Distinct Sites on G Protein βγ Subunits Regulate Different Effector Functions*

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G proteins interact with effectors at multiple sites and regulate their activity. The functional significance of multiple contact points is not well understood. We previously identified three residues on distinct surfaces of Gβγ that are crucial for G protein-coupled inward rectifier K⁺ (GIRK) channel activation. Here we show that mutations at these sites, S67K, S89T, and T128F, abolished or reduced direct GIRK current activation in inside-out patches, but, surprisingly, all mutants synergized with sodium in activating K⁺ currents. Each of the three Gβγ mutants bound the channel indicating that the defects reflected mainly functional impairments. We tested these mutants for functional interactions with effectors other than K⁺ channels. With N-type calcium channels, Gβγ wild type and mutants all inhibited basal currents. A depolarizing pre-pulse relieved Gβγ inhibition of Ca²⁺ currents by the wild type and the S89T and T128F mutants but not the S67K mutant. Both wild type and mutant Gβγ subunits activated phospholipase C β2 with similar potencies; however, the S67K mutant showed reduced maximal activity. These data establish a pattern where mutations can alter the Gβγ regulation of a specific effector function without affecting other Gβγ-mediated functions. Moreover, Ser-67 showed this pattern in all three effectors tested, suggesting that this residue participates in a common functional domain on Gβγ that regulates several effectors. These data show that distinct domains within Gβγ subserve specific functional roles.

G protein βγ subunits interact with several effectors including G protein-gated K⁺ channels, N-type calcium channels and phospholipase Cβ (1). Several reports have investigated the molecular sites of interactions between Gβγ and their effectors, but the exact molecular mechanism by which Gβγ alters effector function remains unclear. In one case, the co-crystal structure of the Gβγ/γ2 and phosphoducin has been determined (2). This structure points to multiple interactions between the two proteins concentrated in at least two distinct domains. Other studies using indirect approaches including mutagenesis have corroborated this design for Gβγ interaction with its effectors (2–5). Most of these studies have identified multiple sites on Gβγ as well as on each effector, where such interactions may occur.

We have recently reported multiple sites of interactions between GIRK channels and Gβγ subunits based on chimeric analysis and mutagenesis studies (6–8). First, we showed that there are at least three interaction sites on the GIRK channel that are crucial both for binding to Gβγ and for transducing the Gβγ effects on channel activity. Recently, we also identified eight residues on Gβγ that are important in functional interactions with GIRK1 channels (8). These amino acids reside on distinct domains of the G protein as assessed from the known crystal structure (9, 10), consistent with the notion of multiple interactions between the two proteins.

Although several interaction points between Gβγ and effectors have been identified, the functional significance of these multiple interactions remains unclear. Furthermore, effector-specific interaction domains on Gβγ have not been defined. Here, we have tested three mutants located on separate surfaces of Gβγ for interactions with GIRK channels, N-type calcium channels, and PLCβ2. One of these mutants, Gβγ(S67K)γ2 showed specific functional defects in regulating each of these three effectors without affecting other Gβγ functional interactions with these effectors.

EXPERIMENTAL PROCEDURES
cDNAs and Mutants—All of the cDNAs used were subcloned into pGEMHE to accommodate sufficient expression in oocytes. Mutations were made using the QuickChange method (Stratagene, La Jolla, CA) using high fidelity Pfu polymerase for 12–16 cycles only. All mutations were confirmed by DNA sequencing (Cornell University Sequencing Facilities, Ithaca, NY).

GST-GIRK Pulldown Assays—Recombinant baculoviruses encoding wild type β1, β1(S67K), β1(S89T), and β1(T128F) were generated using the Bac to Bac Expression System (Invitrogen). G protein βγ complexes were purified by co-expression of βγ with hexahistidine-tagged γ2. The C-terminal (amino acids 191–419) and the N-terminal (amino acids 1–92) domains of GIRK4 were expressed as GST fusion proteins in Escherichia coli BL21(DE3). GIRK4 protein was induced with 0.5 mM isopropyl-1-thio-β-d-galactopyranoside at 37 °C for 3 h, and the protein was purified from the soluble extract. GIRK4 protein was induced with 1.0 mM isopropyl-1-thio-β-D-galactopyranoside at 37 °C for 3 h and purified from inclusion bodies. After solubilization with 5 mM urea, the protein was refolded by dialysis against phosphate-buffered saline (PBS) containing 20% glycerol followed by dialysis against PBS containing 5 mM EDTA and 0.15 mM phenylmethylsulfonyl fluoride. Proteins were purified using glutathione-agarose beads (Sigma). Binding of Gβγ to GST-GIRK4 or GST-GIRK4 was assayed essentially as described (6). Recombinant GST-GIRK (10 μM) was incubated with glutamic acid.

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The abbreviations used are: GIRK, G protein-coupled inward rectifier K⁺; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PLC, phospholipase Cβ; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
tathione-agarose beads for 30 min at 4 °C in PBS containing 2 mM EDTA, 1 mM dithiothreitol, and 0.1% Lubrol. Unbound protein was removed by centrifugation. Gβγ(1 μM) was added to glutathione fusion protein and allowed to incubate for an additional 30 min at 4 °C. Unbound Gβγ was removed by centrifugation, and the glutathione-bound protein complexes were washed with PBS containing 2 mM EDTA, 1 mM dithiothreitol, and 0.01% Lubrol. Protein was eluted by heating at 70 °C for 10 min in SDS-PAGE sample buffer. Gβγ was detected by immunoblot using an antibody directed against the C-terminal peptide of Gβγ.

Expression in Oocytes—cDNA constructs were linearized and subjected to in vitro transcription using the mMessage mMachine kit (Ambion, Austin, TX). The resulting cRNAs were quantified by comparison of two dilutions to a standard on a formaldehyde gel. Oocytes were isolated from Rana pipiens frogs, enzymatically digested with collagenase and incubated in ND-96 solution containing calcium and nutrients (11). Oocytes were injected with cRNAs, 2 ng each of GIRK4/S143T and 2 ng of each G protein subunit or mutant. Following injection, oocytes were kept for 48–96 h in an 18 °C incubator before recordings.

Gβγ Purification.—The Gβγ subunits used for the PLC assay and inside-out patch experiments were generated using the BaculoGold Expression System (PharMingen) and were purified by co-expression of a hemagglutinin-tagged Gβγ, as described by Kozasa and Gilman (12) except that lubrol was replaced with CHAPS in the final washes. Final protein concentrations were estimated against known amounts of BSA standard on SDS-PAGE following Coomassie staining.

Electrophysiology.—Two-electrode voltage clamp recordings were carried out as previously described (13). Briefly, the oocytes were placed in a chamber and perfused with a solution containing high potassium (96 mM). Currents were recorded using a voltage step protocol from −100 to +50 mV (control current), and barium (3 mM) was used to measure the current that was not inwardly rectifying (barium-insensitive current). We determined the inwardly rectifying current by subtracting barium-insensitive current from the control current.

Macro-patch channel activity was recorded on devitellinized oocytes using the inside-out mode of standard patch clamp methods as described (14). The pipette solution contained 96 mM KCl, 1 mM MgCl2, and 5 mM HEPES, pH 7.4. The bath solutions contained 96 mM KCl, 10 mM HEPES, 5 mM EGTA, 20 mM dithiothreitol, and 0.01% CHAPS, pH 7.4. The purified Gβγ subunits were diluted to 16 nM in the bath solution. Recordings were made at a holding membrane potential of −80 mV. Recordings were made using the EPC-9 patch clamp amplifier and PULSE/PULSEFIT (v.7.6) data acquisition software (Heka Electronik, Lambrecht, Germany). Data were stored on the hard disk of a PC-compatible computer and plotted using Microcal Origin. The sampling rate was 10 Hz for most recordings.

For recordings from N-type channels, α1s, α1β, and β1 subunits of calcium channels were expressed at a ratio of 3:1:4 respectively with a higher level than the initial sodium application before the activation was obtained in 10–12 min. Gβγ activation was observed in a small reduction in the K+ current indicating that most of the Gβγ was still on the channel. A second application of sodium activated the channel to a much higher level than the initial sodium application before the activation of GIRK1/GIRK4* channels in oocytes. Sodium, which interacts directly with GIRK4*, activates independently of Gβγ (13, 17, 18), was used to test current levels before and after application of purified Gβγ. We have previously shown that Gβγ can enhance activation of K+ channels by sodium in a synergistic way. Therefore, measuring activation of the channel by sodium after application of Gβγ can serve as an indicator for interactions between the channel and Gβγ. Fig. 2 shows results from inside-out patches in oocytes. Channel activity was tested by a brief bath application of 30 mM sodium; after washout of sodium, 16 mM purified Gβγ-protein was applied to the patch. Wild type Gβγ enhanced GIRK4* currents significantly, whereas the mutants failed to do so. This is similar to our findings in GIRK1/GIRK4 heteromeric channels (8).

To address whether these functional defects were due to a lack of channel binding, we tested the binding of these three mutants to the N and C termini of GIRK4. Fig. 1C shows the results from one such experiment. GST was used as negative control. Wild type Gβγ, as well as mutants Gβγ(S67K), Gβγ(S88T), and Gβγ(T128F), all bound both the N and C termini of GIRK4. Although some differences in channel binding could be detected between Gβγ(S67K) and wild type Gβγ, precise quantitation of such differences was not attempted. Similar experiments showed proper binding of the wild type and the three mutants to the N and C termini of GIRK1 (data not shown). These data indicate that the functional defects in these mutants were not due to a lack of binding to the channel.

Activation of GIRK4* Channels in Inside-Out Patches—We next tested in inside-out patches the activity of purified wild type and mutant Gβγ subunits on GIRK4* channels expressed in oocytes. Sodium, which interacts directly with GIRK4*, activates independently of Gβγ (13, 16, 17) and activates currents independently of Gβγ (13, 17, 18), was used to test current levels before and after application of purified Gβγ. We have previously shown that Gβγ can enhance activation of K+ channels by sodium in a synergistic way. Therefore, measuring activation of the channel by sodium after application of Gβγ can serve as an indicator for interactions between the channel and Gβγ. Fig. 2 shows results from inside-out patches in oocytes. Channel activity was tested by a brief bath application of 30 mM sodium; after washout of sodium, 16 mM purified Gβγ-protein was applied to the patch. Wild type Gβγ enhanced GIRK4* currents significantly, whereas the mutants failed to do so. This is similar to our findings in GIRK1/GIRK4 heteromeric channels (8).

For recordings from N-type channels, α1s, α1β, and β1 subunits of calcium channels were expressed at a ratio of 3:1:4 respectively with a total concentration of 1 μM. G protein subunits were expressed in a ratio of 1:2:3 relative to those for GIRK channel recordings. Two-electrode voltage clamp recordings from oocytes were performed 5–7 days after injections in external solutions containing: 50 mM NaCl, 0.4 mM CaCl2, 2 mM KOAc, 5 mM HEPES pH 7.5 (BaOAc). For recordings, the oocytes were held at −100 mV, and a voltage step to +10 mV was used to activate N-type channels. For pre-pulse facilitation, a depolarizing step to +100 mV was used 1 min after the first activating pulse, followed by a 5-s return to −100 mV and a +10 mV activating pulse. Basal currents were measured at the maximal current level during the first activation pulse. Facilitation is defined as the percentage of the amplitude of the second activating pulse compared with the first.

PLC Assay—The PLC assay was performed as described (3). SF9 cell membranes expressing PLCβ2 were incubated with a mixture of [3H]PIP2 and excess cold-phospholipids in the presence or absence of G protein subunits. A control tube with no PLC added was used to measure background. Reactions were started by addition of 0.5 mM calcium and incubated at 32 °C for 15 min. Reactions were stopped by the addition of methanol/chloroform mixture and EGTA. Tubes were centrifuged, the aqueous phase collected, and radioactive counts of 400 μl of the aqueous phase were determined in a scintillation counter. All the counts were collected, and the blank was subtracted. Generation of inositol 1,4,5-trisphosphate was measured as [3H]counts per minute in the aqueous phase. Dose-response curves were fitted using the sigmoidal curve-fitting routines in Microcal Origin 6.0, and EC50 values were calculated.

RESULTS

Gβγ Mutants Confer Functional but Not Binding Defects.—We previously reported that the Gβγ(S67K), T128F, and S98T mutants failed to activate heteromeric GIRK1/GIRK4 channels. Fig. 1A shows the position of these residues on three distinct surfaces of Gβγ as determined from the crystal structure of Gβγ (9). Here, we use GIRK4/S143T (denoted as GIRK4*), a homomeric-active GIRK4 channel with a mutation in the porehelix (11), to test the effectiveness of these Gβγ mutants to enhance basal currents. We have previously shown that homomeric GIRK4* channels behave similarly to the GIRK1/GIRK4 heteromers and are therefore functionally interchangeable (15). Fig. 1B shows the effectiveness of different Gβγ subunits co-expressed with Gγ2 to enhance GIRK4* currents in oocytes. For comparison, currents at −80 mV were normalized to the levels of GIRK4* expressed alone. Wild type Gβγ enhanced basal GIRK4* currents significantly, whereas the mutants failed to do so. This is similar to our findings in GIRK1/GIRK4 heteromeric channels (8).

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Activation of GIRK4* Channels in Inside-Out Patches.—We next tested in inside-out patches the activity of purified wild type and mutant Gβγ subunits on GIRK4* channels expressed in oocytes. Sodium, which interacts directly with GIRK4*, activates independently of Gβγ (13, 16, 17) and activates currents independently of Gβγ (13, 17, 18), was used to test current levels before and after application of purified Gβγ. We have previously shown that Gβγ can enhance activation of K+ channels by sodium in a synergistic way. Therefore, measuring activation of the channel by sodium after application of Gβγ can serve as an indicator for interactions between the channel and Gβγ. Fig. 2 shows results from inside-out patches in oocytes. Channel activity was tested by a brief bath application of 30 mM sodium; after washout of sodium, 16 mM purified Gβγ-protein was applied to the patch. Wild type Gβγ enhanced GIRK4* currents significantly, whereas the mutants failed to do so. This is similar to our findings in GIRK1/GIRK4 heteromeric channels (8).

Mutants S98T and T128F also activated the channel in all patches that were tested; however, the activation was smaller, and mutants could be washed out with faster kinetics compared with the wild type Gβγ (Fig. 2A). Again in both cases, an enhancement in the sodium response was observed after activation with the mutant proteins. The Gβγ(S67K) mutant on the other hand failed to activate the channel in half of the patches tested, whereas the activation in the other half of the patches was minor (Fig. 2B). However, in all patches regardless of the ability of S67K to activate GIRK* currents, the Na+ response was enhanced following application of S67K (Fig. 2A). Sum-
mary data show the direct channel activation by G\(_{\beta\gamma}\) and mutants (Fig. 2B) and the G\(_{\beta\gamma}\) enhancement of the Na\(^+\) activity (Fig. 2C). These results indicate that S67K induces the conformational changes on the channel that are necessary to enhance the Na\(^+\) activation but are not sufficient to cause channel activation. Furthermore, in those patches where channel activity was stimulated by S67K, this activity was smaller (Fig. 2B) and reversed upon washout with faster kinetics compared with the wild type G\(_{\beta\gamma}\) (data not shown), again suggesting a different functional interaction between the channel and this mutant as compared with the wild type G\(_{\beta\gamma}\).

**Serine 67 Is Important in Interactions with N-type Channels**—We next set out to test the possible role of these three residues in interactions with other effectors of G\(_{\beta\gamma}\). G\(_{\beta\gamma}\) normally inhibits N-type calcium channels. A strong depolarizing pre-pulse relieves this inhibition (19). The G\(_{\beta\gamma}\) inhibition of N-type channels plays an important role in neurotransmitter release and neuronal excitability (20). Distinct interactions between N-type channels and different G\(_{\beta\gamma}\) subtypes have been previously reported (21). We tested the interactions of wild type G\(_{\beta\gamma}\) as well as the three mutants S67K, S98T, and T128F with N-type channels expressed in *Xenopus* oocytes. We co-injected in oocytes mRNAs for the \(\alpha_{1B}\), \(\alpha_{1D}\), and \(\beta_3\) subunits of calcium channels along with the different G protein subunit combinations and tested for inhibition of basal currents as well as pre-pulse facilitation of these currents in oocytes. Fig. 3A shows recordings in the absence and presence of a depolarizing pre-pulse for all combinations tested. All expressed G\(_{\beta\gamma}\) proteins, wild type and mutants, led to a significant reduction in basal calcium channel activity (Fig. 3B).
Activated channels in all patches tested. S67K activated channels in half of the patches tested. 

Altered the functional interactions between the G protein but the lack of facilitation suggests that this mutation has

3 min and reached a maximal level after 10–12 min (B). Wild-type Gβγ2 could be partially washed within 2–5 min. Most of the activity of mutants S98T and T128F were washed with faster kinetics (less than 30 s). Mutant S67K did not activate the channel after Gβγ application caused much higher channel activity (C). B, summary data showing channel activation by the purified Gβγ2 and the mutants. Data is expressed as the ratio between maximal activation by purified protein (B) and the initial sodium activation (A). S98T and T128F activated channels in all patches tested. S67K activated channels in half of the patches tested. C, summary of Na⁺ activation of channel following exposure to Gβγ. Data is expressed as the ratio of Na⁺ activation after Gβγ (C) to the initial sodium activation (A).

Because Gβγ interaction with N-type channels leads to current inhibition, this suggested that all tested Gβγ proteins interact with the channel. In the absence of co-expressed Gβγ the depolarizing pre-pulse did not lead to facilitation of the current. Pre-pulse facilitation was observed in oocytes expressing Gβ1γ2, Gβ1(S98T)γ2 and Gβ1(T128F)γ2. A summary of these data is shown in Fig. 3C. Both S98T and T128F mutants showed facilitation that was not significantly different from the wild type Gβ1γ2 (P > 0.05, unpaired t test). In oocytes expressing Gβ1(S67K)γ2 basal current inhibition was similar to other mutants (~50%, Fig. 3B); however, no pre-pulse facilitation was observed (Fig. 3C). The inhibition of basal current by Gβ1(S67K)γ2 clearly shows that it interacts with the channel, but the lack of facilitation suggests that this mutation has altered the functional interactions between the Gβγ and the Ca²⁺ channel.

Activation of Phospholipase C by Gβγ—Gβγ subunits activate several subtypes of phospholipase Cβ through direct protein-protein interactions (22). Several sites both on the G protein and PLCβ have been identified to be crucial for the functional interaction of the two proteins (3, 4). We tested the effectiveness of our three mutants, S67K, S98T, and T128F in activating PLCβ2. Wild type and mutant Gβγ subunits were purified from S99 cells and used in a PLC assay of PIP₂ hydrolysis. Crude S9 cell membranes expressing PLCβ2 were used. This preparation displays some basal activity that can be enhanced by Gβγ subunits (3). Three independent experiments were performed and Fig. 4 shows the results from one such experiment.

FIG. 2. Gβ mutants fail to activate GIRK⁺ currents but do potentate the Na⁺ effects. A, in the inside-out configuration of the patch clamp, channel activity was first tested by a brief pulse of sodium (A), followed by a 1-min washout before 16 nM purified Gβγ was applied. Channel activation was observed within 2–3 min and reached a maximal level after 10–12 min (B). Wild-type Gβγ2 could be partially washed within 2–5 min. Most of the activity of mutants S98T and T128F were washed with faster kinetics (less than 30 s). Mutant S67K did not activate the channel in three patches of six tested, and in those where it did activate, it could be washed almost completely within 30 s. In all cases, a brief Na⁺ pulse after Gβγ application caused much higher channel activity (C). B, summary data showing channel activation by the purified Gβγ2 and the mutants. Data is expressed as the ratio between maximal activation by purified protein (B) and the initial sodium activation (A). S98T and T128F activated channels in all patches tested. S67K activated channels in half of the patches tested. C, summary of Na⁺ activation of channel following exposure to Gβγ. Data is expressed as the ratio of Na⁺ activation after Gβγ (C) to the initial sodium activation (A).

All mutants stimulated enzyme activity with similar potency indicating that all three mutants interact properly with PLCβ2. Interestingly, Gβ1(S67K)γ2 showed reduced maximal activation of PLCβ2 suggesting partial activation of the enzyme and a potential role for this residue in functional interactions between the two proteins.

DISCUSSION

The βγ subunits of G proteins interact at multiple sites with their effectors. Here we provide evidence that there are independent activating domains within Gβγ that control distinct functions within a given effector. Ser-67 is a surface-exposed residue on Gβ1; mutation of this residue to lysine led to alteration in certain Gβγ actions on effectors without affecting others.

Although binding of this mutant to the channel was similar to the control and it showed enhancement of the Na⁺ activation of GIRK channels, it failed to directly activate K⁺ currents in whole-cell or inside-out patch recordings. This clearly demonstrates that although Ser-67 resides in a domain that is critical for direct activation of GIRK channels, its mutation does not interfere with either binding of Gβγ to the channel or Gβγ interactions that are required to enhance the effectiveness of Na⁺. Therefore, the Gβγ domain required for direct activation of the GIRK channels is distinct from regions that are sufficient for binding of the channel. Furthermore, enhancement of the Na⁺ effect by Gβγ requires interactions between Gβγ and the
channel that are distinct from those involved in direct G\(\beta\gamma\)-mediated activation of K\(^+\) currents.

N-type calcium currents are basally inhibited in the presence of G\(\beta\gamma\). A depolarizing pulse applied to the membrane before channel activation can relieve this inhibition. We tested the effects of mutations at three surface-exposed residues on G\(\beta\gamma\) in interactions with N-type channels. All three mutants caused reduction of basal currents similar to the wild type G\(\beta\gamma\). Interestingly, pre-pulse depolarization did not relieve the inhibition by the S67K mutant, whereas the other two mutants behaved like wild type G\(\beta\gamma\). Here, we observe dual interactions of G\(\beta\gamma\) with the channel, first inhibiting basal activity and second a voltage-sensitive “unbinding” and relief of the basal inhibition. The mutant S67K is immune to the voltage-dependent relief although it effectively interacts and inhibits the channel. Again we observe a separation of the two functional effects of G\(\beta\gamma\) and the involvement of Ser-67 in one but not the other.

Finally, each of the three critical surface-exposed residues activated PLC\(\beta_2\) with potencies similar to that of the wild type G\(\beta\gamma\). However, S67K showed reduced maximal activity. Reduced maximal activity indicates that this mutant cannot fully activate PLC even though it binds normally as indicated by its

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potency. This suggests that serine 67 is involved in activation of PLC by Gβγ.

These functionally critical residues that we identified on the three-dimensional structure of Gβ1 are located on multiple surfaces of Gβ1 supporting the biochemical evidence of multiple interaction points between effectors and Gβ1. Multiple interaction sites of Gβγ have also been found with phosducin (2). Using peptides derived from different regions of Gβ as well as mutagenesis it has been suggested that multiple domains of Gβ interact with PLCβ (3, 4). Our data not only support multiple Gβ/effector interactions, but in addition they demonstrate for the first time that unique and separate molecular determinants of Gβ regulate specific effector functions.

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