An Intact N Terminus of the γ Subunit Is Required for the Gβγ Stimulation of Rhodopsin Phosphorylation by Human β-Adrenergic Receptor Kinase-1 but Not for Kinase Binding

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The abbreviations used are: GRK, G-protein receptor kinase; βARK, β-adrenergic receptor kinase; PH, pleckstrin homology; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; Tritcâ, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ROS, rod outer segments; HPLC, high performance liquid chromatography.

G-protein-coupled receptor responses to agonist ligands are modulated at multiple levels along the signal transduction pathway. Regulation includes short term effects on receptor coupling and on internalization of the receptor. Longer term effects include down-regulation of the cellular receptor content and mRNA levels (Tholanikunnel et al., 1995). Several different protein kinases have been shown to phosphorylate some of the seven transmembrane helix receptors on cytosolic portions of the molecule, reducing coupling of the receptors to their associated heterotrimeric G-proteins (reviewed by Koblika (1992)). One family of protein kinases, the G-protein receptor kinase (βARK) family, has been shown to target agonist-occupied receptors, phosphorylating multiple serine/threonine residues adjacent to acidic amino acid residues in the primary amino acid sequence. There are presently six members of this protein kinase family that share a common catalytic domain, diverging in the N- and C-terminal extensions outside of this region.

The GRK2/GRK3 (βARK1 and βARK2) subfamilies are encoded by separate genes (Benovic et al., 1991). They were originally shown to phosphorylate the βγ-adrenergic receptor and thus were given the name β-adrenergic receptor kinases or βARKs. The βARKs are C-terminally extended relative to other members of the GRK family. The C-terminal 222 amino acids of βARK1 and βARK2 contain a domain responsible for the association of the enzyme with heterotrimeric G-protein subunits (Pitcher et al., 1992). Addition of Gβγ subunits to an in vitro phosphorylation system stimulates βARK phosphorylation of receptor substrates (Haga and Haga, 1990, 1992), but not that of peptide substrates (Pitcher et al., 1992). This C-terminal domain of the kinase includes a region homologous to a domain of the platelet protein pleckstrin (PH domain) (Touhara et al., 1993), thought to mediate protein-protein interactions among signaling proteins (Musacchio et al., 1993; Gibson et al., 1994; Ingley and Hemmings, 1994). PH domains may be functionally analogous to the Src homology 2 and 3 domains of tyrosine kinase signaling systems (Mayer et al., 1993). While the portions of the βARK C terminus involved in the association with Gβγ subunits have been delineated (Koch et al., 1993), less is known about the determinants on the Gβγ partner. The multiple subtypes of Gβ and Gγ, the requirement for the Gγi heterodimer for cellular function, (Llulli et al., 1992), and multiple post-translational modifications (Yamane and Fung, 1993) have impeded study. This paper describes the dissociation of Gβγ binding to hβARK1 from Gβγ stimulation of rhodopsin phosphorylation by this kinase after proteolysis with Lys-C. Gγ is cleaved by the protease at lysine 33 in Gγ2 (lysine 36 in Gγ1), and the Gγ fragments remain associated with the Gβ subunit.

EXPERIMENTAL PROCEDURES

Frozen bovine retinas were from George A. Hormel, Austin, MN. Frozen bovine brain was from Pel-Freez, Rogers, AR. ATP, GDP, GTP-S, NAD+, sodium cholate, Lubrol PX, isobutylmethylxanthine, l-propanol, phosphoethanolamine, dimethylphosphotidylcholine, forskolin, and pyruvate kinase were from Sigma. Endoproteinase Lys-C from Lysobacter enzymogenes (catalog no. 476986) was from Boehringer Mannheim. Reagents for SDS-PAGE were from Research Organics. Nitrocellulose (BA83) was obtained from Schleicher and Schuell. DE-52 was from Whatman. Antibodies specific for β2, β3, β7, β12, pan-β, γ2, γ3, γ7, and γ9 of the heterotrimeric G-protein Gβγ and neutralizing peptides were purchased from Santa Cruz Biotechnology. These antibodies are also useful for enzyme-linked immunosorbent assay determinations. Antibodies to the C-terminal 222 amino acids of human βARK1 were raised against the glutathione S-transferase (GST) fusion protein (Cocalico Biologicals, Reamstown, PA) and purified from an IgG fraction following adsorption against immobilized GST-C-terminal 222-amino acid human βARK1, eluting with 0.1 M glycine, pH 2. Secondary antibody (donkey anti-rabbit peroxidase) and ECL detection reagents were purchased from Amersham. [γ-32P]ATP (3000 Ci/mmmol), [γ-35S]GTP (1045 Ci/mmol), and [α-32P]cAMP (30 Ci/mmol) were from Du Pont NEN. S-Adenosyl-L-[3H methyl]methionine (77 Ci/mmol), the 125I-cAMP Scintillation Proximity Assay Kit, Rainbow prelabeled weight...
markers, and Amplify were acquired from Amersham. Purified recombinant human βARK His6 (PH + C) (Gly556-Ser670) protein was generously provided by Dr. Daruka Mahadevan (Mahadevan et al., 1995). DNA encoding the βARK C-terminal domains βARK (PH + C) (Gly556-Ser670) and the C-terminal 222 amino acids (Pro560-Leu781) were cloned by PCR from the human βARK cDNA provided by Dr. A. DeBlasi (Champagne et al., 1997) and their nucleotide sequences confirmed by us. Applied Biosystems model 373A automated sequencer. The glutathione S-transferase fusion proteins were purified in Escherichia coli strain BL21(DE3) using the pGEX-2T vector system (Pharmacia Biotech Inc.) and purified according to standard protocols. Peptides corresponding to residues 8–34 of Gβ2 or residues 3–29 of Gβ6 were kindly provided by Dr. R. Neubig, Department of Pharmacology, University of Michigan.

Heterotrimeric G-proteins were isolated from frozen bovine brain, and the Gβγ subunit complex (mixed subtypes of β and γ subunits) purified by chromatography in sodium cholate on heptylamine-Sepharose (Sternweis and Pang, 1990) in the presence of GDP.α|Gβ|MγF. Further extension of the separated Gβγ from Gβ subunits for ADP-ribosylation studies was achieved by ion exchange chromatography on MonoQ (Pharmacia) column in 0.1% (w/v) Lubrol PX (Sternweis and Pang, 1990). The purified Gβ and Gγ subunits were stored in aliquots at −80°C. Two different batches of Gβγ when assayed for effects of Lys-C proteolysis on rhodopsin phosphorylation and βARK1 binding activity revealed no discernible differences. Carboxymethylation of the C terminus of the γ subunit of Gβγ with S-carboxymethyl-L-(or-L-methyl)me-thionine was accomplished using an S-carboxymethylated brain membrane fraction (Fung et al., 1990). Bovine retinal rod outer segments (ROS) containing rhodopsin were purified under red light illumination and urea-extracted before use as a phosphorylation substrate (Phillips et al., 1989). SDS-PAGE was performed in 10% acrylamide, 0.267% bisacrylamide gels containing 0.1% SDS in the Laemmli buffer system (Laemmli, 1970). Rhodopsin phosphorylation was determined after separation of 32P-labeled proteins by SDS-PAGE. Radioactive gels were fixed for 10 min in 25% methanol, 10% acetic acid, washed with distilled water for 10 min, and the gels dried at 80°C under vacuum before exposure to Kodak XAR-5 or X-Omat-LS film. The 32P radioactivity of the rhodopsin band was quantitated from the film using a BioImage detection system (Immunodynamics, Inc., La Jolla, CA) was carried out with the same batches of Gβγ used in the phosphorylation reactions. The resolution of the separated Gβγ subunits for ADP-ribosylation of rhodopsin (asurea-treated ROS), 5 μl of 50 μM [γ-32P]ATP, 50 μM ATP, 10 μM GTP, and 160 pmol of rhodopsin were spotted onto Whatman 3MM plates and dried. Autoradiography revealed no discernible differences. Carboxymethylation of the thionine was accomplished using a cholate-extracted brain membrane protein for 5 min. The tubes were then transferred to a 30°C water bath and the incubation continued for 15 min. One hundred microliters of ice-cold 5% (w/v) trichloroacetic acid were added to stop the reaction on ice. Cyclic AMP content was determined in aliquots of the supernatant after centrifugation at 16,000 × g for 10 min at room temperature to remove precipitated material. Adenylate cyclase activity was linear in both membrane protein and time over the ranges used.

Protolysis of Gβγ by Endoprotease Lys-C—Cleavage of the Gβγ complex by Lys-C protease was performed in 50 mM Tris-HCl, pH 8.6, 1 mM dithiothreitol, 0.1 mM EDTA, 0.05% Lubrol PX for 30 min at 30°C using a protase:protein ratio of 1:10 (w/w). The digestion was terminated by the addition of 20 μg/ml leupeptin. To facilitate comparison of the effects of Gβγ subunits with the different functional assays used, each reaction was carried out with the same batches of Gβγ and stored at −70°C that had been kept on ice, incubated without protease at 30°C, incubated with leupeptin-inactivated protease at 30°C, or incubated with active Lys-C at 30°C for 30 min. Thirty minutes of incubation of Gβγ at 30°C without protease had no discernible effect on its measured biochemical properties.

Binding of Gβγ Subunits to PH Domain-containing C-terminal βARK1 Fragments—Gβγ subunit binding to immobilized PH domains was determined by immunoblot analysis with pan-anti-β subunit antibodies as described by Mahadevan et al. (1995).

Amino Acid Sequencing of Lys-C-digested Gβγ—Separation of the Gβγ subunits was achieved by C4 reverse phase chromatography on a C18 column (Parish and Rando, 1994). Western blot analysis showed that the Gβ and Gγ subunits were separated under these conditions (data not shown). Fifty μg of Lys-C-digested Gβγ (1.2 nmol) was diluted with an equal volume of 6 M guanidine HCl, 50 mM dithiothreitol, and injected onto a Brown 24.5 × 30-cm C4 300-A pore size C4 reverse phase column equilibrated with 5% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid at 0.75 ml/min. After a 10-min wash period, the protein was eluted with acetonitrile which was linearly increased to 65% over 40 min and held at 65% for 5 min. The eluent was monitored during the separation at 214 nm, and 0.75-ml fractions were collected. Fractions containing the γ subunits as judged by comigration of digested γ subunit or γ3–29 peptide into the functional assay were concentrated to dryness by vacuum and subjected to C4 reverse phase chromatography. The fractions were subjected to 15 cycles of automated Edman degradation on an Applied Biosystems 477A protein sequencer and the phenylthiohydantoin-amino acids identified with the integral HPLC unit of the sequencer.

RESULTS

The Gβγ subunits of the heterotrimeric G-proteins form a heterodimer (β = 35 kDa or 36 kDa, γ = 6748–8321 kDa), depending on the subunit subtypes and post-translational modifications. This non-covalent complex is not dissociable under non-denaturing conditions and associates reversibly with an undefined variety of Gα subtypes and with a protein kinase A substrate, phosducin (Bauer et al., 1992), as well as a host of
β-Adrenergic Receptor Kinase Stimulation by Gγ N Terminus

Methylation of the C-terminal cysteine (Cys71) carboxyl moiety of the purified brain Gβγ with S-adenosyl-[3H]-methyl]methionine (Fung et al., 1990) provided a marker for the C-terminal fragment of the Gγ subunit. Lys-C treatment of the tritiated Gβγ released a labeled fragment of Gγ, 3500 (Fig. 2), mirroring the shift on SDS-PAGE to lower Mr, seen with silver staining (Fig. 1). This implies that the extreme C terminus of the Gγ subunit remains intact. Immunoblotting after SDS-PAGE showed that N-terminal Gγ subunit immunoreactivity (residues 2-17) was eliminated by Lys-C treatment (Fig. 3). By contrast, for the Gβγ subunit, neither the N-terminal immunoreactivity (data not shown) nor the size on SDS-PAGE (Fig. 1) was affected by Lys-C treatment. Gel permeation chromatography of the [3H]carboxymethylated Gβγ on a Superose 12 column in 0.8% cholate before or after Lys-C treatment demonstrated that the fragments of the cleaved Gγ subunit remained complexed with the Gβγ subunit (Table I). When the Superose 12 column fractions were assayed by enzyme-linked immunosorbent assay and slot blotting onto nitrocellulose, virtually no loss of the immunoreactive N-terminal fragment(s) of Gβγ was observed. By contrast, denaturing SDS-PAGE and subsequent Western blot analysis of the same fractions revealed a significant loss of N-terminal Gγ subunit immunoreactivity (Table I).

Amino Acid Sequence of the Lys-C-treated Gγ Subunit—Gβγ subunits were digested with Lys-C, dissociated with 3 M guanidine hydrochloride + 50 mM dithiothreitol, and separated by C4 reverse phase chromatography as described under “Experimental Procedures.” Fig. 4 shows the migration of the [3H]carboxymethyl marker of the C terminus of the Gγ subunit on a separate HPLC run. The arrow marks the position of the truncated C-terminal Gγ sequences (Ala33–Cys67 for Gγ2; Ala37–Cys71 for Gγ3). The two Gγ subtypes nearly comigrate on reverse phase chromatography so their sequences were determined simultaneously. This was possible because both sequences are known, they were present in different amounts, and they differ at several positions. The asterisk shows the position of the undisected Gγ2 and Gγ3 polypeptides, while the cluster of radioactive peaks around fraction 40 represent undissociated Gβγ and the unidentified carboxymethylated Mγ24,000 protein in brain membranes (see Fig. 1 and Fung et al.)

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**Fig. 1.** 10% acrylamide, 8 M urea Tris-Tricine SDS-PAGE analysis of the cleavage of Gβγ by Lys-C (silver-stained). 2 μg of Gβγ were digested with the indicated ratio of Lys-C protease:Gβγ under the conditions described under “Experimental Procedures.” Lane 1, no protease; lane 2, 1:400; lane 3, 1:200; lane 4, 1:100; lane 5, 1:50; lane 6, 1:25; lane 7, 1:12.5. The Gβ and Gγ subunit positions are indicated by brackets.

**Fig. 2.** Reduction in Mr of C-terminal [3H]carboxymethylated bovine brain Gβγ by Lys-C proteolysis visualized by 3H autoradiography. 100 μg of bovine brain Gβγ were carboxymethylated with 0.5 mg of cholate-extracted bovine brain membrane protein and 50 μCi of S-adenosyl-[3H]-methyl]methionine (77 Ci/mmol) in 0.5 ml of carboxymethylation buffer and the labeled proteins re-extracted with cholate as described under “Experimental Procedures.” Lane 1, [3H]carboxymethylated proteins incubated for 30 min at 30 °C in the absence of protease; lane 2, + 2 μg of endoprotease Lys-C. The Mγ24,000 labeled protein is an endogenous substrate for the carboxymethyltransferase derived from the brain membrane enzyme source. This contaminant represents a minor fraction of the [3H] label incorporated. Lys-C treatment leads to a Mγ → 3500 decrease in size of the labeled Gγ subunit. The leading ion front ran to the very bottom of the gel.
The removal of 32(35) amino acid residues is consistent with the cleavage specificity of Lys-C after lysine. The N-terminal residues (2–17) of the Gαg subunit immunoreactivity were not detected. Minor 214 nm absorbance peaks contained short N-terminal Gαg sequences corresponding to other potential Lys-C proteolytic fragments in the initial brain preparation. Small amounts of sequences corresponding to N-terminal methionine-containing and non-acetylated Gα2 and Gα3 were also detected. The same sequencing results were obtained for two independent preparations of Gαβ subunits.

Functional Effects of Cleavage of the Gα Subunit: Biochemical Activities of Gαβ Subunits—Since Gαβ subunits do not possess an intrinsic enzymatic activity, the effect of Lys-C truncation on several extrinsic measures of functionality of the treated and control Gαβ subunits was evaluated. Their ability to bind to ROS and to modulate rhodopsin phosphorylation by full-length recombinant hARK1 was measured. Their ability to bind to the GST-βARK C-terminal domain of 222 amino acids (Pro346-Leu560) and to bind to a more restricted region of the kinase, GST- or His6-tagged βARK PH+C domain (βARK G556-S670), was determined. Finally, the ability of the treated and control Gαβ subunits to associate with and to modulate Gα subunit activities and to regulate the activity of an effector, Type I adenylate cyclase, were also assessed.

Modulation of βARK1-mediated Receptor Phosphorylation—The Lys-C-treated Gαβ subunit preparation supported substantial translocation of human βARK1 to isolated retinal rod outer segments (Fig. 5A), but failed to stimulate phosphorylation of rhodopsin (Fig. 5B). Gαβ subunits treated with leupeptin-inhibited Lys-C fully supported both translocation and phosphorylation. Fig. 5C shows that Lys-C proteolysis of Gα2 and Gα3 abrogates stimulation of βARK1 phosphorylation of receptor substrates without abolishing binding of the kinase. The saturation of the dose response of phosphorylation observed at low concentrations of Lys-C-digested Gαβ contrasts with the roughly linear increase with control incubated Gαβ. The low stimulation seen in the Lys-C-treated Gαβ may be residual undigested Gαβ, or it may represent an intrinsically lower stimulatory activity. Phosphorylation of rhodopsin was linearly related to the amount of βARK1 and Gαβ present in
the assay. The lack of effect of Gβγ subunits on synthetic peptide substrate RRREEEEEEAAA was unaltered by proteolysis (data not shown).

Binding of Gβγ Subunits to the βARK1 PH Domain—There was no notable difference between Lys-C-treated and untreated Gβγ subunit association with the PH domain contained within the C-terminal 222-amino acid fragment of hβARK1 (GST-(P466-L689)) (Fig. 6), or the shorter (PH + C-terminal helix) (G556-S670) GST-fusion or His6-tagged (PH + C) domains (data not shown) over a range of Gβγ concentrations. Thus, Lys-C proteolysis of Gγ does not detectably alter association of Gβγ subunits with the kinase in the absence of the catalytic and N-terminal sequences of hβARK1.

Effects of N-terminal Gβ and Gγ Synthetic Peptides on Gβγ Subunit Interactions with hβARK1—Antibodies (Santa Cruz Biotechnology) to N-terminal residues (2–17) of Gγ (γ2, or γ3) or the N-terminal region of Gβ (residues 25–40 of β2 or β6 20 μg/ml antibody) or the synthetic peptides in 1 mg/ml gelatin supplied by Santa Cruz Biotechnology (corresponding to these regions (40 μg/ml, 25 μm) were used to try to block or enhance βARK1 binding to Gβγ to immobilized PH domains or to block or stimulate βARK1 phosphorylation of rhodopsin in ROS without significant effect (data not shown). Similar results were seen for peptides corresponding to residues 8–34 of Gγ (Garritsen and Simonds, 1994) or residues 3–29 of Gβ (Katz and Simonds, 1995) (data not shown) over a range of Gβγ concentrations. Thus, there is a region between residues 45–59 in the Gγ subunit that are involved in dimerization with Gβγ (Mende et al., 1995).

At high concentrations trypsin will cleave the Gγ subunit at Arg129 generating two proteolytic fragments of Mr 26,000 and 15,000 without noticeable effect on the Gγ subunit in native Gβγ subunits (Tamir et al., 1991). The accessibility of the cleavage site in the Gγ subunit to Lys-C proteolysis demonstrated in the present work illuminates the quaternary structure of the Gβγ dimer. To minimize possible interactions of the prenyl moiety with lipid bilayers and potential steric hindrance, the proteolysis was performed in cholate, an anionic detergent with the small aggregation number of 2 cholate molecules/micelle (Calbiochem, 1993). There are 7 (Gγ3) or 6 (Gγ2) lysines in the Gγ subunit, which could potentially be available for Lys-C proteolysis. The major identified Gγ products of this protease begin with Ala33 (Gγ2) and Ala37 (Gγ3). Monitoring of the migration of the C-terminal [3H]carboxymethylated Gγ on HPLC gave no evidence for specific smaller or larger cleavage products. Thus, the majority of the treated Gγ retains the prenylated C-terminal cysteine residue. This part of the molecule is thought to interact with the seven-transmembrane helix receptor in the fashion of transducin of the visual system. The farnesylated C terminus of Gβ1γ3 stabilizes the active MII form of rhodopsin, which activates Gt (transducin) in the presence of GTP (Kisselev et al., 1994). Hints as to the relative positioning of the β and γ subunits come from several sources. Copper o-phenanthroline-mediated cross-linking of Gβγ and Gγγ through proximal intersubunit cysteine residues in transducin indicates that β Cys25 and γ Cys36 or Cys37 are nearby in the three-dimensional protein structure (Bubus and Khorana, 1990). This was interpreted as a point of close contact between the subunits. Alignment of transducin Gγγ, with Gγγ and Gγγ places the reactive Cys36/Cys37 at the site of Lys-C digestion determined here. Perhaps lysines C-terminal to this position are not accessible to the protease because of subunit-γ subunit interactions or interactions involving the prenyl group. A
series of sequential amino acid replacements in $\gamma_\gamma(35-37)$ and $\gamma_\gamma(38-40)$ are sufficient to specify the appropriate $\beta-\gamma$ selectivity (Lee et al., 1995). Interestingly, this motif is immediately C-terminal to the Lys-C cleavage site.

$\gamma_\beta$ subunits have been shown to mediate GRK2 and GRK3 ($\beta$ARK1 and $\beta$ARK2) translocation to retinal ROS containing the light receptor substrate rhodopsin, cell membranes (Pitcher et al., 1992), or to phospholipid vesicles (Kim et al., 1993). In addition, they robustly stimulate the activity of the kinase 10–20-fold toward receptor substrates but much less so toward synthetic peptide substrates (Pitcher et al. 1992). Cleavage of the $\gamma_\beta-\gamma$ complex with Lys-C retained the two fragments 1–32(36) and 33(37)-68(71) non-covalently associated with the $\gamma_\beta$ subunit. Tryptic fragments of the $\gamma_\beta$ complex cleaved within $\gamma_\beta$ similarly remain attached (Fung and Nash, 1983; Thomas et al., 1993). $\gamma_\gamma$ is not cleaved by trypsin in the $\gamma_\beta$ complex (Tamir et al., 1991). The Lys-C $\gamma_\beta$ fragment complex retained the ability to function as a binding site for the PH

graphic data are shown at the top. The first five lanes are $\gamma_\beta$ subunits treated with Lys-C in the presence of 10 $\mu$g/ml leupeptin, while the final four lanes are Lys-C-digested $\gamma_\beta$ subunits. Lane 1, no $\gamma_\beta$ subunits; lanes 2 and 6, 27.9 $\mu$g/ml $\gamma_\beta$-subunits; lanes 3 and 7, 55.8 $\mu$g/ml; lanes 4 and 8, 112 $\mu$m; lanes 5 and 9, 223 $\mu$m.

**FIG. 6.** Effect of Lys-C proteolysis of $\gamma_\beta$ subunits on binding to the isolated PH domain of $\gamma$ARK1. The indicated concentrations of treated $\gamma_\beta$ subunits were incubated in a 50-ml reaction volume with 0.5 $\mu$M GST-$\gamma$ARK1 C-terminal (Pro$^{466-469}$) fusion protein immobilized on glutathione-Sepharose as indicated under “Experimental Procedures.” The beads were washed and subjected to SDS-PAGE, and the proteins transferred to nitrocellulose for immunodetection of the $\gamma_\beta$ with a pan-$\gamma_\beta$-specific antibody. A, raw film data. Lanes 1, 5, and 9, 106 nm $\gamma_\beta$-subunits; lanes 2, 6, and 10, 319 nm; lanes 3, 7, and 11, 957 nm; lanes 4, 8, and 12, 2874 nm. Lanes 1–4 represent $\gamma_\beta$ subunits incubated on ice, lanes 5–8 are $\gamma_\beta$ subunits treated with Lys-C at 30 °C for 30 min in the presence of 10 $\mu$g/ml leupeptin and lanes 9–12 are $\gamma_\beta$ subunits treated at 30 °C with Lys-C for 30 min. B, quantitation by densitometry. Circle $\gamma_\beta$ subunits incubated on ice; square, $\gamma_\beta$ subunits treated with Lys-C at 30 °C for 30 min in the presence of 10 $\mu$g/ml leupeptin to inhibit the protease; triangle, $\gamma_\beta$ subunits treated at 30 °C with Lys-C for 30 min. Data are representative of four experiments with single determinations.

**FIG. 5.** Modulation of $\gamma$ARK1-mediated receptor phosphorylation. Incubations of $\gamma_\beta$ subunits with ROS and $\gamma$ARK1 and phosphorylation were performed as described under “Experimental Procedures.” A, translocation of $\gamma$ARK1 to rod outer segment membranes containing rhodopsin. The amount of $\gamma$ARK1 binding to light-activated urea-extracted ROS in the absence, presence of $\gamma_\beta$ subunits, and presence of Lys-C digested $\gamma_\beta$ subunits was determined after SDS-PAGE by immunoreactivity with 0.1 $\mu$g/ml anti-$\gamma$ARK1 antibody. Quantitation of the ECL reaction was by densitometry. Raw film data are shown at the top. Lane 1, control incubated $\gamma_\beta$ subunits; lanes 2 and 3, Lys-C digested $\gamma_\beta$ subunits; lanes 4 and 5, $\gamma_\beta$ subunits treated with Lys-C in the presence of 10 $\mu$g/ml protease inhibitor leupeptin. The experiment was repeated three times with similar results. B, lack of stimulation of rhodopsin phosphorylation by proteolyzed $\gamma_\beta$ subunits. $\gamma$ARK1 phosphorylation of rhodopsin in ROS was determined after translocation of the kinase in the absence, presence, and presence of Lys-C-digested $\gamma_\beta$ subunits. Following SDS-PAGE, the $^{32}$P incorporated into rhodopsin was quantitated by densitometry of the autoradiogram. C, dose response of $\gamma_\beta$ subunits to rhodopsin phosphorylation. ROS were phosphorylated with $\gamma$ARK1 and the indicated concentrations of control incubated or Lys-C digested $\gamma_\beta$ subunits as indicated under “Experimental Procedures” without removing unbound proteins. Following SDS-PAGE the $^{32}$P incorporated into rhodopsin was quantitated by densitometry of the autoradiogram. Circle, control incubated $\gamma_\beta$ subunits; square, Lys-C digested $\gamma_\beta$ subunits. Data are single determinations, and the experiment was repeated twice with similar results. The raw autoradiographic data are shown at the top. The first five lanes are $\gamma_\beta$ subunits treated with Lys-C in the presence of 10 $\mu$g/ml leupeptin, while the final four lanes are Lys-C-digested $\gamma_\beta$ subunits. Lane 1, no $\gamma_\beta$ subunits; lanes 2 and 6, 27.9 $\mu$g/ml $\gamma_\beta$-subunits; lanes 3 and 7, 55.8 $\mu$g/ml; lanes 4 and 8, 112 $\mu$m; lanes 5 and 9, 223 $\mu$m.
binding may be mediated through the C-terminal five WD40 motifs of Gβ̄ rather than through Gγ.

There may be multiple points of interaction between Gβγ, βARK, and other molecules. Some regions may specify binding to target molecules while others may modulate the catalytic activity of βARK in conjunction with the ligand-activated receptor substrate. The modulation of βARK activity could also be occurring at the level of Gβγ interaction with the receptor substrate. While the fragments of Lys-C-digested Gγ appear to remain non-covalently associated in solution as the Gβγ dimer, they either are displaced in the receptor complex with Gβγ, or they fail to assume the appropriate relationship to the rest of the members of the complex to activate βARK phosphorylation of the receptor. Nicking of the Gγ subunit by proteolysis with Lys-C indicates that the Gβγ dimer provides active modulation rather than a passive kinase binding scaffold for the phosphorylation of G-protein-coupled receptor substrates by βARK1.

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REFERENCES

Bauer, P. H., Muller, S., Puzicha, M., Pippig, S., Obermaier, B., Helmreich, E. J. M., and Lohe, M. J. (1992) Nature 358, 73–76
Benovic, J. L., Onorato, J. J., Ariza, J. L., Stone, W. C., Lohse, M. J., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Caron, M. G., and Lifshitz, R. J. (1991) J. Biol. Chem. 266, 14939–14946
Bubus, J., and Khorana, H. G. (1990) J. Biol. Chem. 265, 12995–12999
Calbiochem (1993) A Guide to the Properties and Uses of Detergents in Biology and Biochemistry, Calbiochem-Novabiochem Corp., San Diego
Chuang, T. T., Sallese, M., Ambrosini, G., Parruti, G., and De Blasi, A. (1992) J. Biol. Chem. 267, 6886–6892
Clapham, D. E., and Neer, E. J. (1993) Nature 365, 403–406
Cook, P. F., Neville, M. E., Jr., Vrana, K. E., Hartt, F. T., and Roskoski, R., Jr. (1982) Biochemistry 21, 5794–5799
Fung, B. K., and Nash, C. R. (1983) J. Biol. Chem. 258, 10503–10510
Fung, K.-K., Yamane, H. K., Ota, I. M., and Clarke, S. (1990) FEBS Lett. 260, 313–317
Garratt, A., and Simonds, W. F. (1994) J. Biol. Chem. 269, 24418–24423
Gilson, T. J., Hyvonen, M., Musacchio, A., and Saraste, M. (1994) Trends Biochem. Sci. 19, 349–353
Haga, K., and Haga, T. (1990) FEBS Lett. 268, 43–47
Haga, K., and Haga, T. (1992) J. Biol. Chem. 267, 2222–2227
Huff, R. M., and Neer, E. J. (1986) J. Biol. Chem. 261, 1105–1110
Inglese, J., Freedman, N. J., Koch, W. J., and Lifshitz, R. J. (1993) J. Biol. Chem. 268, 23725–23730
Inglese, E., and Hemmings, B. A. (1994) J. Cell. Biochem. 56, 436–443
Iniguez-Lluich, A., Simon, M. I., Robishaw, J. D., and Gilman, A. G. (1992) J. Biol. Chem. 267, 23409–23417
Katz, A., and Simon, M. I. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1998–2002
Kim, C. M., Dion, S. B., and Benovic, J. L. (1993) J. Biol. Chem. 268, 15412–15418
Kleuss, C. G., Ermoleeva, M. V., and Gautam, N. (1994) J. Biol. Chem. 269, 21399–21402
Kleuss, C., Scherubl, H., Hescheler, J., Schultz, G., and Wittig, B. (1993) Science 259, 832–834
Kleuss, C., Scherubl, H., Hescheler, J., Schultz, G., and Wittig, B. (1992) Nature 358, 424–426
Koblika, B. (1992) Annu. Rev. Neurosci. 15, 87–114
Koch, W. J., Inglese, J., Stone, W. C., and Lifshitz, R. J. (1993) J. Biol. Chem. 268, 8256–8260
Kwon, G., Remmers, A. E., Datta, S., and Neubig, R. G. (1993) Biochemistry 32, 2400–2408
Laemmli, U. K. (1970) Nature 227, 680–685
Lee, C., Murakami, T., and Simonds, W. F. (1993) J. Biol. Chem. 270, 8779–8784
LeViné, H., III, and Sahyoun, N. E. (1988) Brain Res. 439, 47–55
Mahadevan, D., Thakur, N., Singh, J., McPhie, P., Zanigrilli, D., Wang, L.-M., Guerrero, C., LeViné, H., III, Humbert, C., Saldana, J., Gutkind, J. S., and Najmabadi-Hasek, T. (1995) Biochemistry 34, 9111–9117
Maye, L., Ren, R., Clark, K. L., and Baltimore, D. (1993) Cell 73, 629–630
Mende, U., Schrödt, C. J., Yi, F., Spring, D. J., and Neer, E. J. (1995) J. Biol. Chem. 270, 15892–15898
Muntz, K. H., Sternweis, P. C., Gilman, A. G., and Mumbey, S. M. (1992) Mol. Biol. Cell 3, 49–61
Musacchio, A., Gilson, T., Rice, P., Thompson, J., and Saraste, M. (1993) Trends Biochem. Sci. 18, 343–348
Neer, E. J., Loé, J. M., and Waf, G. L. (1984) J. Biol. Chem. 259, 14222–14229
Parish, C. A., and Rando, R. R. (1994) Biochemistry 33, 9986–9991
Phillips, W. J., Tukrawinski, S., and Cerone, R. A. (1999) J. Biol. Chem. 264, 16679–16688
Pitcher, J. A., Inglese, J., Higgins, J. B., Ariza, J. L., Casey, P. J., Kim, C., Benovic, J. L.
β-Adrenergic Receptor Kinase Stimulation by Gγ N Terminus

J. L., Kwatra, M. M., Caron, M. G., and Lefkowitz, R. J. (1992) Science 257, 1264–1267
Premont, R. T., Inglese, J., and Lefkowitz, R. J. (1995) FASEB J. 9, 175–182
Sallese, M., Lombardi, M. S., Haske, T. N., LeVine, H., III, and DeBlasi, A. (1995) J. Receptor Signal Transduction Res. 15, 81–90
Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
Simonds, W. F., Butrynski, J. E., Gautam, N, Unson, C. G., and Spiegel, A. M. (1991) J. Biol. Chem. 266, 5363–5366
Sternweis, P. C., and Pang, I.-H. (1990) Receptor-Effector Coupling: A Practical Approach (Hulme, E. C., ed) pp. 1–31, New York, IRL Press at Oxford University Press, New York
Tamir, H., Fawzi, A. B., Tamir, A., Evans, T., and Northup, J. K. (1991) Biochemistry 30, 3929–3936
Taussig, R., Tang, W.-J., Hepler, J. R., and Gilman, A. G. (1994) J. Biol. Chem. 269, 6093–6100
Tholanikunnel, B. G., Granneman, J. G., and Malbon, C. C. (1995) J. Biol. Chem. 270, 12787–12793
Thomas, T. C., Sladek, T., Yi, F., Smith, T., and Neer, E. J. (1993) Biochemistry 32, 8628–8635
Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 10217–10220
Wang, D.-S., Shaw, R., Winkelman, J. C., and Shaw, G. (1994) Biochem. Biophys. Res. Commun. 203, 29–35
Wilcox, M. D., Schey, K. L., Dingus, J., Mehta, N. D., Tatum, B. S., Halushka, M., Finch, J. W., and Hildebrandt, J. D. (1994) J. Biol. Chem. 269, 12508–12513
Winslow, J. W., Van Amsterdam, J. R., and Neer, E. J. (1986) J. Biol. Chem. 261, 7571–7579
Yamane, H. K., and Fung, B. K.-K. (1993) Annu. Rev. Pharmacol. Toxicol. 32, 201–241