutant of GRK6, their internalization is not (51). Thus, it appeared that in SK-N-SH cells, Gβγ subunits rather than GRK-β-arrestin might be involved in internalization of m3 receptors with clathrin-coated vesicles. It is noteworthy in this regard that, although Gq-coupled muscarinic receptors interact with Gβγ (22), they do not appear to interact directly with tubulin (37). Gβγ could bridge these molecules.

The possible link between internalization of Gβγ-tubulin complexes and m3 muscarinic receptors was investigated. Although tubulin did not colocalize with m3 muscarinic receptors of unstimulated cells, both were found in vesicles in the cytosol 15 min after carbachol stimulation (Fig. 7). Moreover, m3 receptors coimmunoprecipitated with Gβγ, tubulin, and clathrin from cytosol of agonist-stimulated cells (Fig. 6). This suggested that m3 muscarinic receptor-Gβγ-tubulin complexes internalized through the clathrin-mediated endocytic pathway. These observations supported the view that Gβγ subunits might serve as docking units within larger protein assemblies involved in sequestration of m3 muscarinic receptors (22).

Internalization of m3, m13, and m4 muscarinic as well as 5-hydroxytryptamine 2A (5-HT2A) or gonadotropin-releasing hormone (GnRH) receptors is reported to depend on dynamin but not β-arrestin (11, 13, 52). The third intracellular loop of m3 receptors appears responsible for this arrestin-resistant, dynamin-sensitive internalization (11). Because Gβγ binds to this region of m3 receptors (22), it might assist receptor sequestration in the clathrin-coated pits instead of β-arrestin. Both Gβγ subunits and β-arrestins have scaffolding properties (22, 53). We propose that tubulin is an indispensable part of m3 muscarinic receptor sequestration since it binds both Gβγ (5, 8) and dynamin (33). The finding that dominant-negative dynamin inhibits tubulin translocation in response to agonist stimulation (Fig. 8) supports this hypothesis. Thus, tubulin-Gβγ complexes might recruit m3 muscarinic receptors to the clathrin-coated pit assembly instead of β-arrestin. It should be noted, however, that β-arrestin- and dynamin-dependent internalization of m3-m3 muscarinic receptors has also been reported (54).

Cell type- or receptor type-specific differences in intracellular signaling mechanisms might account for this discrepancy. It is also possible that both mechanisms operate in the cell depending on the stimulus or specific experimental conditions. It is also noteworthy that phosphoinositide-binding proteins like β-arrestin tend to concentrate in clathrin-coated vesicles (55). Tubulin binds phosphatidylinositol 4,5-bisphosphate, and this interaction is instrumental in tubulin association with the plasma membrane and regulation of PLCγ1 signaling (7). Such interaction might also contribute to the m3 receptor-Gβγ-tubulin sequestration in the clathrin-coated pits of the plasma membrane. Phosphatidylinositol 4,5-bisphosphate dependence of agonist-evoked endocytosis of m3 receptors in SH-SY5Y neuroblastoma cells has been demonstrated (56).

In conclusion this study presents evidence that tubulin involved in the regulation of PLCγ1 signaling after agonist stimulation of SK-N-SH neuroblastoma cells internalized through a clathrin-mediated endocytic mechanism. This tubulin was apparently involved in the β-arrestin-independent, but dynamin-dependent internalization of m3 muscarinic receptors through its interaction with both Gβγ and dynamin. In turn, Gβγ interaction with both m3 muscarinic receptors and tubulin might scaffold internalizing complexes. Gαq did not appear to undergo clathrin-mediated internalization, although it is transactivated by tubulin after agonist stimulation. It is proposed that Gβγ and tubulin are instrumental in β-arrestin-independent mechanism of receptor internalization that involves dynamin. This novel regulatory mechanism might add to the understanding of receptor internalization as well as cross-regulation between intracellular signaling and the dynamics of the microtubule cytoskeleton.

Acknowledgments—We thank Drs. R. Vallee and M. von Zastrow for the generous gift of material. S. A. K. Chowdhury and C. Khercis are thanked for valuable technical assistance.

REFERENCES
1. Wang, N., Yan, K., and Rasenick, M. M. (1990) J. Biol. Chem. 265, 1239–1242
2. Roychowdhury, S., and Rasenick, M. M. (1994) Biochemistry 33, 9809–9815
3. Popeva, J., Johnson, G. L., and Rasenick, M. M. (1994) J. Biol. Chem. 269, 21748–21754
4. Popeva, J. S., and Rasenick, M. M. (2000) J. Neurosci. 20, 2774–2782
5. Roychowdhury, S., and Rasenick, M. M. (1997) J. Biol. Chem. 272, 31576–31581
6. Roychowdhury, S., Panda, D., Wilson, L., and Rasenick, M. M. (1999) J. Biol. Chem. 274, 13485–13490
7. Popeva, J. S., Greene, A. K., Wang, J., and Rasenick, M. M. (2002) J. Neurosci. 22, 1668–1678
8. Popeva, J. S., and Rasenick, M. M. (2003) J. Biol. Chem. 278, 34299–34308
9. Goodman, O. B., Krupnick, J. G., Santini, F., Gurevich, W., and Penn, R. B., Gagnon, A. W., Keen, J. H., and Benovic, J. L. (1996) Nature 383, 447–450
10. Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S., and Caron, M. G. (2000) J. Biol. Chem. 275, 23120–23126
11. Lee, K. B., Pals-Rylaarsdam, R., Benovic, J. L., and Hosey, M. M. (1998) J. Biol. Chem. 273, 12967–12972
12. Veel, M., Anderson, J., Haryaloglu, A., McGрегor, A. M., Groarke, A. D., Milligan, G., Taylor, P. L., and Eidne, K. A. (1998) Mol. Endocrinol. 12, 1812–1829

Tubulin and Phospholipase C Signaling

30417
Clathrin-mediated Endocytosis of m3 Muscarinic Receptors: ROLES FOR Gβγ AND TUBULIN
Juliana S. Popova and Mark M. Rasenick

J. Biol. Chem. 2004, 279:30410-30418.
doi: 10.1074/jbc.M402871200 originally published online April 26, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402871200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 56 references, 39 of which can be accessed free at http://www.jbc.org/content/279/29/30410.full.html#ref-list-1