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

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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.

Roughly 2% of the human genome encodes G-protein-coupled receptors (1). Agonist binding to these G-protein-coupled receptors catalyzes the activation of Gα and Gβγ subunits of heterotrimeric G-proteins. The free Gα and Gβγ subunits can then interact independently or in concert with numerous effectors. Gβγ regulates processes as diverse as the yeast pheromone response (2–4) and mammalian heart rate (5). The increasing list of Gβγ effectors includes ion channels (6–12), phospholipase C β (13), adenyl cyclases (14), G-protein-coupled receptor kinases (15), PI 3-kinase (16), plasma membrane Ca²⁺ pumps (17), Bruton’s tyrosine kinase (18), and calmodulin (19). Little is known about how Gβγ interacts with its effectors. The repeating WD40 motif of Gβγ 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), γ-aminobutyric acid (GABA B), D₂-dopamine, α₂-adrenergic, opiate, somatostatin, and adenosine all employ the Gαi-Gβγ signal transduction system to activate GIRK channels via direct Gβγ 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 IKACh 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 Gβγ binds the native IKACh complex with a Kd of 55 nM (9). Gβγ binds both recombinant GIRK1 (Kd = 125 nM) and GIRK4 (Kd = 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 C-terminal tail of GIRK1 and GIRK4 subunits bind Gβγ (9, 32, and 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 Gβγ that bind GIRK channel subunits (43, 47) and about how many Gβγ subunits can bind the tetrameric channel complex.

We have used a biochemical approach to determine how many Gβγ 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 Gβγ subunits in their natural membrane environment.

EXPERIMENTAL PROCEDURES

Isolation, Solubilization, and Purification of GIRK1/GIRK4 Heteromultimers (IKACh) from Native Atrial Membranes—Bovine atrial plasma membranes were isolated (48) and solubilized as described (31). Native GIRK1/GIRK4 heteromultimers (IKACh) 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 and 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 h with 100 μM dithiothreitol and then dialyzed against 20–50 mM HEPES, 100 mM NaCl, pH 7.4–7.5 (Gβγ
binding buffer). Individual membrane aliquots were preincubated with purified bovine brain Gβγ and rotated for 20 min 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 h with 100 mM dithiothreitol 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 min at room temperature prior to cross-linking.

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. 5–10% separating, 3% stacking, and pre-cast gels (20%) were utilized. Samples were analyzed by immunoblotting with anti-GIRK4 antibodies (generated against amino acids 19–32, Ref. 31) and/or anti-GIRK1 antibodies (generated against the last 156 amino acids of GIRK1, Ref. 31). Several antibodies were tested for use in the anti-Gβγ immunoblotting experiments. Only one anti-Gβγ antibody (anti-KTREGNVRVSREL, Chemicon International, 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 h 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 masses were calculated using densitometry profiles from a combination of prestained high molecular mass markers (Bio-Rad) and low and high molecular mass markers (Amersham Pharmacia Biotech). In a portion of the gels, thyroglobulin (Amersham Pharmacia Biotech) was added to molecular mass markers (Amersham Pharmacia Biotech). In a portion of prestained high molecular mass markers (Bio-Rad) and low and high molecular weight proteins were treated for 1 h with 100 mM dithiothreitol and then cross-linked. Gas 19–32, Ref. 31) and/or anti-GIRK1 antibodies (generated against amino acids 19–32, Ref. 31) and/or anti-GIRK1 antibodies (generated against the last 156 amino acids of GIRK1, Ref. 31). Several antibodies were tested for use in the anti-Gβγ immunoblotting experiments. Only one anti-Gβγ antibody (anti-KTREGNVRVSREL, Chemicon International, 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 h 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 masses were calculated using densitometry profiles from a combination of prestained high molecular mass markers (Bio-Rad) and low and high molecular mass markers (Amersham Pharmacia Biotech). In a portion of the gels, thyroglobulin (Amersham Pharmacia Biotech) was added to ensure 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_{KACH} is composed of 2 GIRK1 and 2 GIRK4 subunits (31). Complete cross-linking of purified atrial I_{KACH} formed a single adduct with a total molecular mass that was most consistent with a tetramer. In addition, partial cross-linking of purified I_{KACH} 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 Gβγ molecules can be cross-linked to GIRK tetramers.

Gβγ Cross-linking to Purified Native I_{KACH}—To test whether GIRK1/GIRK4 heterotetramers could be directly and specifically cross-linked to purified Gβγ, we used isolated native atrial GIRK1 and GIRK4 subunits (31) and bovine brain Gβγ (9). Isolated GIRK1 and GIRK4 heterotetramers were preincubated with isolated Gβγ, followed by cross-linking with DTSSP (Fig. 1). Although the predicted molecular masses 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 Gβγ, a band corresponding to a molecular mass of 390 kDa was detected. Because Gβγ, 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 mass of Gβγ is 42 kDa, suggesting that the 160-kDa shift was because of cross-linking of several Gβγ molecules to the GIRK1/GIRK4 heterotetramers. Because Gβγ 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 because phosphatidylinositol bisphosphate (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 mass 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 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 and then analyzed by SDS-PAGE and immunoblotting. Untreated GIRK4, treated as a 47-kDa trichloroacetic acid-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 and 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 heterotetramers (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 mass, 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 Gβγ and GIRK4-containing solutions with DTSSP represent the binding of zero, one, two, three, and four Gβγ molecules to GIRK4 homotetramers.

Multiple lines of evidence suggest that Gβγ is directly cross-linked to GIRK channels in our experiments. Gβγ has been coimmunoprecipitated with GIRK subunits under the conditions used in our experiments (9). The ~45-kDa increments between cross-linked GIRK-Gβγ adducts are consistent with the stepwise addition of 42-kDa Gβγ subunits to the channel. Finally, similar results were obtained even when I_{KACH} and Gβγ were purified to ~95% homogeneity prior to cross-linking. Because, I_{KACH} and Gβγ are the predominant proteins in solution, the molecular mass shift with Gβγ addition strongly suggests that Gβγ is being directly cross-linked to the channel. As a final precaution, we tested whether the putative GIRK-Gβγ adducts are recognized by anti-Gβγ antibodies. COS7 cells were transiently transfected with GIRK4 and their membranes were isolated. The membranes were treated with Gβγ and DTSSP, followed by SDS-PAGE analysis. Immunoblots were probed with anti-GIRK4 antibodies then stripped and reprobed with anti-Gβγ antibodies (Fig. 4, lanes 1 and 2, respectively). The anti-Gβγ antibodies recognized bands at molecular masses that correspond to the putative GIRK-Gβγ 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 GIRK binding in membranes 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). Nonprenylated 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 I_{KACH}-Gβγ interaction has been repeatedly estimated by using the Hill equation to fit the Gβγ-I_{KACH} dose-response curve. Estimates of the Hill coefficient for I_{KACH} activation varied from 1.5 (9) to 3 (56, 57) whereas it was ~1 in the study of the direct binding of purified I_{KACH} 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. For the Hill coefficient to equal the Gβγ binding stoichiometry, two criteria need to be met or approximated. The Gβγ molecules must bind...
of magnitude higher than the three, and four G

Hill equation to the G

into account the increasing open probability of the channel cooperativity (58). In addition, the Hill equation does not take

detected. We were unable to cross-link more than four G

more complicated Monod, Wyman, and Changeux (MWC) formalism for purified G biopsy, anti-GIRK1 and anti-GIRK4 antibodies. Heidi Chial for critical reading of the manuscript and Dr. Eva Neer for helpful discussions.

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A 230-kDa band was observed in the G

binding pockets were formed within subunits or between subunits. Short of direct structural determination, in future experiments it may be possible to cross-link G biopsy to GIRKs during patch clamp recording. Such a technique has proven valuable in evaluating cyclic nucleotide binding to cyclic nucleotide-gated channels (59).

the channel simultaneously and G biopsy 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 G biopsy binding to I

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activation (9, 56, 57) and the G biopsy-dependent shifts in its gating modes (60, 61) suggest that GIRK channels have multiple G biopsy binding sites.

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