Although the β-adrenergic receptor kinase (βARK) mediates agonist-dependent phosphorylation and desensitization of G protein-coupled receptors, recent studies suggest additional cellular functions. During our attempts to identify novel βARK interacting proteins, we found that the cytoskeletal protein tubulin could specifically bind to a βARK-coupled affinity column. In vitro analysis demonstrated that βARK and G protein-coupled receptor kinase-5 (GRK5) were able to stoichiometrically phosphorylate purified tubulin dimers with a preference for β-tubulin and, under certain conditions, the βIII-isotype. Examination of the GRK/tubulin binding characteristics revealed that tubulin dimers and assembled microtubules bind GRKs, whereas the catalytic domain of βARK contains the primary tubulin binding determinants. In vitro interaction of GRK and tubulin was suggested by the following: (i) co-purification of βARK with tubulin from brain tissue; (ii) co-immunoprecipitation of βARK and tubulin from COS-1 cells; and (iii) co-localization of βARK and GRK5 with microtubule structures in COS-1 cells. In addition, GRK-phosphorylated tubulin was found preferentially associated with the microtubule fraction during in vitro assembly assays suggesting potential functional significance. These results suggest a novel link between the cytoskeleton and GRKs that may be important for regulating GRK and/or tubulin function.

Tubulin, along with a variety of associated proteins, constitutes microtubules, a major component of the cytoskeleton. Tubulin exists principally in two forms, as either cytosolic soluble tubulin heterodimers consisting of various α- and β-tubulin isotypes or as insoluble assembled tubulin polymers (microtubules) (1). The ability of tubulin to cycle between these two states is one of its most fundamentally important features. In cells, microtubules are involved in such diverse and dynamic functions as maintaining cell shape, endocytosis, exocytosis, vesicle trafficking, cellular transport, and mitosis (1). These dynamic functions require the coordinated regulation of microtubule function and assembly, a process thought to reflect integration of various extracellular signals (2). Indeed, many studies have revealed functional relationships between tubulin and various cellular signaling molecules. For example, tyrosine kinases such as the insulin receptor and c-Src, as well as second messenger-responsive kinases such as the cAMP-dependent and Ca2⁺/calmodulin-dependent kinases, can regulate microtubule function and/or assembly through phosphorylation (Ref. 3 and references therein). Moreover, a series of recent studies have demonstrated direct binding interactions between Gα and Gβγ subunits and tubulin (4–6) suggesting new modes of regulation for microtubule assembly. Other studies provide examples of tubulin or microtubules regulating either the activity or the localization of signaling molecules such as the A1 adenosine (7) and γ-aminobutyric acid (8) receptors, phospholipase C-β1 (9), and Ki-Ras (10). Taken together, these studies provide support for a novel paradigm whereby information flow appears to be bi-directional between signaling and cytoskeletal molecules.

β-Adrenergic receptor kinase (βARK)† is a member of a family of G protein-coupled receptor kinases (GRKs) that includes rhodopsin kinase (GRK1), βARK (GRK2), βARK2 (GRK3), GRK4, GRK5, and GRK6. GRKs phosphorylate the agonist-activated form of G protein-coupled receptors which in turn promotes the high affinity binding of a second family of proteins, called arrestins (11). This process functions to both uncouple the G protein from the receptor and to promote receptor endocytosis via clathrin-coated pits. βARK is one of the best characterized GRKs and has been shown to be important for the phosphorylation and desensitization of a variety of receptors (11). Moreover, βARK activity appears to be regulated through interactions with both free heterotrimeric Gβγ subunits (12, 13) and negatively charged membrane phospholipids (14–16). These interactions are thought to be important largely for their ability to promote translocation of βARK to the plasma membrane, facilitating interaction with receptor substrates. Recent studies have provided novel information regarding the function and cellular localization of βARK. Specifically, it was shown that βARK can traffic along with β2-adrenergic receptors to the endosome following receptor activation (17). Mayor and co-workers (18) have also demonstrated an association of βARK with microsomes that appears to be mediated via an unidentified βARK-binding protein. Furthermore, βARK has been implicated to function in developmental processes involved in cardiogenesis through currently undefined mechanisms (19). Given these and other areas of inquiry, it seems likely that additional cellular substrates and regulators of βARK exist. Thus, in this study a biochemical approach was employed to explore this possibility. We describe here the identification of tubulin as a novel GRK substrate and present biochemical and cellular characterization of GRK/tubulin interaction.

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1 The abbreviations used are: βARK, β-adrenergic receptor kinase; GRK5, G protein-coupled receptor kinase-5; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; HA, hemagglutinin; PPT, Partially purified tubulin; PIPES, 1,4-piperazinediethanesulfonic acid; PBS, phosphate-buffered saline.
**EXPERIMENTAL PROCEDURES**

**Materials**—Dodecylmaltoside, urea, and protein A-agarose were from Boehringer Mannheim, and [32P]ATP was from NEN Life Science Products. Anti-α-tubulin monoclonal antibody and enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech. Polyvinylidene difluoride membrane was purchased from Amersham Life Systems. Nitrocellulose was from MSI, and LipofectAMIN™ and cell culture medium were from Life Technologies Inc. Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies and Affi-Prep 10 affinity chromatography matrix were from Bio-Rad. Horseradish peroxidase-conjugated horse anti-mouse antibody was from Vector Laboratories. Hemagglutinin (HA)-specific polyclonal antibody was from Babco. GRK5-specific polyclonal antibody was from Santa Cruz Biotechnology. Fluorescein isothiocyanate-conjugated anti-mouse antibody, peroxidase-conjugated antihuman antibody, and Slowfade™ mounting medium were from Molecular Probes Inc. PGEX-2T GST gene fusion vector was from Amersham Pharmacia Biotech. ATP, GTP, glutathione-agarose beads, paclitaxel (Taxol), crude soybean phosphatidylcholine, β-mercaptoethanol, iodoacetic acid, and all other chemicals were from Sigma.

**Protein Expression and Purification**—βARK and GRKs were overexpressed in and purified from Sf9 insect cells (20, 21). Purified rhodopsin kinase was generously provided by Drs. J. Pitcher and R. Lefkowitz, and purified αGβγ2 was generously provided by Dr. S. P. Kennedy. Partially purified tubulin (PT) containing microtubule-associated proteins was prepared from porcine brain by one three cycles of temperature-dependent microtubule assembly/disassembly as described previously (6, 22). Purified tubulin, prepared from bovine brain by assembly/disassembly by phosphorylation-conjugated chromotography, and an agarose-free of microtubule associated proteins (23), was from Cytokeleton and microSuppliers.

**Gel Electrophoresis and Immunoblotting**—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using standard methods (24). Following electrophoresis, proteins were electroblotted onto nitrocellulose. The membrane was then incubated in blocking buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02% Tween 20, and 5% non-fat dried milk) at 22 °C for 1 h followed by probing with either an α-tubulin-, βARK-, GRK5-, or arsatin-specific primary antibody diluted in blocking buffer. Membranes were subsequently probed with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody in blocking buffer for 20–60 min at 22 °C. All blots were developed by ECL following the manufacturer’s guidelines.

**Synthesis of βARK-coupled Affinity Resin**—50 ml of Affi-Prep 10 affinity chromatography matrix (20% w/v) was incubated with or without βARK at a final concentration of 0.3 mg/ml in 20 ml HEPES, pH 7.2, 5 mM EDTA, 0.02% Triton X-100 for 5 h at 4 °C. The coupling reaction was stopped by addition of 5 ml of 1 M Tris-HCl, pH 7.5, and incubation overnight at 4 °C. A trace amount of [35S]methionine-labeled ARK was included in the initial coupling reaction to enable a determination of the coupling efficiency (~1% of ARK resin). The ARK resin was then washed extensively with 20 ml HEPES, pH 7.2, 200 mM NaCl, 5 mM EDTA, 0.02% Triton X-100 and stored at 4 °C.

**Identification of βARK-binding Proteins**—Fresh bovine calf brain was stripped of connective tissue and miced in 200 ml of homogenization buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 mM NaCl, 5 mM benzamidine, 5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.02% Triton X-100) using a Brinkman Polytron (14,000 rpm, 30 s). The homogenate was centrifuged at 45,000 × g for 20 min and the supernatant was centrifuged at 300,000 × g for 60 min. The final supernatant was aliquoted and stored at −70 °C until use. One ml of βARK-coupled affinity resin (20% w/v) or control resin was incubated with 10 ml of the soluble brain extract (or buffer) for 2 h at 4 °C. The incubation mixture was centrifuged at 1000×g for 1 min, and the supernatant was washed four times with buffer (20 mM HEPES, pH 7.5, 5 mM EDTA, 100 mM NaCl, 0.02% Triton X-100). Bound proteins were released from the matrix by addition of 50 μl of SDS sample buffer and 100 μl of water to the final pellet followed by boiling for 10 min. The samples were then subjected to 7.5% SDS-PAGE, and the separated proteins were transferred to a polyvinylidene difluoride membrane. A specific 55-kDa protein band was identified by Ponceau S staining, excised, and then submitted to the Harvard Microchemistry Facility for sequence analysis.

**Phosphorylation Assay**—Phosphorylation reactions contained, in a total reaction volume of 20–50 μl, 0.025–0.2 μM βARK, GRK5, or rhodopsin kinase, 0.025–8 μM purified tubulin dimers, 100 μM [γ-32P]ATP (2.0–7.5 cpm/μmol), 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 7.5 mM MgCl2 in the absence or presence of 0.2 μM αGβγ2 and phosphatidylcholine (20 μg)-dodecylmaltoside (10 μM) mixed micelles (16). Reactions were incubated at 30 °C for 5–90 min, stopped with SDS sample buffer, and subjected to 10% SDS-PAGE. After autoradiography, the 32P-labeled tubulin bands were excised and counted to determine the picomoles of phosphate transferred. The 32P-labeled tubulin bands were excised and counted to determine the picomoles of phosphate transferred. Enzymes were immobilized on glutathione-agarose beads were incubated with 0.4 μg of soluble tubulin dimers in 100 μl of binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.02% Triton X-100, 5% GTP) for 45 min. Reactions were then chilled on ice for 5 min followed by precipitation in a microcentrifuge for 10 s. The pellet was washed three times with 400 μl of binding buffer and then boiled with SDS sample buffer. Samples were subjected to 10% SDS-PAGE and Western blotting using a monclonal α-tubulin-specific antibody.

**Binding and Phosphorylation of Taxol-stable Microtubules by GRKs**—Tubulin assembly reactions contained, in a total volume of 50 μl, 60 μg of tubulin (or control buffer), 1 mM GTP, 30% glycerol, and 10 μM Taxol in 100 mM PIPES, pH 6.9, 1 mM EGTA, 1 mM MgCl2. Reactions were incubated at 37 °C for 30 min followed by pelleting at 150,000 × g for 10 min at 37 °C. The supernatant was removed and the Taxol-stable microtubule pellets were gently resuspended in 50 μl of reaction buffer (100 mM PIPES, pH 6.9, 1 mM EGTA, 7.5 mM MgCl2, 100 μM [γ-32P]ATP (5 cpm/μmol), 1 μM Taxol) in the absence or presence of 5 μg of βARK or GRK5 and incubated for 30 min at 30 °C. Tubulin was then repelleted, and both the supernatant and pellet fractions were boiled with SDS sample buffer and subjected to 10% SDS-PAGE, Coomassie Blue staining, and densitometry. After autoradiography, the 32P-labeled tubulin bands were excised and counted to determine the picomoles of phosphate transferred.

**Cell Culture and Transfection**—COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate at 37 °C in a humidified atmosphere containing 5% CO2. Cells grown to 75–95% confluency were transfected with 10 μg of either pcDNA3, pcDNA3-βARK, pcDNA3-pBC12BI, or pBC-GRK5 DNA using 65 μl of LipofectAMIN™ per T75 flask according to the manufacturer’s instructions. For immunoprecipitation experiments, cells were harvested 60 h after transfection. For immunofluorescence experiments, cells were trypsinized and plated onto 12-mm glass coverslips in a 24-well plate dish 2 h after transfection and analyzed at 24–36 h after transfection.

**Immunoprecipitation**—COS-1 cells were transfected either with pcDNA3 or pcDNA3-βARK as described above. At 60 h after transfection, T75 flask were rinsed with PBS, and the cells were scraped into 1 ml of extraction buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM diithiothreitol, 100 mM NaCl, 0.02% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin) and lysed by freeze-thaw at −70 °C. Lysates were then centrifuged at 55,000 × g for 1 h at 4 °C, and the supernatant was stored at −70 °C. For immunoprecipitation, 100 μl of lysate was incubated with either a βARK- or HA-specific polyclonal antibody or in the absence of antibody for 30 min at 4 °C followed by addition of 50 μl of 50% protein A-agarose pre-equilibrated in extraction buffer and an additional 60 min incubation at 4 °C. Samples were then centrifuged at 2000 rpm for 30 s in a microcentrifuge. The pellet was washed three times with extraction buffer for 30 min at 4 °C and fixed in 4% paraformaldehyde in PBS sample buffer. Samples were subjected to 10% SDS-PAGE and Western blot analysis using either βARK-specific or α-tubulin-specific monoclonal antibodies.

**Immunofluorescence Microscopy**—Transfected COS-1 cells grown on glass coverslips were rinsed twice with PBS at 37 °C and fixed with methanol at −20 °C for 10 min. Alternatively, some cells were preincubated with Dulbecco’s modified Eagle’s medium containing 10 μM (−)
isoproteonol for either 20 or 60 min at 37 °C prior to fixation. Fixed cells were subsequently rinsed three times with phosphate-buffered saline (PBS) and permeabilized with PBS containing 0.2% Triton X-100 for 10 min at 22 °C. These cells were blocked for 30 min at 37 °C with PBS containing 0.05% Triton X-100 (PBS/Triton) and 5% non-fat dried milk and subsequently incubated with an α-tubulin-specific mouse monoclonal antibody and either a βARK-GRK5, or arrestin-3-specific rabbit polyclonal antibody diluted in the same buffer for 1 h at 37 °C. Cells were rinsed three times with PBS/Triton and then incubated at 37 °C for 30 min. The cells were then incubated with a tetramethylrhodamine-conjugated goat anti-mouse antibody and a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody both diluted 1:100 in PBS/Triton and 5% non-fat dried milk for 1 h at 37 °C. Cells were rinsed five times with PBS/Triton and then incubated at 37 °C for 30 min followed by fixing with methanol at −20 °C for 5 min. Cells were rinsed with PBS and mounted on a slide with Slowfade TM mounting medium. Fluorescence microscopy was performed on a Bio-Rad MRC600 laser scanning confocal microscope (Hemmelholsteadt, UK) attached to a Zeiss Axiovert 100 microscope, using a Zeiss Plan-Apo 63×1.40 NA oil immersion objective at a zoom factor of 1.4.

Assembly of GRK-phosphorylated Tubulin Dimers—Purified tubulin dimers were subjected to a 10 min pre-spin at 150,000 × g at 4 °C to precipitate any existing tubulin aggregates. The supernatant was removed and utilized in the subsequent phosphorylation assay. Phosphorylation reactions (40 μl total) were incubated at 30 °C for 45 min and contained 60 μg of tubulin dimers, 200 μM GTP, 100 mM PIPES, pH 6.9, 1 μM EGTA, and 3 mM MgCl₂, in the absence or presence of 100 μM GDPγS or GDPβS and 4 μg of βARK or GRK5. Assembly of phosphorylated or control tubulin was initiated by the addition of glycerol (5–30% final concentration) and raising the temperature to 37 °C for 5–60 min. Assembly was quantitated by pelleting the microtubules at 150,000 × g for 10 min at 37 °C. The supernatant and pellet fractions were then subjected to 10% SDS-PAGE, Coomassie Blue staining, and densitometry. Distribution of the 32P-labeled tubulin was assessed by autoradiography, followed by excision and counting of the radiolabeled bands.

RESULTS AND DISCUSSION

Identification of Tubulin as a βARK-interacting Protein—In an effort to identify novel GRK-interacting proteins, a crude bovine brain cytosolic extract was chromatographed over a βARK affinity column. The column was washed extensively, and bound proteins were eluted with boiling SDS sample buffer and subsequently subjected to SDS-PAGE and Coomassie Blue staining. This strategy identified a total of six proteins that specifically bound to the βARK column (Fig. 1). The predominant interacting protein migrated at ~55 kDa, and additional proteins of ~50, ~46, ~44, ~38, and ~35 kDa were also observed. Since βARK is known to bind Gβγ subunits, we initially probed this blot using a Gβγ common antibody. This revealed that the ~35-kDa protein was a Gβ subunit (data not shown).

In an effort to identify the 55-kDa protein, it was transferred to a polyvinylidene difluoride membrane and subjected to tryptic digestion and sequence analysis. Two tryptic peptides were sequenced (AFHVXYVFEGMEEEGFSXAR and SGPFGQIFRPDNVFQGSGAGGN). A data base search revealed that the first peptide is 100% conserved with human α4-, mouse α3- and α6-, and chicken α1-, α3-, α5-, and α8-tubulin isotypes and corresponds to a highly conserved region in α-tubulin (residues 404–423 in human α4). The second peptide is 100% conserved with several class I (β1) (human β1, mouse β3, and chicken β7), class II (βIII) (human β2, chicken β1 and β2, rat β1, and pig βA), class III (βIII) (pig βB), and class IVa (βIVa) (human β5) α-tubulin isotypes (residues 79–101 of human β1-tubulin). Although bovine tubulin sequences remain relatively poorly characterized, based on these two peptides and their alignment with known tubulin sequences, the 55-kDa protein has been unambiguously identified as tubulin although the specific α- and β-tubulin isotypes present remains unclear.

Importantly, tubulin is known for its ability to cycle between soluble αβ dimers and insoluble assembled microtubules (1), and it is of interest to consider which form is responsible for the observed βARK binding. Although this was not assessed directly, the temperature and buffering conditions used to generate the brain extract, as well as those used in the binding experiment, inhibit tubulin assembly. Thus, it is likely that the tubulin was principally in the soluble form implying that βARK can bind soluble tubulin dimers.

Phosphorylation of Tubulin by GRKs—The βARK/tubulin interaction was confirmed by the ability of purified βARK to rapidly phosphorylate highly purified bovine brain soluble tubulin dimers to a stoichiometry of ~1.0 mol of P/mol of tubulin dimer (Fig. 2). Addition of βARK activators such as GβγG or phosphatidylcholine-dodecylmaltoside mixed micelles increased the stoichiometry to ~1.5 and ~1.6 mol/mole, respectively, or to ~2.0 mol/mole when added together. Addition of 10 mM dodecylmaltoside alone, however, was without effect (data not shown).

We also investigated the ability of tubulin to serve as a substrate for other members of the GRK family (Fig. 2). Rho- dopasin kinase weakly phosphorylated tubulin (stoichiometry of ~0.45 mol of P/mol of tubulin dimer), whereas GRK5 rapidly phosphorylated tubulin to a stoichiometry of ~1.6 mol/mol. Interestingly, whereas both GRK5 and βARK appear capable of phosphorylating at least two sites on the tubulin dimer, there are apparent differences in the nature of this phosphorylation as demonstrated by a lower mobility phosphorylated species generated in the presence of GRK5 that is not observed in the presence of βARK (Fig. 2A). This difference suggests specificity in the molecular nature of the GRK/tubulin interaction and allows the potential for functional differences to be attributed to each of these phosphorylation reactions.

In order to identify which tubulin isotypes (α or β) are substrates for GRKs, tubulin was initially phosphorylated by either βARK or GRK5 and then carboxymethylated. Tubulin α- and β-isotypes are differentially susceptible to carboxymethylation allowing for their electrophoretic separation (27). Moreover, among the various β-isotypes, βIII has a distinct susceptibility to carboxymethylation that resolves it from both the α- and other β-isotypes (β*) (27), as seen in the Coomassie Blue-stained lanes in Fig. 3. Phosphorylation of tubulin by 0.025 μM kinase for 15 min resulted in a preferential phosphorylation of...
Fig. 2. Phosphorylation of tubulin by GRKs. A, phosphorylation reactions were performed at 30 °C for 60 min and contained 200 nM βARK, GRK5, or rhodopsin kinase (RK), or buffer (No Kinase) and 100 nM tubulin dimers, in the absence or presence of 200 nM purified Gβγ (βγ) and phosphorylcholine-dodecylmaltoside mixed micelles (PL), and were performed at 30 °C for 5 min as described under "Experimental Procedures." Arrow on right indicates a slower mobility phosphorylated tubulin species observed in the presence of GRK5. B, time course of tubulin phosphorylation by rhodopsin kinase (□), GRK5 (○), βARK (●), or βARK with Gβγ and PL (♦). Phosphorylation was performed as described above with incubation times from 5 to 90 min. The proteins were subjected to SDS-PAGE and visualized by autoradiography, and the stoichiometry of phosphorylation determined by excising and counting the 32P-labeled bands. Values are mean ± S.E. from four separate experiments.

Fig. 3. Phosphorylation of β-tubulin isotypes by GRKs. Phosphorylation reactions contained either 0.025 or 2.5 μM βARK or GRK5 and 4.5 μM tubulin dimers and were performed at 30 °C for 30 min followed by carboxymethylation and SDS-PAGE as described under "Experimental Procedures." Right and left end lanes represent Coomassie staining of 2.5 μg of carboxymethylated tubulin and demonstrate the distinct migration of α-, βIII-, and ββ (all other ββ)-tubulin isotypes as indicated on the right. The center four lanes represent autoradiography of 0.65 μg of tubulin phosphorylated by the indicated kinases.

β-tubulin isotypes by both βARK and GRK5 (Fig. 3). Interestingly, at higher GRK levels (2.5 μM kinase), a qualitative difference in the phosphorylation selectivity was observed with βARK and particularly GRK5, both exhibiting a preference for the βIII-isotype (even though βIII represents only ~25% of the total tubulin (28)). Although we have not more thoroughly investigated the molecular basis of this apparent switch in isotype specificity, it is possible that the formation of GRK-tubulin hetero-oligomers at higher GRK concentrations contributes to this process. Total cellular concentrations of βARK and GRK5 were estimated to be in the range of 0.01–0.2 μM based on Western analysis of several cell lines (data not shown). Thus, while 2.5 μM kinase may not seem physiological it is likely that GRKs can exist in much higher local concentrations in various cellular micro-domains (e.g., at the plasma membrane/cytoskeleton interface), suggesting that both concentrations of GRK used in this experiment may be physiologically relevant.

The above data permit a comparison of our findings with previously characterized tubulin phosphorylation studies. Most striking is the fact that the brain-specific βIII-tubulin isotype appears to be the principal tubulin isotype phosphorylated in brain tissue in vivo (27, 29). Additional studies have revealed that Ser444 of βIII-tubulin is a critical phosphorylation site (29) and that casein kinase II may be partly responsible for this in vivo phosphorylation (29–31). Interestingly, casein kinase II and βARK have previously been shown to preferentially phosphorylate substrates containing acidic residues N-terminal to the phosphorylation site (32). Moreover, these kinases are also both effectively inhibited by micromolar concentrations of heparin, which was previously used to demonstrate that a microtubule-associated kinase activity was likely casein kinase II (20, 30, 31). Thus, GRK-mediated tubulin phosphorylation appears to be consistent with several aspects of the physiological phosphorylation of brain tubulin previously characterized (27, 29–32).

It is important to note that our studies have been limited to analysis of brain tubulin. Although it is clear that the neuronal-specific βIII-isotype is the preferred physiological phosphorylation substrate in brain, relatively few studies have characterized the tubulin isotypes phosphorylated in non-neuronal tissues. Since GRKs are expressed ubiquitously, it is of interest to consider which tubulin isotypes may be phosphorylated in other tissues. Of specific interest is the cardiac myocyte, where the levels of some β-tubulin isoforms have been shown to increase significantly in congestive heart failure (33–35). The increased levels of microtubules are thought to be, at least in part, a consequence of increased microtubule stability (34, 35) and might be related to the extent of post-translational modification such as phosphorylation. Interestingly, both βARK and GRK5 levels have also been shown to increase (~2–3-fold) in the failing heart and are thought to contribute to the pathology of this condition (36, 37). Thus, further study of the specific β-tubulin isotypes phosphorylated by GRKs, as well as the possible functional consequences, in non-neuronal cells such as cardiac myocytes is warranted.

In order to characterize the GRK-mediated phosphorylation better, kinetic analysis was performed by varying the tubulin concentration in the presence of the various GRKs (Table I).

### Table I

| Kinase  | Additions | Vₚₚ | Vₘₐₓ | Vₘₐₓ/Kₘ |
|--------|-----------|-----|------|--------|
| βARK   | 0.42 ± 0.10 | 17.4 ± 1.0 | 41.4   |
| βARK PL| 0.56 ± 0.17 | 25.8 ± 2.1 | 46.0   |
| βARK βγ| 0.23 ± 0.05 | 15.0 ± 0.6 | 64.3   |
| βARK PL + βγ| 0.28 ± 0.01 | 21.1 ± 0.2 | 74.4   |
| GRK5   | 0.064 ± 0.019 | 8.66 ± 0.71 | 135   |
| RK     | 4.41 ± 1.6 | 53.3 ± 0.94 | 1.21   |
Phosphorylation reactions were performed at 30 °C for 5 min, a time within the linear region of phosphorylation time courses for all kinases examined (Fig. 2). These studies revealed a $K_m$ of 0.42 μM and a $V_{\text{max}}$ of 17.4 nmol of P/min/mg for the βARK-mediated tubulin phosphorylation reaction. Although addition of Gβγ had little effect on the maximum velocity of this reaction ($V_{\text{max}} = 15.0$ nmol of P/min/mg), the apparent affinity of βARK for tubulin was increased nearly 2-fold ($K_m = 0.23$ μM). Conversely, the primary effect of the phospholipid addition was on the catalytic activity of βARK yielding a $V_{\text{max}}$ of 25.8 nmol P/min/mg. Concomitant addition of both Gβγ and phospholipid was additive yielding a combined enhancement of both $K_m$ and $V_{\text{max}}$. These two regulators of βARK activity have previously been suggested to elicit their effects largely by facilitating membrane translocation of βARK, thereby achieving closer proximity to membrane-bound receptor substrates (12–15). Importantly, the effects of Gβγ and phospholipid on the kinetic parameters of tubulin phosphorylation are apparent in this soluble system underscoring their potential role in the direct activation of βARK. Interestingly, however, membrane-bound forms of tubulin have been observed in a variety of systems (10, 38–40), and tubulin palmitoylation has recently been characterized in detail (41). Thus, it is likely that the properties of membrane translocation also are important aspects of the βARK/tubulin interaction in cells. For example Gβγ and phospholipid may direct the specific phosphorylation of the palmitoylated membrane-bound forms of tubulin, in a fashion analogous to that of G protein-coupled receptors. Alternatively, palmitoylated tubulin itself may be capable of supporting βARK membrane translocation thus regulating the localization and/or function of βARK.

The kinetic analysis of GRK5 revealed a relatively modest catalytic activity toward tubulin with a $V_{\text{max}}$ of 8.7 nmol of P/min/mg, approximately one-third that of activated βARK. However, the affinity of GRK5 for tubulin was observed to be ~4.5-fold greater ($K_m = 0.064$ μM) than that of activated βARK. These distinctions in kinetic properties support the observed differences in βARK- and GRK5-mediated tubulin phosphorylation (Fig. 2), although the significance of these observations remains unclear. Rhodopsin kinase phosphorylated tubulin with a $K_m$ of 4 μM and a $V_{\text{max}}$ of 5.3 nmol of P/min/mg making it a relatively inefficient kinase for tubulin. Importantly, however, the affinities exhibited by βARK, GRK5, and rhodopsin kinase are all within physiological ranges. For example, the affinities of βARK and GRK5 for an in vivo substrate, the β2-adrenergic receptor, have previously been assessed to be 0.25 (42) and 0.54 μM (21), respectively. Moreover, the total cellular concentration of tubulin in fibroblasts has been estimated to be ~20 μM (1). Finally, measured affinities for several well characterized tubulin kinases have revealed $K_m$ values for tubulin of 20 μM for casein kinase II (30) and 11.3 μM for Ca2+/calmodulin-dependent kinase (43). Taken together, these data suggest that tubulin may serve as a physiological substrate for GRK-mediated phosphorylation.

**In Vitro Interaction of GRKs with Soluble and Assembled Tubulin**—Since the GRK/tubulin association was discovered via their direct binding (Fig. 1), we were interested in further characterizing this aspect of the interaction. Thus, we assessed the binding of soluble tubulin dimers to GST-βARK fusion proteins containing either the N-terminal, central catalytic, or C-terminal domains of βARK. When these GST-βARK fusions were incubated with purified soluble tubulin dimers and subsequently precipitated with glutathione-agarose beads, it was found that the central catalytic domain of βARK (residues 185–467) contained the major binding determinant for tubulin (Fig. 4). These data are consistent with the ability of multiple GRKs to phosphorylate tubulin as the catalytic domain is the most highly conserved region among the GRKs (11). Whereas the N-terminal domain possessed a small amount of specific binding, the C-terminal GST-βARK fusion which includes the PH domain and Gβγ binding determinants demonstrated no tubulin binding capacity. This raises the interesting possibility that βARK, which appears to have distinct regions for interaction with Gβγ (the C-terminal domain) and tubulin (the catalytic domain), may be able to concomitantly associate with both tubulin and Gβγ to form a βARK-Gβγ-tubulin ternary complex. This is consistent with our observation that the apparent affinity of βARK for tubulin is increased ~2-fold by the presence of Gβγ (Table 1) and is further supported by the recent demonstration that Gβγ can directly associate with microtubules (6). These observations suggest potentially interesting implications for βARK regulation.

Although the data presented in Figs. 1–4 demonstrate that GRKs can bind and phosphorylate soluble tubulin, these data do not test whether assembled microtubules may also serve as substrates for GRK-mediated phosphorylation. In order to directly address this question, stable microtubules were formed by assembly in the presence of Taxol and then tested for their ability to specifically bind and be phosphorylated by βARK or GRK5. Taxol-stable microtubules were sedimented, and the pellet was resuspended in buffer containing Taxol, [γ-32P]ATP, and either βARK or GRK5. After incubation at 30 °C for 30 min, the microtubules were repelleted and examined by SDS-PAGE and Coomassie Blue staining and densitometry. The stable microtubules were found to specifically sediment a significant portion of βARK (54 ± 3%; n = 8) and GRK5 (69 ± 5%; n = 4). When subjected to autoradiography it was found that the stable microtubules also served as a substrate for βARK-mediated (0.13 ± 0.01 mol P/mol tubulin dimer; n = 3) and GRK5-mediated (0.19 ± 0.01 mol P/mol tubulin dimer; n = 3) phosphorylation. Thus, similar to previous findings with casein kinase II (30), GRKs can directly bind and phosphorylate preformed Taxol-stabilized microtubules. Whereas the form of tubulin (soluble dimers or assembled microtubules) that is the preferred substrate for GRKs remains unclear, it is noteworthy that under similar conditions, the phosphorylation stoichiometries of assembled (above) and unassembled (βARK = 0.14 ± 0.02 mol P/mol tubulin dimer and GRK5 = 0.10 ± 0.01 mol P/mol tubulin dimer) tubulin are comparable.

**In Vivo Interaction of GRKs with Tubulin**—To address the potential physiological relevance of the GRK/tubulin interaction, we assessed the in vivo association of GRKs and tubulin using three distinct approaches. First, a partially purified tubulin preparation (PPT) was generated from porcine brain tissue by two successive cycles of tubulin assembly (at 37 °C) and...
disassembly (at 4 °C) each followed by centrifugation. In this way tubulin, as well as microtubule-associated proteins, are significantly enriched within one or two cycles (22). This enrichment was quantitated by subjecting varied amounts of PPT to SDS-PAGE, Coomassie Blue staining, and densitometry revealing that tubulin represents ~93% of the protein in this preparation, and the remaining ~7% is composed of microtubule-associated proteins and other associated proteins (data not shown). We then tested various amounts of PPT for the presence of βARK by Western blotting using a βARK-specific antibody. This analysis revealed a significant amount of βARK present in the PPT preparation (0.05–0.1% of the total protein, Fig. 5A). A similar analysis of the initial soluble porcine brain extract revealed that βARK is present at ~0.02% of the total protein (data not shown) suggesting a 2.5-5-fold enrichment of βARK in PPT. Analysis of equal amounts of the warm (37 °C) and cold (4 °C) supernatant and pellet fractions revealed that βARK is associated to a similar extent with the “microtubule fractions” (i.e. the warm pellet and cold supernatant) and the “non-microtubule fractions” (i.e. warm supernatant and cold pellet). Given the above data that suggest βARK may bind to both soluble and assembled forms of tubulin, it is not surprising that βARK does not strictly associate with the microtubule fraction. Even so, a significant amount of βARK is present in the 2-cycle PPT (Fig. 5A) as well as in a 3-cycle PPT (data not shown). Thus, the presence of βARK in these tubulin preparations is suggestive of an in vivo association between βARK and tubulin that by necessity includes association with microtubules, as βARK does not precipitate on its own under these conditions (data not shown). In order to demonstrate the specific nature of the βARK/tubulin association, the arrestins present in the brain extract and PPT were also quantitated. While arrestins were found to be expressed at ~10-fold higher levels than βARK (~0.2% of the protein in the pig brain extract), no arrestin was detected in 40 µg of PPT (data not shown).

A second approach to examine the GRK/tubulin interaction in vivo involved the immunoprecipitation of either endogenous or overexpressed βARK from COS-1 cells using a βARK-specific polyclonal antibody. COS-1 cells were transfected either with pcDNA3 or pcDNA3-βARK DNA using LipofectAMINE Transfection reagents. Western analysis revealed detectable levels of endogenous tubulin and βARK, as well as a robust overexpression of βARK in cells transfected with pcDNA3-βARK (Fig. 5C). Immunoprecipitation of βARK from cell lysates using the βARK-specific polyclonal antibody was detectable in both cell lysates and was proportional to the respective βARK expression levels (data not shown). When the βARK immunoprecipitates were blotted and probed with an α-tubulin-specific antibody, a significant coprecipitation of tubulin was detected in lysates from both the pcDNA3- and pcDNA3-βARK-transfected cells (Fig. 5B), clearly demonstrating the in vivo association of βARK and tubulin. In contrast, no tubulin was found in control immunoprecipitations performed either in the absence of the βARK antibody or with an HA-specific polyclonal antibody.

We also assessed GRK/tubulin interaction by immunofluorescence microscopy of COS-1 cells overexpressing either βARK or GRK5. Transfected cells were plated on glass coverslips, fixed, permeabilized, and then incubated with tubulin- and βARK- or GRK5-specific primary antibodies followed by fluorescently labeled secondary antibodies. This analysis revealed that GRKs are found associated with the microtubule cytoskeleton (Fig. 6). Specifically, βARK is found enriched in a perinuclear region of the cells coincident with the microtubule organizing center (top panels) as well as in other regions containing dense microtubule structures (middle and bottom panels), a pattern comparable with that of casein kinase II microtubule co-localization in NRK cells (31). A generally similar co-localization of GRK5 with microtubules was also observed, with the exception that GRK5 was not significantly localized to the perinuclear region (data not shown). Although co-localization is apparent at areas of high microtubule content, GRKs are not strictly associated with microtubules. This observation may, in part, be due to the relatively high level of GRK expression in these cells or the apparent ability of GRKs to interact with both soluble and assembled forms of tubulin. In addition, the effect of endogenous microtubule-associated proteins on GRK/microtubule interactions is presently unknown. In any case, it appears that at least a portion of the GRKs are specifically associated with distinct microtubule structures in cells. Taken together, the co-purification (Fig. 5A) and co-immunoprecipitation (Fig. 5B) of βARK and tubulin, as well as the co-localization of βARK and GRK5 with microtubules (Fig. 6 and data not shown), demonstrates that GRK/tubulin interactions occur in vivo.

Analysis of the Functional Role of GRK/Tubulin Interaction—While providing important in vitro and in vivo evidence
for GRK/tubulin association, the above experiments fail to
define any functional role for this interaction. Formally, three
principal possibilities exist with respect to the functional sig-
nificance of this interaction: (i) GRKs might regulate the as-
sembly of tubulin; (ii) GRKs might regulate one or more of the
many diverse functional properties of microtubules (i.e.
independently of effects on tubulin assembly); or (iii) tubulin and/or
microtubules may effect GRK function by regulating its cellular
localization. Importantly, none of these possibilities are mutu-
ally exclusive, allowing the potential for complex functional
relationships in cells.

Whereas the cellular functions of tubulin are diverse and
complex, the ability of tubulin to dynamically cycle between
soluble and assembled states is a fundamental property readily
amenable to in vitro analysis (1, 2, 22, 23). Indeed, tubulin
assembly is known to be subject to a wide variety of regulatory
post-translational modifications including phosphorylation (3).
Therefore, we initially focused on tubulin assembly as the most
experimentally tractable and likely property of tubulin to be
influenced by GRK-mediated phosphorylation. In this regard,
we attempted to examine whether βARK- or GRK5-mediated
tubulin phosphorylation could affect the properties of tubulin
assembly in vitro. Unfortunately, since the assembly assay is
highly sensitive to buffering conditions and requires high tu-
bulin concentrations, suboptimal phosphorylation conditions
were required resulting in phosphorylation stoichiometries of
only 0.1–0.25 mol of P_i/mol tubulin dimer with either βARK or
GRK5. Thus, as might be expected, we did not observe any
effect of this phosphorylation on the rate or extent of tubulin
assembly. However, although we observed no obvious effects of
GRK-mediated tubulin phosphorylation on assembly, the
above experiments did reveal one striking and extremely re-
producible observation. When we examined the relative distri-
bution of the GRK-phosphorylated tubulin species, we found a
disproportionate amount of the radiolabeled tubulin associated
with the pellet fraction consistently in over 70 separate reac-
tions under a variety of conditions. This observation is illus-
trated in Fig. 7 using tubulin phosphorylated by either βARK

![Fig. 6. Co-localization of βARK with microtubules. COS-1 cells were
transfected with pcDNA3−βARK and fixed and permeabilized at 24–36 h after
transfection. Cells were probed with both βARK- and tubulin-specific primary anti-
bodies followed by fluorescently labeled secondary antibodies as described under
"Experimental Procedures." βARK was labeled with fluorescein isothiocyanate-
conjugated anti-rabbit antibody and is shown in the left panels (βARK), and tu-
bulin was labeled with tetramethylrhodo-
damine-conjugated anti-mouse antibody
and is shown in the center panels (MT). Tubulin and βARK immunofluorescence
are overlaid in the panels on the right (βARK/MT). Yellow represents co-local-
ization of tubulin and βARK.](image-url)
or GRK5 to average stoichiometries of 0.14 ± 0.02 and 0.10 ± 0.01 mol of P1/mol of tubulin dimer, respectively. When assembly was promoted by 30% glycerol for 30 min, −90% of the total tubulin and −95% of the phosphorylated tubulin were found associated with the pellet fraction. However, when assembly was promoted by 5% glycerol, only −20% of the total tubulin was pelleted, whereas −90% of the βARK- or GRK5-phosphorylated tubulin was pelleted.

These differences in the extent of total versus phosphorylated tubulin pelleted in the presence of 5% glycerol might be explained by one of two possibilities. First, the observed differences could be generated by a preferential phosphorylation of pre-formed microtubules. Importantly, the tubulin preparation is subjected to centrifugation prior to the phosphorylation reaction to remove any insoluble tubulin aggregates, ensuring that the tubulin added to the phosphorylation assay is at least initially in a soluble form. However, the phosphorylation assay is by design compatible with, although not optimal for, tubulin assembly. Thus, it is likely that during the phosphorylation reaction a small fraction of the tubulin assembles into microtubules. Indeed, centrifugation immediately after the phosphorylation step generally pellets 5–10% of the tubulin. In addition, the glycerol and GTP added to initiate assembly do not inhibit GRK activity. Thus, phosphorylation can continue as microtubules form, and it is conceivable that microtubules become preferentially phosphorylated by GRKs leading to the observed increase in radiolabeled tubulin in the pellet. A second possibility is that the phosphorylated species could preferentially be driven into the assembled form thus causing a disproportionate increase in phosphorylated tubulin in the pellet. This would suggest a direct functional role of GRK-mediated phosphorylation in promoting tubulin assembly. This possibility is consistent with the finding that under similar phosphorylation conditions βIII-tubulin is preferentially phosphorylated by both βARK and GRK5 (Fig. 3). This is of interest as phosphorylation of βIII has previously been shown to promote the assembly of tubulin and the stability of microtubules (27, 31). Although we have currently been unsuccessful in further discriminating between these two possibilities, this effect is highly reproducible and likely represents an important aspect of the GRK/tubulin interaction.

An additional consideration in our in vitro functional analysis was that additional components not present in the assembly assay may be critical. Therefore, we also examined the effects of overexpression of GRKs on microtubule stability in COS-1 cells by immunofluorescence confocal microscopy. These experiments did not reveal any major GRK-dependent alterations in microtubule stability. Similarly, co-expression of the β2-adrenergic receptor and βARK or GRK5 in COS-1 cells also had no effect on the microtubule cytoskeleton even after a 20- or 60-min activation with β-agonist (data not shown). The possibility remains that potential changes are confined to distinct microdomains in the cell, perhaps involving regions of the plasma membrane/cytoskeleton interface, and therefore go undetected by this relatively insensitive method of analysis. Additionally, we currently have no appreciation of how microtubule-associated proteins, important physiological regulators of tubulin assembly, may affect the GRK/tubulin interaction. Whereas the data presented in Fig. 7 suggests a potential role for GRKs in regulating tubulin assembly, more sophisticated analysis will be required to establish the precise role of GRKs in this process.

As stated above, a second possible result of the GRK/tubulin interaction might be the disruption or enhancement of some aspect of microtubule function. For example, phosphorylation has been demonstrated to regulate the ability of tubulin to interact with membranes (40), the ability of microtubules to cross-link with actin microfilaments (44), and the ability of microtubule motor proteins to function in transport (45). Thus, βARK-mediated phosphorylation of microtubules could conceivably regulate any one of the many diverse microtubule functions in cells. However, identification and characterization of such a regulatory function would be limited by the diversity and complexity of the various possibilities.

Finally, a third possible result of the GRK/tubulin interaction is the tubulin- or microtubule-mediated regulation of the cellular localization and/or function of GRKs. It has been demonstrated that tubulin and microtubules are capable of influencing cellular signaling through direct interaction with a variety of signaling molecules (4–9). The data presented in this article demonstrate the binding of GRKs to both soluble tubulin dimers and to assembled microtubules in vitro. Importantly, our immunofluorescence data reveal the association of βARK and GRK5 with microtubules in cells suggesting that GRKs may be sequestered by the microtubule cytoskeleton, perhaps directing currently unappreciated GRK interactions. Such regulation has recently been demonstrated for the A2 adenosine receptor (7) and K-Ras (10). Additionally, tubulin is known to become palmitoylated and to associate with a variety of cellular membranes including the plasma membrane, endosomes, microsomes, and various organelles (10, 38–40). Thus, the potential exists that tubulin, perhaps in coordination with membrane phospholipids and/or Gβγ subunits, could be responsible for directing GRKs to defined membrane microdomains in the cell. Finally, a particularly intriguing possibility is that such...
interactions may be dynamically regulated in coordination with dynamic functions of the microtubule cytoskeleton during processes such as mitosis and cell differentiation.

In summary, these data identify a novel interaction between GRKs and tubulin in which both βARK and GRK5 are capable of phosphorylating tubulin in a stoichiometric fashion with a preference for β-tubulin and specifically the βIII-isotype under certain conditions. Importantly, these observations are suggested to be physiologically relevant due to the ability of βARK to co-purify with tubulin from brain tissue, the ability of tubulin to co-immunoprecipitate with βARK from cell lysates, and the observed co-localization of βARK and GRK5 with microtubules in cells. Whereas the potential functional consequences of GRK/tubulin interaction are largely unknown, GRKs appear to either selectively interact with microtubules (compared with soluble tubulin) and/or play a role in regulating tubulin assembly. Thus, GRK/tubulin interaction may represent a novel link between cell signaling and cytoskeletal molecules. As further investigations proceed into both the molecular nature and functional consequences of these interactions, it may be anticipated that GRKs will be capable of coordinating specific signaling events with changes in the stability and/or function of the cytoskeleton. Additionally, it is likely that just as tubulin has been shown to regulate a variety of other signaling molecules, tubulin may similarly affect the localization and/or function of GRKs, possibly directing distinct signaling events that remain to be identified.

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