The Stoichiometry of Gβγ Binding to G-Protein-Regulated Inwardly Rectifying K⁺ Channels (GIRKs)

Running title: GIRK-Gβγ binding stoichiometry

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SUMMARY

G-protein-coupled inwardly rectifying K⁺ (GIRK; Kir3.x) channels are the primary effectors of numerous G-protein-coupled receptors. GIRK channels decrease cellular excitability by hyperpolarizing the membrane potential in cardiac cells, neurons, and secretory cells. Although direct regulation of GIRKs by the heterotrimeric G-protein subunit Gβγ has been extensively studied, little is known about the number of Gβγ-binding sites per channel. Here we demonstrate that purified GIRK (Kir 3.x) tetramers can be chemically cross-linked to exogenously purified Gβγ subunits. The observed laddering pattern of Gβγ attachment to GIRK4 homotetramers was consistent with the binding of one, two, three, or four Gβγ molecules per channel tetramer. The fraction of channels chemically cross-linked to four Gβγ molecules increased with increasing Gβγ concentrations and approached saturation. These results suggest that GIRK tetrameric channels have four Gβγ-binding sites. Thus, GIRK (Kir 3.x) channels, like the distantly related cyclic nucleotide-gated channels, are tetramers and exhibit a 1:1 subunit:ligand binding stoichiometry.
INTRODUCTION

Roughly 1 percent of the human genome encodes G-protein-coupled receptors (1). Agonist binding to these G-protein-coupled receptors catalyzes the activation of $G\alpha$ and $G\beta\gamma$ subunits of heterotrimeric G-proteins. The free $G\alpha$ and $G\beta\gamma$ subunits can then interact independently or in concert with numerous effectors. $G\beta\gamma$ regulates processes as diverse as the yeast pheromone response (2-4) and mammalian heart rate (5). The increasing list of $G\beta\gamma$ effectors includes ion channels (6-12), phospholipase C$\beta$ (13), adenylyl cyclases (14), G-protein-coupled receptor kinases (15), PI3 kinase (16), plasma membrane Ca$^{2+}$ pumps (17), Bruton’s tyrosine kinase (18), and calmodulin (19). Little is known about how $G\beta\gamma$ interacts with its effectors. The repeating WD40 motif of $G\beta\gamma$ gives it a rigid propeller-like structure, which does not appear to be altered upon its interaction with effectors (20-23).

Homotetrameric and heterotetrameric combinations of the four known mammalian GIRK subunits are activated by neurotransmitters in the nervous system, pancreas, and heart. Muscarinic (m2, m4), $\gamma$-aminobutyric acid (GABA$_B$), D2-dopamine, $\alpha$2-adrenergic, opiate, somatostatin, and adenosine all employ the $G\alpha_i$-$G\beta\gamma$ signal transduction system to activate GIRK channels via direct $G\beta\gamma$ binding to the tetrameric channel. GIRK4 knockout mice have irregularities in heart rate variability (5) and difficulties with spatial learning (24). GIRK2 knockout mice are prone to seizures (25). Weaver mice have a mutation in the pore domain of
the GIRK2 subunit (26) that renders the channel nonselective (27) and results in the degeneration of cerebellar granule cells (28) and the dopaminergic neurons of the substantia nigra (29,30).

The native atrial I_{K_ACh} channel is composed of two GIRK1 subunits and two GIRK4 subunits (31-33) that comprise a channel that mediates neuronal regulation of heart rate. Biochemical studies indicate that G\beta\gamma binds the native I_{K_ACh} complex with a K_d of 55 nM (9). G\beta\gamma binds both recombinant GIRK1 (K_d =125 nM) and GIRK4 (K_d = 50 nM) (9). GIRK1 subunits are unable to form functional homomultimers (34), whereas GIRK4 homomultimers have been biochemically isolated from bovine atria (35). GIRK2/3 and GIRK1/2 heteromultimers have also been isolated from brain (1,36). The carboxyl terminal tail of GIRK1 and GIRK4 subunits bind G\beta\gamma (9,32,37-46), but the detailed steps of how this binding leads to channel gating is not known. Furthermore, there is limited data about the areas of G\beta\gamma that bind GIRK channel subunits (43,47) and about how many G\beta\gamma subunits can bind the tetrameric channel complex.

We have used a biochemical approach to determine how many G\beta\gamma subunits bind GIRK tetramers. By extending our previous chemical cross-linking studies (31), which indicated that GIRKs form tetramers, we demonstrate that GIRK4 homotetramers bind four G\beta\gamma subunits in their natural membrane environment.
EXPERIMENTAL PROCEDURES

Isolation, solubilization and purification of GIRK1/GIRK4 heteromultimers (I_{KACH}) from native atrial membranes - Bovine atrial plasma membranes were isolated (48) and solubilized as described (31). Native GIRK1/GIRK4 heteromultimers (I_{KACH}) were purified to greater than 90% homogeneity as described (31). The protease inhibitors leupeptin (50 µg/ml Sigma-Aldrich Inc.), phenylmethylsulfonyl fluoride (100 µg/ml, Sigma-Aldrich Inc.), aprotinin (1 µg/ml, Sigma-Aldrich Inc.), and pepstatin (2 µg/ml, Sigma-Aldrich Inc.) were used during all steps of the purification.

Expression and isolation of GIRKs from COS7 and CHO cells - Plasma membrane proteins containing GIRK1-AU5 and GIRK4-AU1 were isolated from COS7 cells and solubilized as described (49).

Gβγ purification - G-proteins were isolated from bovine brain, separated into Gα and Gβγ subunits (50), and were further purified by affinity chromatography using immobilized Gα (51).

Gβγ-binding in membranes - Isolated COS7 cells or native atrial membranes were treated for 1 hour with 100 mM DTT and then dialyzed against 20-50 mM HEPES, 100 mM NaCl at pH 7.4 -7.5 (Gβγ-binding buffer). Individual membrane aliquots were preincubated with purified bovine brain Gβγ and rotated for 20 minutes at room temperature prior to cross-linking.
The Gβγ stock solution was in Gβγ-binding buffer containing 0.1% CHAPS (0.1% Gβγ-binding buffer). The final CHAPS concentration was less than or equal to 0.1%.

*Gβγ-binding to solubilized protein* - Solubilized COS7 membrane proteins were treated for 1 hour with 100 mM DTT and then dialyzed against 0.1% Gβγ-binding buffer. Individual aliquots were preincubated with purified brain Gβγ (supplied in 0.1% Gβγ-binding buffer) and rotated for 20 minutes at room temperature prior to cross-linking.

*Chemical cross-linking* - 5 mM dithiobis[sulfosuccinimidylpropionate] (DTSSP, Pierce Chemical, Rockford, Illinois) was prepared as an 11X stock solution immediately prior to use in 100 mM HEPES containing buffer at pH 7.5. Iodine was added only to solutions containing purified I*KACH*. Cross-linking reactions were allowed to proceed for 30 min at room temperature and then quenched with 50 mM Tris. Typically 5-10 µg of membrane proteins were used per reaction in a final volume of 15 µl.

*SDS-PAGE and immunoblotting* - Atrial membrane proteins or recombinant GIRK proteins were resuspended in Laemmli sample buffer containing 100 mM dithiothreitol (or 30 mM iodoacetamide when a cross-linking agent was used) for 15 min at 50 °C, 30 min at room temperature, and 15 min at 50 °C. 3-10% separating, 3% stacking, and pre-cast 2-15% (ISS) gels were utilized. Samples were analyzed by immunoblotting with anti-GIRK4 antibodies (generated against amino acids 19-32) (31) and/or anti-GIRK1 antibodies (generated against the last 156 amino acids of GIRK1) (31). Several antibodies were tested for use in the anti-Gβγ immunoblotting experiments. Only one anti-Gβγ antibody (anti-KTREGNVRVSREL, Chemicon International, Inc. Temecula, CA) reacted with Gβγ after DTSSP treatment.
Typically, DTSSP treatment reduced total antigenic signal by > 90%, > 60% and > 95% for anti-GIRK4, anti-GIRK1 and anti-Gβγ antibodies, respectively. Transfer times for immunoblot analysis were extended to > 2 hr at 15 V to improve transfer of the high molecular weight complexes. A GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, California) was used to analyze the protein gels and immunoblots. Molecular weights were calculated using densitometry profiles from a combination of pre-stained high molecular weight markers (Bio-Rad Laboratories, Hercules, California) and low and high molecular weight markers (Pharmacia Biotec Inc., Uppsala, Sweden). In a portion of the gels, thyroglobulin (Pharmacia Biotech Inc., Uppsala, Sweden) was added to insure linearity up to 330 kDa.
RESULTS

Previous chemical cross-linking studies demonstrated that GIRK subunits form tetrameric channels and that the native atrial channel $I_{KACa}$ is composed of 2 GIRK1 and 2 GIRK4 subunits (31). Complete cross-linking of purified atrial $I_{KACa}$ formed a single adduct with a total molecular weight that was most consistent with a tetramer. In addition, partial cross-linking of purified $I_{KACa}$ produced subsets of molecular weight adducts consistent with monomers, dimers, trimers, and tetramers. In this study, we extended our previous experiments to determine how many $G\beta\gamma$ molecules can be cross-linked to GIRK tetramers.

$G\beta\gamma$ cross-linking to purified native $I_{KACa}$. To test whether GIRK1/GIRK4 heteromultimers could be directly and specifically cross-linked to $G\beta\gamma$, we used isolated native atrial GIRK1 and GIRK4 subunits (31) and bovine brain $G\beta\gamma$ (9). Isolated GIRK1 and GIRK4 heterotetramers were preincubated with isolated $G\beta\gamma$, followed by cross-linking with DTSSP (Fig. 1). Although the predicted molecular weights of GIRK1 and GIRK4 subunits are 56 and 45 kDa, respectively, the glycosylated GIRK1 migrates in a broad band between 67 - 72 kDa (9). In the absence of $G\beta\gamma$, a band formed at 230 kDa, consistent with the total molecular weight of two GIRK1 (56, 67-72 kDa) and two GIRK4 (45 kDa) subunits. In the presence of $G\beta\gamma$, a band corresponding to a molecular weight of 390 kDa was detected. Since $G\beta\gamma$, GIRK1 and GIRK4 were the predominant proteins present, we interpreted the 160 kDa shift as the result of direct cross-
linking of Gβγ to GIRK channels. The molecular weight of Gβγ is 42 kDa, suggesting that the 160 kDa shift was due to cross-linking of several Gβγ molecules to the GIRK1/GIRK4 heterotetrarmers. Since GIRK4 can form homotetramers, we repeated the previous cross-linking experiment using recombinant GIRK4 subunits. In the absence of Gβγ, cross-linking of recombinant GIRK4 resulted in a band at 170 kDa, corresponding to GIRK4 homotetramers. When Gβγ was added to recombinant GIRK4, cross-linking yielded a band at ~320 kDa (not shown). This banding pattern is most consistent with four specific and saturable Gβγ-binding sites per GIRK4 homotetramer.

**Cross-linking of membrane confined GIRK4 homotetramers.** It is important to study GIRK-Gβγ binding in its membrane environment since phosphatidylinositol-bis-phosphate (PIP2)(52,53) is involved in the Gβγ-mediated activation of GIRK channels. Our previous GIRK cross-linking studies employed isolated, solubilized channels (31). In this study, we tested whether GIRK subunits could be cross-linked into tetramers in membranes. After DTSSP cross-linking of membranes from COS7 cells expressing either recombinant GIRK4, GIRK1, or GIRK1 and GIRK4, SDS-PAGE yielded 180-220 kDa bands (Fig. 2B, lanes 1-3). These bands are similar in molecular weight to those produced when solubilized GIRKs are cross-linked into tetramers (31). Of the GIRK tetramers, the chemically cross-linked GIRK4 homotetramers produced the narrowest band, around 190 kDa (Fig. 2B, lane 1). In addition, the GIRK4 band cross-linked directly in membranes (Fig. 2B, lane 1) was narrower than that of GIRK4 that had been solubilized before cross-linking (31).
Partial Gβγ cross-linking to membrane-confined GIRK tetramers. GIRK4 homotetramers were used in our membrane-confined GIRK-Gβγ binding experiments because cross-linking of GIRK4 homotetramers in membranes yielded the narrowest bands. We altered our cross-linking conditions in order to verify that there were indeed four Gβγ binding sites in the GIRK tetramer. DTSSP and Gβγ concentrations were adjusted so that variable numbers of Gβγ molecules were cross-linked to the GIRK4 homotetramers. COS7 cells were transiently transfected with GIRK4, and their membranes were divided into separate aliquots. Each aliquot was treated with variable amounts of Gβγ and DTSSP then analyzed by SDS-PAGE and immunoblotting. Untreated GIRK4, migrated as a 47 kDa TCA disruptable monomer (Fig. 3B, lane 1). GIRK4 cross-linked with DTSSP migrated as a 170 kDa tetramer (Fig. 3B, lane 2). GIRK4 preincubated with Gβγ and then cross-linked with DTSSP, resulted in a laddering pattern of four main adducts (in addition to the GIRK4 homotetramer adduct) with consistent 40-45 kDa increments between bands (Fig. 3B, lanes 3 & 4). The proportion of high molecular weight adducts increased with increasing Gβγ concentrations. Unlike our previous experiments that used solubilized GIRK protein, a population of the membrane-confined GIRK4 homotetramers (166 kDa) remained resistant to any Gβγ binding. One possible explanation for this observation is that a subpopulation of GIRK4 homotetramers may not have been accessible to the exogenously applied Gβγ. In five independent trials, four GIRK-Gβγ adducts consistently appeared. In some trials, high molecular weight, lower intensity smears formed, but these bands were not consistently reproducible. A laddering pattern was not formed when Gβγ was boiled prior to its addition to membranes (data not shown). We hypothesize that the five adducts formed by
treatment of G\(\beta\gamma\) and GIRK4-containing solutions with DTSSP represent the binding of zero, one, two, three and four G\(\beta\gamma\) molecules to GIRK4 homotetramers.

Multiple lines of evidence suggest that G\(\beta\gamma\) is being directly cross-linked to GIRK channels in our experiments. G\(\beta\gamma\) has been coimmunoprecipitated with GIRK subunits under the conditions used in our experiments (9). The ~45 kDa increments between cross-linked GIRK-G\(\beta\gamma\) adducts are consistent with the stepwise addition of 42 kDa G\(\beta\gamma\) subunits to the channel. Finally, similar results were obtained even when I\(_{\text{K}_{\text{ACh}}}\) and G\(\beta\gamma\) were purified to >95% homogeneity prior to cross-linking. Because, I\(_{\text{K}_{\text{ACh}}}\) and G\(\beta\gamma\) are the predominant proteins in solution, the molecular weight shift with G\(\beta\gamma\) addition strongly suggests that G\(\beta\gamma\) is being directly cross-linked to the channel. As a final precaution, we tested whether the putative GIRK-G\(\beta\gamma\) adducts are recognized by anti-G\(\beta\gamma\) antibodies. COS7 cells were transiently transfected with GIRK4 and their membranes were isolated. The membranes were treated with G\(\beta\gamma\) and DTSSP, followed by SDS-PAGE analysis. Immunoblots were probed with anti-GIRK4 antibodies then stripped and reprobed with anti-G\(\beta\gamma\) antibodies (Fig. 4, lanes 1 & 2, respectively). The anti-G\(\beta\gamma\) antibodies recognized bands at molecular weights that correspond to the putative GIRK-G\(\beta\gamma\) adducts.
DISCUSSION

The present study of Gβγ binding to channel proteins has several advantages over other approaches. First, we ensured that we were using intact tetramers throughout our Gβγ-binding experiments. In addition, we purposely studied Gβγ binding in membranes in order to approximate physiological conditions. This is especially important because PIP₂, a component of the cell membrane, plays a role in Gβγ-mediated activation of GIRKs (52,53). Non-prenylated Gβγ mutants do not activate GIRK channels (54,55), indicating that Gβγ association with cell membranes may be a prerequisite for Gβγ-binding. We have paid careful attention to detergent concentrations, because low detergent conditions can potentially expose hydrophobic patches on GIRKs, producing nonspecific binding. Indeed, we found it difficult to prevent GIRK and Gβγ aggregation in low detergent concentrations.

The stoichiometry of the Iₖₐₜₐₜ-Gβγ interaction has been repeatedly estimated by using the Hill equation to fit the Gβγ-Iₖₐₜₐₜ dose-response curve. Estimates of the Hill coefficient for Iₖₐₜₐₜ activation varied from 1.5 (9) to 3 (56,57) while it was ~1 in the study of the direct binding of purified Iₖₐₜₐₜ and Gβγ proteins (9). Although often used to infer binding stoichiometry of Gβγ with GIRK subunits, the Hill coefficient is a measure of cooperativity, not the number of binding sites. In order for the Hill coefficient to equal the Gβγ-binding stoichiometry, two criteria need to be met or approximated. The Gβγ molecules must bind the channel simultaneously and Gβγ must bind with infinite cooperativity (58). In addition, the Hill equation
does not take into account the increasing open probability of the channel with each ligand molecule bound. Thus, the stoichiometry of Gβγ binding to I_{KACH} is not adequately determined by fits of the Hill equation to the Gβγ dose-response relations. Even the more complicated Monod, Wyman, and Changeux (MWC) does not properly describe the subunit gating of the cyclic nucleotide-gated channel (59). Nevertheless, the cooperativity in I_{KACH} activation (9,56,57) and the Gβγ-dependent shifts in its gating modes (60,61) suggest that GIRK channels have multiple Gβγ-binding sites.

Given the inadequacy of available models, a direct biochemical approach was used to determine GIRK-Gβγ-binding stoichiometry. Solutions containing purified GIRK1 and GIRK4 were treated with the cross-linking reagent DTSSP in the presence or absence of Gβγ. A 230 kDa band was observed in the absence of Gβγ, compared to a 390 kDa band when Gβγ was present. We concluded that the 160 kDa shift was the result of covalent linkage of multiple 42 kDa Gβγ molecules to the channel. Next, solubilized recombinant GIRK4 homotetramers were treated with DTSSP in the presence and absence of Gβγ. A 170 kDa band formed without Gβγ in contrast to the 320 kDa band in the Gβγ-containing experiment. The 150 kDa shift in the presence of Gβγ is most consistent with the chemical cross-linking of four 42 kDa Gβγ molecules to the GIRK4 homotetramers with complete Gβγ binding site saturation and cross-linking. Finally, a variety of membrane-associated GIRK channels were treated with DTSSP and analyzed by SDS-PAGE. In each case, completely cross-linked GIRK tetramers resulted. To verify that there were four Gβγ binding sites in GIRK tetramers, we altered our cross-linking conditions. DTSSP and Gβγ concentrations were adjusted so that variable numbers of Gβγ
molecules were cross-linked to the GIRK4 homotetramers. Five adducts, representing zero, one, two, three, and four Gβγ molecules cross-linked to the channel, were detected. We were unable to cross-link more than four Gβγ molecules to the channel, even with Gβγ concentrations two orders of magnitude higher than the $K_d$ for Gβγ binding to GIRK subunits. We conclude that four Gβγ subunits can bind to a GIRK tetramer.

Currently, the Gβγ binding site on GIRK subunits is thought to reside primarily on the cytoplasmic carboxyl terminal region shortly after the second transmembrane domain. But a detailed description of the GIRK/Gβγ binding site will undoubtedly require direct structural determination. For example, it is not possible to determine with our experiments whether the Gβγ binding pockets were formed within subunits or between subunits. Short of direct structural determination, in future experiments it may be possible to cross-link Gβγ to GIRKs during patch clamp recording. Such a technique has proven valuable in evaluating cyclic-nucleotide binding to cyclic-nucleotide-gated channels (59).

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FOOTNOTE

The abbreviations used are:

GIRK, G-protein gated inwardly rectifying K\(^+\) channel;

DTSSP, dithiobis(sulfosuccinimidylpropionate);

PAGE, polyacrylamide gel electrophoresis;

SDS, sodium dodecyl sulfate;

PVDF, polyvinylidene difluoride;

TCA, trichloroacetic acid;

Kir, inwardly rectifying K\(^+\)-selective channel;

\(I_{\text{KACH}}\), Native Atrial G-Protein-Gated K\(^+\) Channel;
FIGURE LEGENDS

Figure 1. Purified native atrial GIRK1&4 heterotetramers can be directly and specifically cross-linked to purified Gβγ. A, Schematic depicting procedure used to generate (B). Squares represent GIRK subunits. B, 2-15% SDS-PAGE and anti-GIRK1 immunoblotting of solubilized GIRK1 and GIRK4 heterotetramers. Lane 1, pure solubilized native atrial GIRK1 and GIRK4 heterotetramers treated with 1 mM DTSSP. Lane 2, pure solubilized GIRK1 and GIRK4 heterotetramers preincubated with 3 µm Gβγ and treated with 1 mM DTSSP. Exposure times for Lane1 and Lane2 were not identical.

Figure 2. Membrane-confined GIRK channels can be completely chemically cross-linked into tetramers. A, Schematic depicting procedure used to generate (B). B, CHO membrane proteins were cross-linked with DTSSP and analyzed by 2-15% SDS-PAGE and immunoblotting. Lanes 1-3, membranes from CHO cells transfected with the indicated GIRK subunit and cross-linked with 2.5 mM DTSSP.

Figure 3. DTSSP treatment of membrane-confined GIRK4 homotetramers in the presence of Gβγ creates a laddering pattern consistent with 4 Gβγ sites per GIRK tetramer. 3-10% SDS-PAGE analysis, anti-GIRK4 immunoblotting and cross-linking of membranes from COS7 cells that were transfected with GIRK4. A, Schematic depicting procedure used to generate (B). B, Lane 1, GIRK4-containing membranes that were TCA precipitated prior to SDS-PAGE. Lane 2, GIRK4-containing membranes cross-linked with 5 mM DTSSP. Lanes 3&4, GIRK4-
containing membranes pretreated with 0.13 µM and 0.63 µM Gβγ, respectively, followed by cross-linking with 5 mM DTSSP. **Squares** represent GIRK subunits. **Circles** represent Gβγ molecules. The molecular weight markers at left correspond to panel 1. The molecular weight markers on the right correspond to adducts in panel 2. **C**, GIRK4-containing membranes pretreated with 3.1 µM Gβγ and then cross-linked with 5 mM DTSSP, enlarged view. **D**, In a separate trial, densitometry profiles were created by scanning each lane of the gel top to bottom, which is now represented as top to bottom on the graph. **Profile 1**, GIRK4-containing membranes cross-linked with 5 mM DTSSP (0.0 µM Gβγ). **Profile 2**, GIRK4-containing membranes preincubated with 0.6 µM Gβγ and cross-linked with DTSSP (0.6 µM Gβγ). **Profile 3**, GIRK4-containing membranes preincubated with 3.1 µM Gβγ and cross-linked with 5 mM DTSSP (3.1 µM Gβγ).

**Figure 4.** Anti-Gβγ antibodies recognize bands at molecular weights that correspond to the putative GIRK-Gβγ adducts. 3-10% SDS-PAGE analysis and immunoblotting of membranes from COS7 cells that were transfected with GIRK4, pretreated with Gβγ, and then treated with DTSSP. **Lane 1**, GIRK4-containing membranes pretreated with 6.3 µM Gβγ and cross-linked with 4 mM DTSSP probed with anti-GIRK4 antibodies. **Lane 2**, (Lane 1) stripped and reprobed with anti-Gβγ antibodies.
Figure 1
Figure 3
Figure 4
The stoichiometry of $G_{\beta\gamma}$ binding to G-protein-regulated inwardly rectifying K+ channels (GIRKs)
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