Binding of the G protein βγ subunit to multiple regions of G protein-gated inward-rectifying K⁺ channels

Chou-Long Huang*, Yuh Nung Jan, Lily Y. Jan

Howard Hughes Medical Institute, Departments of Physiology and Biochemistry and Biophysics, the University of California at San Francisco, CA 94143-0724, USA

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Abstract We have previously shown that direct binding of the βγ subunit of G protein (Gβγ) to both the N-terminal domain and the C-terminal domain of a cloned G protein-gated inward-rectifying K⁺ channel subunit, GIRK1, is important for channel activation. We have now further localized the Gβγ binding region in the N-terminal domain of GIRK1 to amino acids 34–86 and the Gβγ binding region in the C-terminal domain of GIRK1 to two separate fragments of amino acids 318–374 and amino acids 390–462. Of the four cloned mammalian GIRK subunits, GIRK1–4, GIRK1 and 4 form heteromeric K⁺ channels in the heart and similar channels in the brain include heteromultimers of GIRK1 and 2, and possibly other GIRK homomultimers and heteromultimers. We found that the N-terminal and the C-terminal domains of all four GIRKs bound Gβγ. The Gβγ binding activities for the C-terminal domains of GIRK2–4 were lower than that for the C-terminal domain of GIRK1. The higher Gβγ binding activity for the C-terminal domain of GIRK1 is due to amino acids 390–462 which are unique to GIRK1. We also found that the N-terminal and C-terminal domains of GIRKs interacted with each other, and the N-terminal domain of either GIRK1 or GIRK4 together with the C-terminal domain of GIRK1 exhibited much enhanced binding of Gβγ. These results are consistent with the idea that the N- and C-terminal domains of the cardiac G protein-gated K⁺ channel subunits may interact with each other to form higher affinity binding site(s) for Gβγ.

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Key words: Direct protein-protein interaction; Weaver mutation; G-protein-gated inwardly rectifying K⁺ channel; Fusion protein; Gβγ binding; Glutathione-S-transferase

1. Introduction

Activation of G protein-gated inwardly rectifying K⁺ channel by G protein-coupled receptors represents one mechanism of inhibitory synaptic transmission [1]. In the heart, activation of the muscarinic K⁺ channel by acetylcholine results in slowing of the heart rate [2–4]. In the nervous system, activation of similar G protein-activated K⁺ channels may mediate the inhibitory action of neurotransmitters such as somatostatin, adenosine, serotonin, opioid peptides and γ-aminobutyric acid (GABAγ) [5,6]. The βγ subunit of G protein (Gβγ) activates these K⁺ channels via a membrane-delimited pathway that does not involve water-soluble cytoplasmic factors [7–9].

*Corresponding author (present address): University of Texas Southwestern Medical Center at Dallas, H5-112, MC 8856, 5323 Harry Hines Blvd, Dallas, TX 75235-8856, USA. Fax: (1) (214) 648-2071.
E-mail: chuan1@mednet.swmed.edu

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2. Materials and methods

2.1. Materials

IPTG and molecular biological reagents were from Boehringer Mannheim. Glutathione 4B Sepharose beads, reduced glutathione and pGEX-2T vector were from Pharmacia. Iminodiacetic acid coupled-Sepharose beads charged with nickel ion were from Invitrogen. Donkey anti-rabbit IgG coupled with horseradish peroxidase and enhanced chemiluminescence (ECL) system for western blotting were from Ameraham. Other chemicals including imidazole were from Sigma. E. coli DE 3 strain was used for fusion protein expression.

2.2. Construction of plasmid and expression of GST fusion protein

PGEX-2T vector was used for expression of recombinant bacterial fusion proteins. Nucleotide sequences corresponding to the peptide fragment (suspended in an equal volume of PBS) was incubated with nickel affinity beads and eluted with 10 mM reduced glutathione according to the Pharmacia product instruction manual as previously described [33, 34]. Fusion proteins were dialyzed in phosphate-buffered saline (PBS) at 4°C and stored at -70°C. The purity and integrity of fusion proteins were checked by SDS-PAGE and Coomassie Blue staining. The concentration of fusion protein is determined using a Bio-Rad protein assay kit (Bradford).

2.3. Co precipitation assay and western blotting

For binding of Gβγ to GST fusion proteins, purified bovine brain Gβγ (40 nM) [34] was incubated with GST fusion proteins at the indicated concentrations and 20 μl of glutathione Sepharose beads and eluting with 10 mM reduced glutathione according to the Pharmacia product instruction manual as previously described [33, 34]. Fusion proteins were dialyzed in phosphate-buffered saline (PBS) at 4°C and stored at -70°C. The purity and integrity of fusion proteins were checked by SDS-PAGE and Coomassie Blue staining. The concentration of fusion protein is determined using a Bio-Rad protein assay kit (Bradford).

For binding of Gβγ to H6-tagged fusion protein of GγKs, Gβγ (40 nM) was incubated with GST-GKC-H6 at the indicated concentrations and 20 μl of GST-GKC-H6 beads were incubated with GγK at room temperature for 1 h. The GST-GKC-H6 beads were washed with PBS and the bound proteins were eluted with 10 mM reduced glutathione.

2.4. Coprecipitation assay and western blotting

Using the coprecipitation assay to detect interaction between purified Gβγ and hexahistidine (H6) tagged fusion proteins containing the fragment of GIRK1, we have previously shown that Gβγ binds to the C-terminal domain of GIRK1 [25]. Using glutathione-S-transferase (GST) fusion protein of GIRK1, Inanobe et al. [26] and Kunkel and Peralta [27] have also demonstrated binding of Gβγ to the C-terminal domain of GIRK1. Previously, we localized the GγK binding site on the C-terminal domain of GIRK1, and made additional fusion proteins containing smaller fragments of GIRK1 and examined coprecipitation of these proteins with the purified Gβγ.

3. Results

3.1. Gβγ binding site in the C-terminal domain of GIRK1

Using the coprecipitation assay to detect interaction between purified Gβγ and hexahistidine (H6) tagged fusion proteins containing the fragment of GIRK1, we have previously shown that Gβγ binds to the C-terminal domain of GIRK1 [25]. Using glutathione-S-transferase (GST) fusion protein of GIRK1, Inanobe et al. [26] and Kunkel and Peralta [27] have also demonstrated binding of Gβγ to the C-terminal domain of GIRK1. Previously, we localized the Gβγ binding site on the C-terminal domain of GIRK1, and made additional fusion proteins containing smaller fragments of GIRK1 and examined coprecipitation of these proteins with the purified Gβγ.

In analyzing a series of deletion constructs, we found that deletion of A.A. 373-317 (as in GST-GKCI80-374) did not affect binding of the fusion protein to Gβγ (Fig. 1). Further deletion of A.A. 318-339 (as in GST-GKC318-339), however, reduced the Gβγ binding activity of the fusion protein, indicating that A.A. 318-339 either was part of the binding site or was important for the stability of the fusion protein. A fusion protein containing A.A. 180-374 (GST-GKC180-374) showed reduced Gβγ binding activity. Amino acids from 374-384 of GIRK1 have been suggested to be important for binding with Gβγ based on its limited sequence homology with adenylyl cyclase 2 [36]. We therefore made a GST fusion protein of the C-terminal domain of GIRK1 with an internal deletion of A.A. 375-389 (GST-GKC180-374; 300-402) with the fragment of amino acids 180-374 fused to the fragment of amino acids 390-462 and found that the binding of Gβγ to this fusion protein was comparable to that of Gβγ to GST-GKC180-374, indicating that A.A. 375-389 is not critical for the Gβγ interaction. Taken together, these results indicate that the regions of A.A. 318-374 and A.A. 390-462 of the C-terminal domain of GIRK1 are important for Gβγ binding. A smaller fusion protein containing A.A. 390-462 (GST-GKC390-462), however, did not show detectable Gβγ binding activity (not shown). We do not know whether this fusion protein is folded properly. Previously, we have shown that a peptide derived from amino acids 434-462 of GIRK1 (peptide GC) [25] partially inhibited binding of Gβγ to the entire C-terminal domain of GIRK1. We found that peptide GC partially inhibited the binding of Gβγ to GST-GKC340-462, but not that of Gβγ to GST-GKC180-374 (Fig. 1C). This partial inhibition could be an indication that other amino acids besides 434-462 in the region of amino acids 390-462 are also important for Gβγ binding.

3.2. Gβγ binding activity of GIRK2-4

The Hill coefficient for Gβγ activation of atrial muscarinic K+ channel is > 1, indicating that more than one Gβγ is required for full activation of the G protein-gated K+ channels [37]. Since the atrial and neuronal G protein-gated inward-rectifying K+ channels may include heteromultimers of GIRK1 and 4 and heteromultimers of GIRK1 and 2, respectively, it would be important to determine whether GIRK2 and 4 also bind Gβγ. The findings that Gβγ can activate the
homomeric GIRK2 or GIRK4 channels [12,13,16-19] suggest that both GIRK2 and GIRK4 contain G\(\beta\)\(\gamma\) binding site. Given that the region of A.A. 318-374 of GIRK1 is highly conserved in GIRK1^l (Fig. 2A), we wonder whether the C-terminal domain of GIRK2-4 would also interact with G\(\beta\)\(\gamma\). Indeed, GST fusion proteins containing the C-terminal domains of GIRK2 (GST-G2C), GIRK3 (GST-G3C), and GIRK4 (GST-G4C) all bound G\(\beta\)\(\gamma\) (Fig. 2B). The G\(\beta\)\(\gamma\) binding activity of GST-G2C, GST-G3C or GST-G4C is equivalent to that of GST-GKC\(_{180-462}\), but is less than that of GST-GKC\(_{390-462}\). The control fusion protein containing the C-terminal domain of IRK1, GST-IRK, did not show significant binding to G\(\beta\)\(\gamma\). The greater G\(\beta\)\(\gamma\) binding activity of GST-GKC\(_{180-462}\) could be accounted for by the finding that both the regions of A.A. 318-374 and A.A. 390-462 of the C-terminal domain of GIRK1 contribute to G\(\beta\)\(\gamma\) binding activity.

### 3.4. The N-terminal and C-terminal domains of GIRKs interact with each other

Our finding of G\(\beta\)\(\gamma\) binding to both the N- and C-terminal
domains of the GIRKs raises the possibility that the two domains may interact with each other to form a stronger binding site for G\(\beta\gamma\). We therefore looked for interaction between GST-GKN and GST-GKC using the co precipitation assay. A GST-GKC-H6 fusion protein was constructed by adding a hexahistidine affinity tag to the C-terminus of GST-GKC\(_{180-462}\). A control GST-H6 was also constructed. By incubating a solution containing 1 \(\mu\)M GST-GKN and 200 nM of either GST-GKC-H6 or GST-H6 with nickel affinity beads, we found that GST-GKN co precipitated with

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**Fig. 2.** Binding of G\(\beta\gamma\) to C-terminal domains of GIRK2-4. (A) Sequence comparison between amino acid 318-374 of GIRK1 and homologous regions of GIRK2, 3 and 4. The number in parentheses ( ) indicates amino acids for each channel shown for comparison. Amino acids are shown in single-letter code. Letter in bold indicates identity or similarity of amino acids among GIRK1-4. (B) Co-precipitation of G\(\beta\gamma\) with C-terminal domains of GIRK1-4. GST fusion protein containing C-terminal domain of GIRK1 (GST-GKC\(_{180-462}\); GST-GKC\(_{180-374}\)), GIRK2 (GST-G2C), GIRK3 (GST-G3C) or GIRK4 (GST-G4C) (200 nM) as indicated were incubated with G\(\beta\gamma\) (40 nM) and processed for co-precipitation as described in Fig. 1 and Section 2. Filter was probed with antibodies against G\(\beta\). The immunoreactivity for G\(\beta\) is indicated by the arrow. Similar results were observed in 2 other experiments.

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**Fig. 3.** G\(\beta\gamma\) binding region in the N-terminal domain of GIRK1. (A) Schematic diagram of GST fusion protein constructs of the N-terminal domain of GIRK1 and G\(\beta\gamma\) binding activities for these constructs. (+) Indicates maximal activity; ( ) denotes reduced activity; (-) indicates no detectable activity. (B) Localization of G\(\beta\gamma\) binding region. GST fusion proteins containing fragments of N-terminal domain of GIRK1 as indicated (200 nM) or the control GST alone were incubated with G\(\beta\gamma\) (40 nM) and processed for coprecipitation. Nitrocellulose filter was probed with antibodies against G\(\beta\). The immunoreactivity for G\(\beta\) is indicated by the arrow. Similar results were observed in 2 other experiments.
Fig. 4. Binding of Gβγ to N-terminal domains of GIRK2-4. (A) Sequence comparison between amino acids 34–86 of GIRK1 and homologous regions of GIRK2-4. The number in parentheses ( ) indicates amino acids for each channel shown for comparison. Amino acids are shown in single-letter code. Letter in bold indicates identity or similarity of amino acids among GIRK1-4. (B) Co-precipitation of Gβγ with N-terminal domains of GIRK1-4. GST fusion proteins containing fragments of the N-terminal domain of GIRK1 (GST-GKN), GIRK2 (GST-G2N), GIRK3 (GST-G3N) or GIRK4 (GST-G4N) (200 nM) as indicated were incubated with Gβγ (40 nM) and processed for co-precipitation. Filter was probed with antibodies against Gβ. The immunoreactivity for Gβ is indicated by the arrow. Similar results were observed in 3 other experiments.

GST-GKC-H6, but not with GST-H6 (Fig. 5A), indicating a specific protein-protein interaction between the N- and C-terminal domains of GIRK1. The fusion protein GST-G4N also coprecipitated specifically with GST-GKC-H6 (5B), indicating that the C-terminal domain of GIRK1 also interacts with the N-terminal domain of GIRK4, consistent with the previous reports that GIRK1 and GIRK4 form heteromultimer in the heart [13].

To see if the interaction of the N-terminal domain with the C-terminal domain of the channel alters the binding of Gβγ to the channel fragments, we used nickel affinity beads to precipitate GST-GKC-H6, which was present at 50, 100 or 200 nM together with GST, GST-GKN or GST-G4N and with or without Gβγ. GST-GKN or GST-G4N coprecipitated with GST-GKC-H6 in a dose-dependent manner using nickel affinity beads (not shown). In the presence of GST, GST-GKN or GST-G4N, Gβγ coprecipitated with increasing concentrations of GST-GKC-H6 in a dose-dependent manner (Fig. 6). However, the amount of Gβγ coprecipitating with 100 nM GST-GKC-H6 in the presence of 1 μM GST-GKN or GST-G4N was equivalent to that with 200 nM GST-GKC-H6 in the presence of 1 μM control GST. This increase in the binding of Gβγ to GST-GKC-H6 is more than an additive effect of the binding of Gβγ to GST-GKC-H6 and that of Gβγ to GST-GKN or GST-G4N. The N-terminal domain has a lower affinity for binding Gβγ [25]. Given that at most one tenth of the GST-GKN or GST-G4N coprecipitated with GST-GKC-H6 using nickel affinity beads under the experimental condi-

Fig. 5. Interaction between the C-terminal domain of GIRK1 and the N-terminal domain of GIRK1 (A) or GIRK4 (B). (A) GST-GKN (1 μM) was incubated with GST-GKC-H6 or the control GST-H6 (200 nM) and processed for coprecipitation by nickel affinity beads. Nitrocellulose membrane was probed with antibodies against the GST-GKN. The immunoreactivity for GST-GKN is indicated by arrow. Similar results were obtained in 2 other experiments. (B) GST-G4N (1 μM) was incubated with GST-GKC-H6 or the control GST-H6 (200 nM) and processed for coprecipitation by nickel affinity beads. Filter was probed with antibodies against GST-G4N. The immunoreactivity for GST-G4N is indicated by the arrow. Similar results were observed in 2 other experiments.
4. Discussion

By studying co precipitation of Gβγ with a series of fusion proteins containing fragments of GIRK channel subunits, we have found that all four mammalian GIRKs contain Gβγ binding regions and further identified regions of GIRKs that are important for Gβγ binding. Based upon the findings that fusion proteins GST-GKC180-462, GST-GKC318-462, GST-GKC340-462, GST-GKC390-462 and GST-GKC390-462 all bound Gβγ and the binding activities for GST-GKC180-462, GST-GKC318-462 and GST-GKC340-374, 390-462 were equal but greater than the activity for GST-GKC434-462 or GST-GKC180-374, we conclude that both the fragments of A.A. 318-374 and A.A. 390-462 contribute to the Gβγ binding activity in the C-terminal domain of GIRK1 (Fig. 1). The existence of Gβγ binding site(s) in the fragment of A.A. 318-374 is evident from the finding that both GST-GKC180-374 and GST-GKC318-462 bound Gβγ. Addition of amino acids 390-462 to the fragment of A.A. 318-374 (as in GST-GKC180-374; 390-462 or GST-GKC318-462) markedly enhanced the Gβγ binding activity of the fusion protein, either by providing an additional Gβγ binding site or by increasing the stability of the fusion protein. The finding that a peptide corresponding to A.A. 434-462 of GIRK1 partially inhibited the binding of Gβγ to GST-GKC340-462 suggests that the fragment of A.A. 390-462 also contains binding site(s) for Gβγ. Nevertheless, as GST-GKC390-462 did not show detectable Gβγ binding, it remains possible that A.A. 390-462 contributes to the Gβγ binding by stabilizing the conformation of the fusion protein.

With respect to the N-terminal domain, we found that amino acids 34-86 of GIRK1 bound Gβγ, but its binding activity is lower compared to that of the full-length GKN1-86. The region of A.A. 1-33 may contribute to the maximal Gβγ binding activity of the N-terminal domain through providing an additional binding site or via stabilizing the conformation of the N-terminal domain. The finding that the N-terminal domains of GIRK2-4 (which have significant sequence homology to GIRK1 in the region of A.A. 34-86) bound Gβγ as well as GST-GKN1-86 is consistent with the observation that the region of A.A. 34-86 of GIRK1 binds Gβγ. Our previous finding of partial inhibition of the binding between GKN1-86 and Gβγ by a peptide (GN) derived from A.A. 1-38 of the N-terminal domain of GIRK1 [25], however, indicates that A.A. 1-38 may have weak interaction with Gβγ. Alternatively, GN peptide may interact with the N-terminal domain and thereby interfere with the binding of Gβγ to GST-GKN1-86.

We found that all 4 GIRK isoforms exhibit Gβγ binding activity on both the N- and C-terminal domains of the channel proteins. G protein-gated inward-rectifying K+ channels can be comprised of identical or similar GIRK subunits. The heteromultimers of GIRK1 and GIRK4, and GIRK1 and GIRK2 correspond to some of the cardiac muscarinic G protein-gated inward-rectifying K+ channels, I_{KACO} [13] and the G protein-gated K+ channel in the brain [16,20], respectively. While the homomultimers of GIRK2 or GIRK4 are functional G protein-gated K+ channels [13,16,18,19], the homomultimers of GIRK1 or GIRK3 do not give rise to K+ current [13,16,21]. Our results indicate that the inability of the homomultimers of GIRK1 or GIRK3 to produce K+ current is not due to a lack of Gβγ binding domain.

Using fusion proteins containing fragments of channel proteins, we found that the Gβγ binding activities for the N-terminal domain of GIRK1-4 are roughly equal and that for the C-terminal domain of GIRK1 is greater than the binding activity for the C-terminal domain of GIRK2, 3 or 4. Binding of Gβγ to both the N- and C-terminal domains of GIRK1 is important for channel activation [25,29,31]. It thus seems likely that binding of Gβγ to both the N- and C-terminal domains of GIRK2, 3 or 4 is also important for channel activation, as the Gβγ binding regions of GIRK2-4 are homologous to regions of A.A. 34-86 and A.A. 318-374 of GIRK1. The relative contribution of each region to the overall binding of Gβγ to the full-length polypeptide or the native multimeric channel, however, is unknown. A previous study using the full-length GIRK1 and GIRK4 proteins expressed in Sf 9 cells reported that the Gβγ binding activity for GIRK4 is slightly greater than that for GIRK1 [32]. Since we do not know how the individual Gβγ binding region on the N- or the C-terminal domain folds together to form the Gβγ binding site in the full-length protein, it is difficult to compare the results of the two studies. Nevertheless, our investigation
clearly demonstrates that residues that are important for Gβγ binding are distributed on both the N- and C-termini of the channel.

It is possible that the Gβγ binding regions on the N- and C-terminal cytoplasmic domains are interrelated. The two cytoplasmic domains interact with each other, and this interaction potentiates the binding of Gβγ to the channel. This finding of synergistic action between the N- and C-terminal domains in the activation of channel by Gβγ is supported by a recent report by Tucker et al. [31]. In that study, they made chimera channels containing the transmembrane domain of the G protein-insensitive subunit K₄.1 and the N-terminal, the C-terminal or both domains of GIRK1, and examined the role of these cytoplasmic domains in activation of channel by Gβγ. They found that K⁺ current through chimeric channels containing the N- or the C-terminal domain of GIRK1 was stimulated by Gβγ by ~1.5- or ~2-fold, respectively; K⁺ current through chimeric channels containing both the N- and C-terminal domains of GIRK1, in contrast, was maximally stimulated by Gβγ by ~7-fold. Our results of a synergistic effect between the N- and C-terminal domains for binding Gβγ reinforce this physiological finding using chimeric channels.

In addition to the G protein-gated inwardly rectifying K⁺ channels, Gβγ regulates many other effectors including phospholipase C-β, phospholipase A₂, type 2 adenylate cyclase, β-adrenergic receptor kinase (βARK), and Ca²⁺ channel [38]. The Gβγ binding sites for βARK [33] and phosphodosh [39] have been mapped. The consensus sequence, if any, for binding Gβγ reinforce this physiological finding using chimeric channels.

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