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Evidence That Direct Binding of $G_{\beta\gamma}$ to the GIRK1 G Protein–Gated Inwardly Rectifying K^+ Channel Is Important for Channel Activation

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Summary

Activation of G protein–gated K^+ channels by G protein–coupled receptors contributes to parasympathetic regulation of heart rate in the atrium and inhibitory postsynaptic potentials in the peripheral and central nervous system. Having found that $G_{\beta\gamma}$ activates the cloned GIRK1 channel, we now report evidence for direct binding of $G_{\beta\gamma}$ to both the N-terminal hydrophilic domain and amino acids 273–462 of the C-terminal domain of GIRK1. These direct interactions are physiologically important because synthetic peptides derived from either domain reduce the $G_{\beta\gamma}$ binding as well as the $G_{\beta\gamma}$ activation of the channel. Moreover, the N-terminal domain may also bind trimeric $G_{\alpha\beta\gamma}$, raising the possibility that physical association of G protein–coupled receptors, G proteins, and K^+ channels partially accounts for their compartmentalization and hence rapid and specific channel activation by receptors.

Introduction

K^+ channel activation by transmitters, via their G protein–coupled receptors, represents a basic mechanism of inhibitory synaptic transmission. Indeed, the first demonstrated chemical synaptic transmission involves the ability of the parasympathetic transmitter acetylcholine to activate the m2 muscarinic receptors and consequently the muscarinic K^+ channels, $I_{K(ACh)}$ in the pacemaker cells, resulting in slowing of the heart rate (Loewi, 1921; Hartzell, 1988; Brown and Birnbaumer, 1990; Szabo and Otero, 1990; Hille, 1992; Kurachi et al., 1992). This activation of K^+ channels requires the activation of G proteins (Breitwieser and Szabo, 1985; Pfaffinger et al., 1985) but does not involve water-soluble cytoplasmic factors (Sakmann et al., 1983; Soejima and Noma, 1984). Similar membrane-delimited activation of inwardly rectifying K^+ channels may mediate the action of inhibitory transmitters such as somatostatin, γ-aminobutyric acid type B ($GABA_B$), adenosine, serotonin, and opioid peptides in central and peripheral neurons (Andrade et al., 1986; Mihara et al., 1987; Nawy and Copenhagen, 1987; North et al., 1987; Trussell and Jackson, 1987; Williams et al., 1988; Miyake et al., 1989; North, 1989; Nicoll et al., 1990; Rainnie et al., 1994).

How G proteins activate muscarinic K^+ channels in a membrane-delimited manner is not known. Both α and βγ subunits of the activated G protein have been reported to activate cardiac muscarinic K^+ channels in excised, inside-out membrane patches (Codina et al., 1987; Logothetis et al., 1987, 1988; Yatani et al., 1987; Cerbai et al., 1988; Kirsch et al., 1988; Kurachi et al., 1989c; Kobayashi et al., 1990; Ito et al., 1992; Kurachi, 1992; Wickman et al., 1994). However, it has not been possible to determine whether the G protein subunits directly bind to the channel protein and cause channel activation (Hille, 1992; Clapham, 1994). Although indirect actions of G proteins involving intermediates of the phospholipase A_2 pathway have been implicated in the activation of muscarinic K^+ channels (Kim et al., 1989; Kurachi et al., 1989b), a recent study revealed that inhibitors of the phospholipase A_2 pathway do not block activation of muscarinic K^+ channels by $G_{\beta\gamma}$ (Ito et al.,1992). Therefore, it remains to be determined whether channel activation is mediated by direct interaction between the G protein and the channel protein.

The cDNA for a G protein–activated K^+ channel has been isolated (GIRK1/KGA) (Dascal et al., 1993; Kubo et al., 1993b). In the heart, GIRK1 has been found to coassemble with cardiac inward rectifier (CIR), another member of the inwardly rectifying K^+ channel family (Krapivinsky et al., 1995). Moreover, coinjection of GIRK1 and CIR, GIRK2, or GIRK3 into Xenopus laevis oocytes results in much increased current expression, whereas certain cultured cells transfected with cDNA for GIRK1 alone fail to express functional G protein–gated K^+ channels (Kofuji et al., 1995; Krapivinsky et al., 1995). These observations strongly suggest that GIRK1 is a subunit of heteromultimeric G protein–gated K^+ channels. However, it remains possible that GIRK1 can form functional homomultimeric channels in the heart and other tissues. In this study, we refer to GIRK1 channels as channels expressed in Xenopus oocytes injected with *GIRK1* cRNA.

While both α (G_α) and βγ ($G_{\beta\gamma}$) subunits of the activated G protein have been reported to activate the atrial muscarinic K^+ channel (see above), only $G_{\beta\gamma}$ has been found to activate the GIRK1 channel (Reuveny et al., 1994; Takao et al., 1994; Kofuji et al., 1995; Lim et al., 1995). Although previous studies have suggested that the C-terminal domain is important for the activation of GIRK1 by $G_{\beta\gamma}$ (Reuveny et al., 1994; Takao et al., 1994), there is no direct evidence that the C-terminal domain or other regions of GIRK1 bind $G_{\beta\gamma}$. To determine whether G protein can activate this G protein–gated K^+ channel via direct protein–protein interactions between G protein and the channel,

we studied the interaction of $G_{\beta\gamma}$ with GIRK1 using combined biochemical and electrophysiological approaches. First, we asked whether $G_{\beta\gamma}$ binds to GIRK1 and, if so, whether purified $G_{\beta\gamma}$ directly binds to purified hydrophilic domains of GIRK1. We then carried out deletion analysis to define more clearly the regions of GIRK1 that bind $G_{\beta\gamma}$. This has enabled us to design peptides of sequences derived from $G_{\beta\gamma}$-binding regions of GIRK1, in order to test whether these peptides interfere with $G_{\beta\gamma}$ binding to GIRK1 as well as $G_{\beta\gamma}$ activation of GIRK1 channels. To our surprise, our studies reveal that two separate regions of GIRK1 directly bind $G_{\beta\gamma}$ and are involved in channel activation by $G_{\beta\gamma}$.

Results

Physical Association of the GIRK1 Channel with $G_{\beta\gamma}$

The cloned GIRK1 channel belongs to a rapidly expanding family of inwardly rectifying K^+ channels, composed of subunits that each contain two putative transmembrane domains (Ho et al., 1993; Kubo et al., 1993a). As control for GIRK1, the inward rectifier IRK1 (Kubo et al., 1993a) was chosen because it shares 43% amino acid identity with GIRK1 (Kubo et al., 1993b) but does not require G protein for channel activation. To determine whether $G_{\beta\gamma}$ specifically associates with the GIRK1 channel, we expressed hexa-histidine-tagged GIRK1 (H_6-GIRK1) or IRK1 (H_6-IRK1) in Sf9 cells using the baculovirus vector, incubated detergent-solubilized channel proteins with detergent-solubilized $G_{\beta\gamma}$ expressed in Sf9 cells, and used nickel resin affinity beads for H_6-tagged proteins to precipitate H_6-GIRK1 or H_6-IRK1 (see Experimental Procedures). We found that H_6-GIRK1 but not H_6-IRK1 coprecipitated $G_{\beta\gamma}$, even when H_6-IRK1 was present at a level 5 times higher than H_6-GIRK1 (Figure 1A). The amount of H_6-GIRK1 protein expressed in Sf9 cells was comparable to that of H_6-IRK1 protein (Figures 1A and 1B, legend). These results clearly indicate that $G_{\beta\gamma}$ is specifically associated with GIRK1.

Direct Binding of $G_{\beta\gamma}$ to Two Regions of GIRK1

To determine whether $G_{\beta\gamma}$ binds directly to GIRK1 and, if so, to localize $G_{\beta\gamma}$ binding regions on GIRK1, we expressed bacterial fusion proteins of the N- or C-terminal hydrophilic domains of GIRK1 with the H_6 tag (H_6-GKN or H_6-GKC_1; Figure 2A), purified them to apparent homogeneity (Figure 2B), and examined their ability to bind purified $G_{\beta\gamma}$. $G_{\beta\gamma}$ coprecipitated with H_6-GKC_1 and H_6-GKN, but not with control H_6-IKC, when H_6 affinity beads were used to precipitate the fusion protein (Figure 2C). The $G_{\beta\gamma}$ binding to H_6-GKN appeared to be less than that to H_6-GKC_1. These experiments demonstrate that both the N- and the C-terminal hydrophilic domains of GIRK1 bind $G_{\beta\gamma}$ directly.

We next examined the affinity of these two regions of GIRK1 for $G_{\beta\gamma}$. A different design for fusion proteins was used for this study because the H_6-tagged GKN and GKC_1 fusion proteins form intracellular inclusion bodies when overexpressed in Escherichia coli and thus require denaturation and renaturation steps during the purification. Some of the fusion proteins solubilized in this manner may

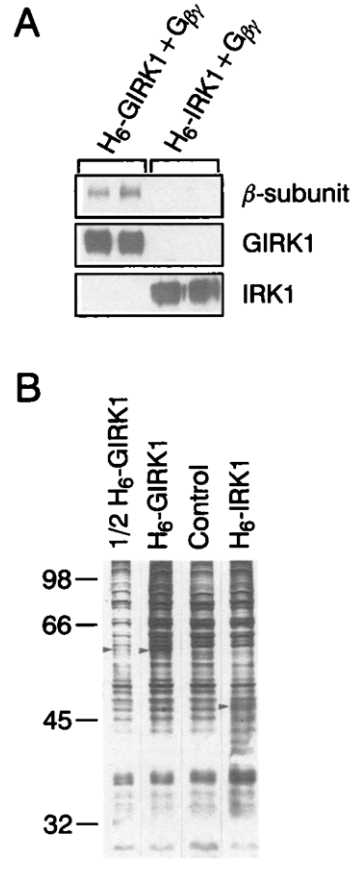

Figure 1. Association of $G_{\beta\gamma}$ with GIRK1

(A) Coprecipitation of $G_{\beta\gamma}$ with H_6-GIRK1 but not H_6-IRK1. Proteins from membrane fractions of Sf9 cells were solubilized by 1% Lubrol at 3.9 mg/ml for H_6-GIRK1 and 4.4 mg/ml for H_6-IRK1. Solubilized membrane fractions containing H_6-GIRK1 (400 μg of protein, left 2 lanes, samples in duplicate) or H_6-IRK1 (400 μg, right 2 lanes) were each incubated with solubilized membrane proteins from Sf9 cells expressing $G_{\beta\gamma}$ (40 μg) in 0.5 ml of reaction buffer. H_6 affinity Sepharose beads were then added to isolate proteins for analysis by SDS–PAGE and sequential immunoblot analysis with antibodies against the β subunit of G protein (top), GIRK1 (middle), or IRK1 (bottom). By comparing the minimal amount of membrane protein required for detection by antibody in immunoblot analysis to the detection limit of the antibody (at least 3.9 μg of membrane protein containing H_6-GIRK1 is required for detection by anti-GIRK1 antibody, which has a detection limit of 44 fmol; at least 6.6 μg of membrane protein containing H_6-IRK1 is required for detection by anti-IRK1 antibody, which has a detection limit of 61 fmol; see Experimental Procedures for detection limit of antibody), we estimate the concentrations of H_6-GIRK1 and H_6-IRK1 used in these experiments to be 8.9 and 8.0 nM, respectively. As shown here, $G_{\beta\gamma}$ associated with H_6-GIRK1 but not with H_6-IRK1. In a separate experiment where an estimated 8.9 nM H_6-GIRK1 and 40 nM H_6-IRK1 were used in parallel binding studies, $G_{\beta\gamma}$ remained differentially associated with H_6-GIRK1 but not with H_6-IRK1 (data not shown).

(B) Silver staining of channel proteins expressed in Sf9 cells. Membrane protein fractions (40 μg) containing H_6-GIRK1 (lane 2 from left) or H_6-IRK1 (lane 4) or neither channel (control; lane 3) were separated by 7.5% SDS–PAGE and visualized using a silver staining kit (Bio-Rad); 1/2 H_6-GIRK1 (lane 1) indicates that 20 μg of membrane protein containing H_6-GIRK1 was loaded. As shown, the amounts of H_6-IRK1 and H_6-GIRK1 proteins contained in 40 μg of membrane protein are comparable. The amount of H_6-IRK1 protein in 40 μg of membrane protein is more than the amount of H_6-GIRK1 protein in 20 μg of membrane protein. Molecular weight markers (in kilodaltons) are indicated on the left. The arrowheads indicate the protein band corresponding to H_6-GIRK1 in lanes 1 and 2 and the protein band corresponding to H_6-IRK1 in lane 4.

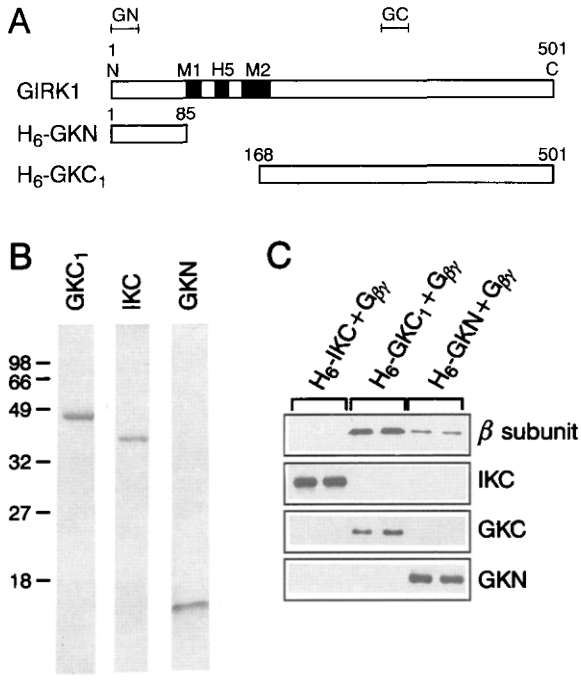

Figure 2. Direct Binding of $G_{\beta\gamma}$ to Both the N- and C-Terminal Hydrophilic Domains of GIRK1

(A) Schematic diagrams of GIRK1 and the fusion proteins. H_6-GKN and H_6-GKC_1 indicate the sequences included in the N- and C-terminal fusion proteins. GN (MSALRRKFGDDYQVVTTSSSGS GLQPQGPG-QGPQQQLV, amino acids 1–38 in single letter code) and GC (PMKLQ-RISSVPGNSEEKLVSKTTKMLSDP, amino acids 434–462) indicate sequences of synthetic peptides used in Figures 5 and 6.

(B) Coomassie blue staining of purified fusion proteins H_6-GKC_1, H_6-IKC, and H_6-GKN. Purified fusion proteins (1 μg each) were separated by 12.5% SDS–PAGE and stained with Coomassie blue. Molecular weight markers (in kilodaltons) are indicated at the left.

(C) Coprecipitation of $G_{\beta\gamma}$ with purified H_6-GKN or H_6-GKC_1 by H_6 affinity beads. Purified H_6-GKN, H_6-GKC_1, or H_6-IKC (amino acids 173–428 of IRK1) (200 nM each, in duplicate) was incubated with purified $G_{\beta\gamma}$ (40 nM) before the addition of H_6 affinity beads. Bound proteins were analyzed by immunoblotting sequentially with antibodies against β subunit of G protein, IKC, GKC, or GKN. In addition to the β subunit, the γ subunit was also detected in either H_6-GKC_1 or H_6-GKN complexes by immunoblot analysis (data not shown).

not be correctly folded. Glutathione S-transferase (GST) fusion proteins of the N-terminal domain of GIRK1 (GST-GKN) and part of the C-terminal domain of GIRK1 (GST-GKC_7; see next paragraph and Figure 4) remained largely soluble and were purified from the soluble fraction of bacterial lysate. Figure 3A shows the saturable binding interaction between $G_{\beta\gamma}$ and GST-GKC_7 as the concentration of $G_{\beta\gamma}$ was increased from 0.1 to 5 μM. The stoichiometry of $G_{\beta\gamma}$ binding to GST-GKC_7 is approximately 1:1 (estimated 9.4 pmol of $G_{\beta\gamma}$ bound to 11.3 pmol of GST-GKC_7; see legend to Figure 3B for details). The estimated K_D for $G_{\beta\gamma}$ binding to GST-GKC_7 is ~0.5 μM.

Binding of $G_{\beta\gamma}$ to GST-GKN was readily detectable by Coomassie blue staining when 1, 3, or 5 μM $G_{\beta\gamma}$ was incubated with GST-GKN (Figure 3A). If we assume the binding of $G_{\beta\gamma}$ to GST-GKN is 1:1, the estimated K_D could be ~3 μM. It has therefore been technically difficult to demonstrate saturation of $G_{\beta\gamma}$ binding, given that the concentration of purified $G_{\beta\gamma}$ stock solutions was 40 μM. The binding of $G_{\beta\gamma}$ to GST-GKN is specific; there was no detectable binding of $G_{\beta\gamma}$ to GST, GST-IKC, or a fusion protein of GST and the C-terminal hydrophilic domain of Kv1.2, even when these fusion proteins were present at 10 times higher concentration than GST-GKN (Figure 4C and data not shown). Together, these studies reveal that both the N- and the C-terminal hydrophilic domains of GIRK1 bind $G_{\beta\gamma}$ directly and that the C-terminal domain has a higher affinity for $G_{\beta\gamma}$.

Mapping of the $G_{\beta\gamma}$-Binding Region in the C-Terminal Hydrophilic Domain

The $G_{\beta\gamma}$-binding region in the C-terminal domain of GIRK1 was further characterized by analyzing a series of smaller fusion proteins, H_6-GKC_2 to H_6-GKC_6 and GST-GKC_7 (Figure 4A). H_6-GKC_1, H_6-GKC_2, and H_6-GKC_6 each bound $G_{\beta\gamma}$ equally well (Figure 4B), indicating that the $G_{\beta\gamma}$-binding domain of H_6-GKC_1 is included in the 190 amino acid segment from 273 to 462. Indeed, the fusion protein containing only this region of the channel, GST-GKC_7, retains full binding activity for $G_{\beta\gamma}$ (Figure 4C). The reduced binding interaction between $G_{\beta\gamma}$ and H_6-GKC_3 could be due to absence of part of the binding site in the fragment containing residues 354–462 or reduced structural stability of this smaller fusion protein. Interestingly, the 190 amino acid segment of the $G_{\beta\gamma}$-binding region overlaps with the GIRK1 fragment that shows sequence similarity with the $G_{\beta\gamma}$-binding domain of the β-adrenergic receptor kinase (βARK1; Reuveny et al., 1994). The latter corresponds to part of a plekstrin homology domain, with sequence similarities to several signaling molecules that may interact with $G_{\beta\gamma}$ (Shaw, 1993; Touhara et al., 1994; Tsukada et al., 1994).

Effect of N- and C-Terminal Peptides on GIRK1 Activation

To determine whether the direct biochemical interaction of $G_{\beta\gamma}$ with the N-terminal domain and/or amino acids 273–462 of the C-terminal domain of GIRK1 is important for the physiological activation of the channel, we asked whether peptides derived from parts of these $G_{\beta\gamma}$-binding domains affect GIRK1 channel activity. We chose peptides GN and GC (see Figure 2A) because they correspond to sequences within the $G_{\beta\gamma}$-binding domains of GIRK1 but not within the segments of GIRK1 showing significant sequence similarity to other K^+ channels such as IRK1 (Kubo et al., 1993b). To examine the effect of these peptides on $G_{\beta\gamma}$ activation of GIRK1, we injected oocytes with cRNAs for GIRK1, β1, and γ2. Under these conditions, the GIRK1 channel activity recorded in excised patches persists in GTP-free solution and has been shown previously to result from $G_{\beta\gamma}$ activation of the channel (Reuveny et al., 1994). When applied to inside-out patches excised from oocytes coexpressing GIRK1 and $G_{\beta\gamma}$, 100 μM GN or 100 μM GC peptide significantly reduced the channel activity by 60% and 65%, respectively (Figures 5A, 5B, and 5F). Application of 100 μM GN and 100 μM GC peptides together

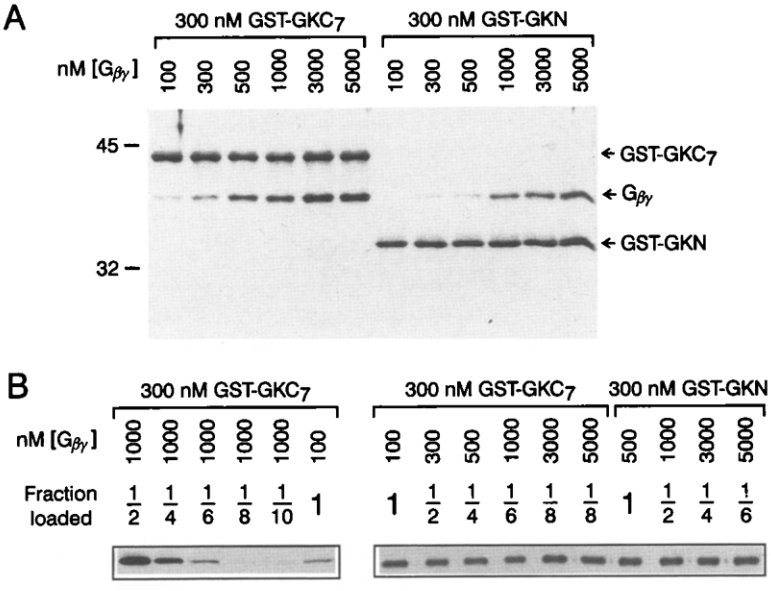

Figure 3. Binding Affinity of GST-GKN and GST-GKC$_7$ for $G_{\beta\gamma}$

(A) Dose-dependent coprecipitation of $G_{\beta\gamma}$ by GST fusion proteins. Purified GST-GKN or GST-GKC$_7$ (300 nM) was incubated with purified $G_{\beta\gamma}$ at 0.1, 0.3, 0.5, 1, 3, or 5 μM in 50 μl of reaction buffer and then processed for coprecipitation by GST beads. After washing beads by resuspension and centrifugation, pellets that contained beads, GST fusion proteins, and $G_{\beta\gamma}$ bound to the fusion proteins were denatured by heating in 20 μl of SDS gel sample loading buffer. Samples (15 μl) were separated by 7.5% SDS–PAGE and visualized by Coomassie blue staining. Since GST beads bind >95% of fusion proteins (see Experimental Procedures), 15 μl of sample would contain ~0.4 μg (11.3 pmol) of Gβ and 0.5 μg (300 nM × 50 μl × 3/4 = 11.3 pmol) of GST-GKC$_7$ if the binding of $G_{\beta\gamma}$ to GST-GKC$_7$ is saturated at the stoichiometry of 1:1. The amount of G_β estimated to be in the sample (at saturation) is 9.4 pmol, as determined by comparing the intensities of serial dilutions of bound and free G_β (see below). Molecular weight standards (in kilodaltons) are indicated at the left. The γ subunit of G protein was run off the 7.5% gel.

(B) Determination of the relative amount of $G_{\beta\gamma}$ binding to GST fusion proteins when increasing concentrations of $G_{\beta\gamma}$ were used. Serial dilutions (1× [no dilution], 2×, 4×, 6×, 8×, and 10×) of a 1 μl sample containing bound $G_{\beta\gamma}$ (pellet obtained as above) for a particular total concentration of $G_{\beta\gamma}$ (e.g., 1 μM, as shown in the left panel) were run on the same gel, along with 1 μl of undiluted (1×) sample containing bound $G_{\beta\gamma}$, for a total $G_{\beta\gamma}$ concentration of 0.1 μM, to determine that a specific fraction of the former (1/6, in the example shown in the left panel) yields a signal on the Western blot of equivalent intensity as the latter. For each binding reaction (at a different $G_{\beta\gamma}$ concentration), the fraction of bound $G_{\beta\gamma}$ deemed to be equivalent in quantity to the amount of bound $G_{\beta\gamma}$ at 0.1 μM total $G_{\beta\gamma}$ concentration was run on the same gel, as shown in the right panel, to confirm that they yield similar intensities on the Western blot. Thus, following incubation of 300 nM GST-GKC$_7$ with 0.3, 0.5, 1, 3, and 5 μM $G_{\beta\gamma}$, the amount of $G_{\beta\gamma}$ bound to GST-GKC$_7$ was 2, 4, 6, 8, and 8, respectively, times the amount of $G_{\beta\gamma}$ bound to GST-GKC in experiments in which 300 nM GST-GKC$_7$ was incubated with 0.1 μM $G_{\beta\gamma}$. At total $G_{\beta\gamma}$ concentrations of 0.5, 1, 3, and 5 μM, the relative amount of $G_{\beta\gamma}$ bound to GST-GKN is 1, 2, 4, and 6, respectively. The concentration of total $G_{\beta\gamma}$ for half-maximal binding of $G_{\beta\gamma}$ to GST-GKC$_7$ is therefore 0.5 μM. At this total $G_{\beta\gamma}$ concentration, serial dilutions of sample containing bound $G_{\beta\gamma}$ or free $G_{\beta\gamma}$ were run on the same gel and treated identically in Western analysis. One-third of free $G_{\beta\gamma}$ gave signal of equivalent intensity to that of bound $G_{\beta\gamma}$. In other words, when 500 nM $G_{\beta\gamma}$ was incubated with 300 nM GST-GKC$_7$, we found that GST-GKC$_7$ precipitated one-fourth of total $G_{\beta\gamma}$. This gives the values for $[G_{\beta\gamma}]_{free}$, $[G_{\beta\gamma}\text{-GST-GKC}_7]_{bound}$, and $[\text{GST-GKC}_7]_{free}$ as 375 nM (500 nM × 3/4), 125 nM (500 nM × 1/4), and 175 nM (300 nM − 125 nM), respectively. Using the equation $K_D = [G_{\beta\gamma}]_{free} \times [\text{GST-GKC}_7]_{free}/[G_{\beta\gamma}\text{-GST-GKC}_7]_{bound}$, K_D is calculated to be 0.52 μM. This K_D may represent an underestimation since nonspecific binding was not substracted from total binding. As shown, nearly equimolar amounts of GST-GKC$_7$ (11.3 pmol) and $G_{\beta\gamma}$ (125 nM × 2 × 50 μl × 3/4 = 9.4 pmol) were coprecipitated at saturating levels of $G_{\beta\gamma}$. If we assume 1:1 binding, we can estimate K_D as the concentration of free $G_{\beta\gamma}$ at half-maximal binding (~0.4 μM for GST-GKC$_7$ and ~3 μM for GST-GKN). In the left panel, the duration of autoradiographic exposure of immunoblot was kept to the minimum (~1 s) to allow comparison of the intensity of the signal over the range of five different dilutions of the sample. The duration of exposure was 5 s for the right panel.

reduced channel activation by 72%, similar to the reduction produced by either peptide alone (Figure 5F). One possible explanation for the lack of additivity is that the same $G_{\beta\gamma}$ binds to the N- and C-terminal domains of a subunit or two adjacent subunits in a multimeric channel. Alternatively, peptides GN and GC may correspond to only part of the $G_{\beta\gamma}$-binding domain and may not abolish the interaction of $G_{\beta\gamma}$ with GIRK1 entirely. In control experiments, 100 μM GN or GC peptide did not inhibit inward current through IRK1 channels (Figures 5E and 5F), suggesting that the inhibition of GIRK1 is not due to nonspecific occlusion of the channel pore. Furthermore, a peptide derived from the C-terminal domain of IRK1 (IC) did not significantly reduce GIRK1 channel activity (Figures 5C and 5F). Thus, the inhibitory effect on GIRK1 channel activity appears to be specific for the GN and GC peptides.

Consistent with their inhibitory effects on $G_{\beta\gamma}$ activation of GIRK1, 100 μM GN and GC peptides partially inhibited $G_{\beta\gamma}$ binding to H_6-GKN and H_6-GKC$_1$ fusion proteins, respectively (Figure 6A). Interestingly, GN peptide did not interfere with $G_{\beta\gamma}$ binding to H_6-GKC$_1$, nor did GC peptide interfere with $G_{\beta\gamma}$ binding to H_6-GKN (Figure 6B). These results suggest that the N- and C-terminal domains bind two separate surfaces of $G_{\beta\gamma}$, and that the reduction of single-channel activity by GN and GC peptides is due to inhibition of $G_{\beta\gamma}$ binding to the N- and C-terminal domains of GIRK1, respectively.

GIRK1 and CIR have been reported to coassemble in the heart (Krapivinsky et al., 1995). We therefore examined whether peptides GN and GC also affected $G_{\beta\gamma}$ activation of channels from oocytes expressing both GIRK1 and CIR and found that they reduced channel activity by 50% ± 10% (n = 4; Figure 5D). The inhibition of channel activity persisted during washout of peptides but could be partially reversed by application of 20 nM $G_{\beta\gamma}$. For comparison, Nair et al. (1995) found that a 28 amino acid peptide (100 μM) derived from the $G_{\beta\gamma}$-binding domain of βARK1 caused a sustained reduction of cardiac $I_{K(ACh)}$ channel activity by 70%, though channel activity could be restored by application of $G_{\beta\gamma}$.

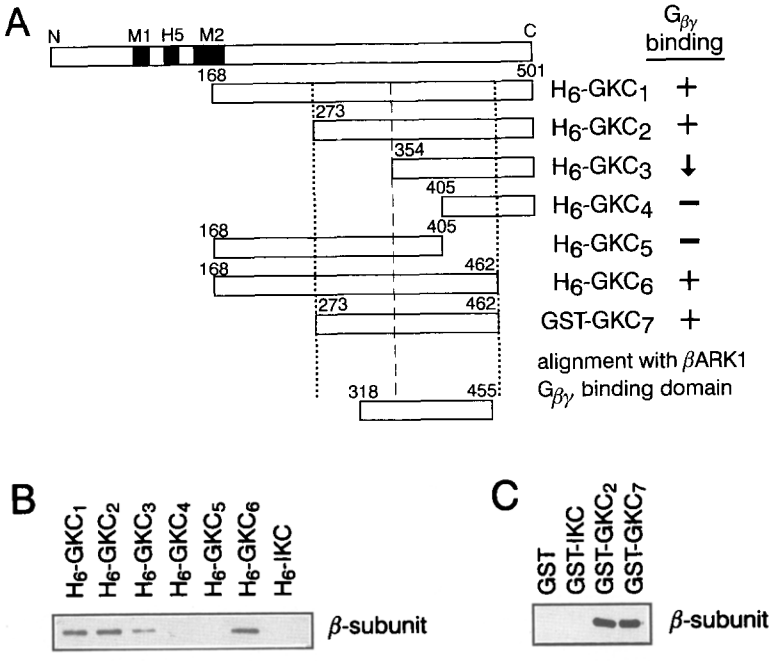

Figure 4. Localization of the $G_{\beta\gamma}$-Binding Region in the C-Terminal Hydrophilic Domain of GIRK1

(A) Schematic diagram of the C-terminal deletion fusion proteins H_6-GKC$_1$ to H_6-GKC6 and GST-GKC$_7$.

(B) Coprecipitation of $G_{\beta\gamma}$ with H_6-GKC$_1$, H_6-GKC$_2$, H_6-GKC$_3$, and H_6-GKC$_5$ but not with H_6-GKC$_4$ and H_6-GKC$_5$ or H_6-IKC by H_6 affinity beads. $G_{\beta\gamma}$ (40 nM) was incubated with H_6-tagged fusion proteins (300 nM) and processed for coprecipitation by H_6 affinity beads as described in Experimental Procedures.

(C) Coprecipitation of $G_{\beta\gamma}$ with GST-GKC$_2$ and GST-GKC$_7$ but not with GST-IKC or GST alone. $G_{\beta\gamma}$ (40 nM) was incubated with GST fusion proteins (300 nM) and processed for coprecipitation by GST affinity beads.

Effect of G_α on $G_{\beta\gamma}$ Binding to the Hydrophilic Domains of GIRK1

Previous electrophysiological studies have shown that G_α-GDP prevents $G_{\beta\gamma}$ activation of muscarinic K^+ channels and GIRK1 channels (Logothetis et al., 1987; Ito et al., 1992; Wickman et al., 1994; Reuveny et al., 1994). We found that G_α-GDP, but not G_α-GTPγS, abolished the binding of $G_{\beta\gamma}$ to H_6-GKC$_1$ (Figures 7A and 7B), consistent with the expectation that, following GTP hydrolysis, G_α-GDP sequesters $G_{\beta\gamma}$ and terminates channel activation. In contrast to H_6-GKC$_1$, H_6-GKN remained associated with $G_{\beta\gamma}$, even if $G_{\beta\gamma}$ was preincubated with a 25-fold excess of G_α-GDP (Figure 7B). Since most of the $G_{\beta\gamma}$ is expected to be in the trimeric $G_{\alpha\beta\gamma}$ form under this experimental condition, H_6-GKN probably binds to the trimeric G protein. Finally, not only was H_6-GKN associated with $G_{\beta\gamma}$ in the presence or absence of G_α-GDP, H_6-GKN also associated with G_α-GDP even in the absence of $G_{\beta\gamma}$ (Figure 7C). Thus, H_6-GKN but not H_6-GKC$_1$ exhibits the capacity to bind G_α-GDP as well as the trimeric G protein, an aspect of G protein interaction that could be involved in the coupling of this channel to G protein–coupled receptors.

Discussion

By analyzing the interaction of $G_{\beta\gamma}$ with the N- or the C-terminal hydrophilic domain of the GIRK1 K^+ channel, both purified to apparent homogeneity, we have obtained evidence for direct binding of G protein subunits to a G protein–gated K^+ channel. The physiological significance of this direct protein–protein interaction is underscored by the ability of peptides derived from the $G_{\beta\gamma}$-binding regions of GIRK1 to disrupt $G_{\beta\gamma}$ binding to these hydrophilic domains of GIRK1 as well as by GIRK1 channel activation by $G_{\beta\gamma}$. We discuss below the finding of two separate regions of GIRK1 involved in direct binding to $G_{\beta\gamma}$, possible implications of the unexpected observation that the N-terminal hydrophilic domain of GIRK1 may bind to G_α-GDP and the trimeric G protein, and the potential physiological significance of transmitter and hormone actions involving direct interactions between G proteins and ion channels.

Two Regions of the GIRK1 K^+ Channel Involved in Activation of the Channel

In the present study, we have shown that either the N- or the C-terminal domain of GIRK1 binds $G_{\beta\gamma}$ independently, most likely in a stoichiometry of one $G_{\beta\gamma}$ for each GKC or GKN fusion protein. The apparent K_D for $G_{\beta\gamma}$ binding to GST-GKC$_7$ and GST-GKN (0.5 and 3 μM, respectively) is higher than the reported K_{act} for $G_{\beta\gamma}$ activation of $I_{K(ACh)}$ ($\sim 10^{-8}$ M; Ito et al., 1992; Wickman et al., 1994). This difference between apparent K_D and K_{act} could be due to the different methods used. K_{act} is measured in recordings of single channels where strong positive cooperativity is observed (Ito et al., 1992), whereas the apparent K_D is measured for binding of $G_{\beta\gamma}$ to fusion proteins containing fragments of the channel protein in a detergent solution. It is also possible that the N- and C-terminal domains together may form a $G_{\beta\gamma}$-binding site of higher affinity in the multimeric channel.

The inhibition of $G_{\beta\gamma}$ binding to H_6-GKN by peptide GN but not by peptide GC (and vice versa) and the ability of G_α-GDP to inhibit $G_{\beta\gamma}$ binding to H_6-GKC$_1$ but not H_6-GKN suggest that the N- and C-terminal domains interact with different surfaces of $G_{\beta\gamma}$. $G_{\beta\gamma}$ activation of the cardiac $I_{K(ACh)}$ exhibits a Hill coefficient greater than 3 (Ito et al., 1992), suggesting that multiple $G_{\beta\gamma}$ subunits are involved in the activation of a muscarinic K^+ channel. This could be due to interaction of three or more $G_{\beta\gamma}$ subunits with multiple subunits of the presumed multimeric channel protein. Further experiments are needed to determine whether a sin-

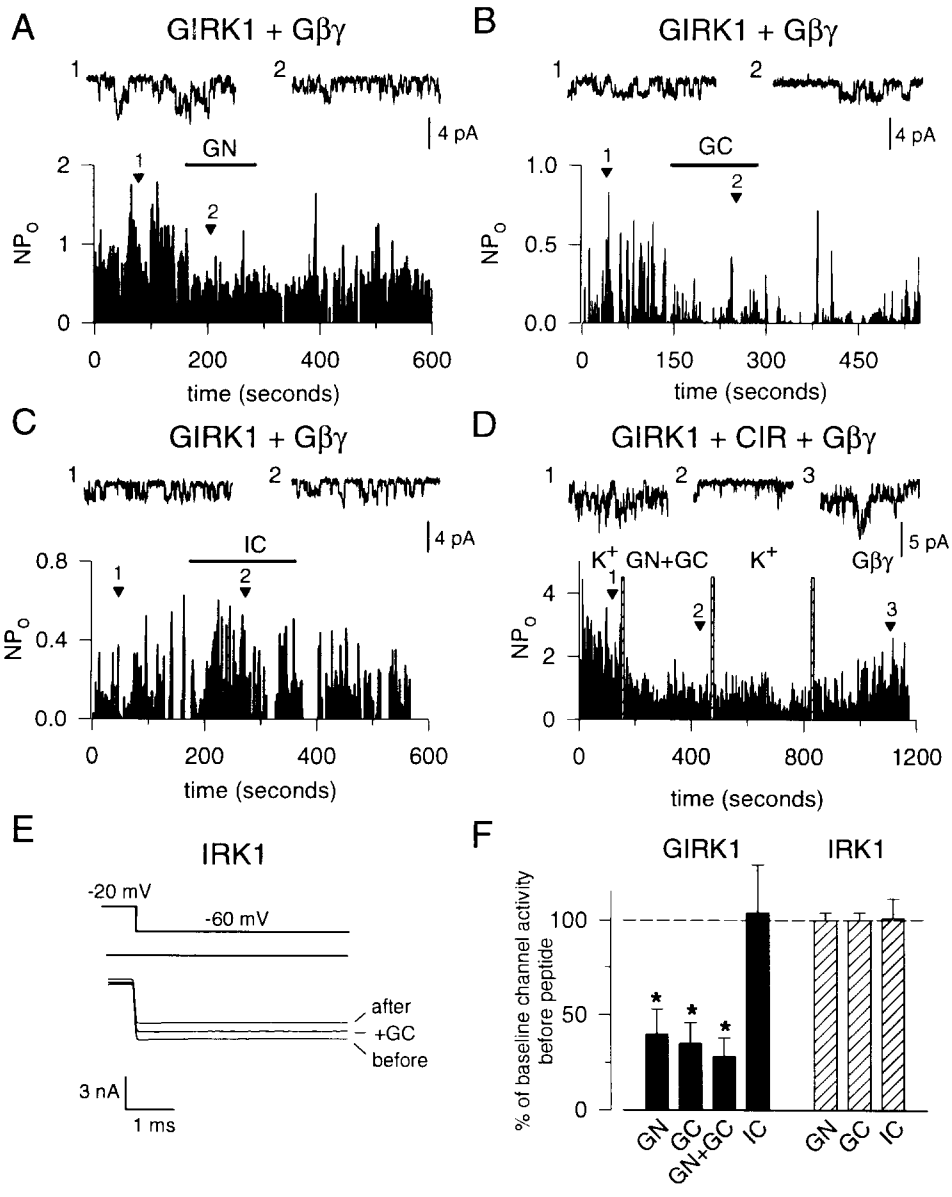

Figure 5. Inhibition of $G_{\beta\gamma}$-Induced GIRK1 or GIRK1/CIR Single-Channel Activity by Peptides Derived from Parts of the N- and C-Terminal Domains of GIRK1

(A–C) Single-channel GIRK1 activity was recorded continuously at −60 mV from oocytes coexpressing GIRK1 and $G_{\beta1\gamma2}$ subunits. All single channels displayed strong inward rectification. After formation of stable inside-out patch recordings, the intracellular surface of the patch was exposed to GN, GC, or IC peptide (at 100 μM). The open-channel probability (NP_o) plotted as a function of time shows the inhibition produced by exposure (bar) to peptides GN (A) and GC (B) but not to the control peptide IC (C). Current recordings (365 ms) illustrating single-channel activity are shown above each plot at the time indicated by arrows. Channel activity continued to be suppressed following washout of GN and GC peptides.

(D) Application of GN and GC peptides (100 μM each) inhibits $G_{\beta\gamma}$ activation of channel activity recorded from ooycytes coexpressing GIRK1, CIR, and $G_{\beta\gamma}$. The NP_o plot shows channel activity recorded in an excised patch during perfusion with internal K^+ (GTP-free), subsequent application of 100 μM GN and GC followed with internal K^+, and application of 20 nM $G_{\beta\gamma}$.

(E) Application of 100 μM GC to a macropatch from an oocyte injected with *IRK1* cRNA produces little change in current at −60 mV. A time-dependent block of outward current was observed for all three peptides (data not shown). Owing to continuous rundown of IRK1 current in the excised membrane patch, the amplitude of inward current decreased steadily from "before" to "after" GC peptide application.

(F) Bar graph shows average (± SEM) percentage inhibition by peptides. GIRK1 single-channel activity was reduced to 40% ± 13% (n = 5), 35% ± 11% (n = 4), 28% ± 10% (n = 4), and 104% ± 25% (n = 7) of control for GN, GC, GN plus GC, and IC peptides, respectively. After adjusting for rundown, IRK1 currents were 100% ± 4% (n = 5), 100% ± 4% (n = 4), and 101% ± 10% (n = 4) of control for GN, GC, and IC peptides, respectively, where "control" represents the baseline channel activity measured over a period of 1–2 min just before application of peptides (asterisk indicates $p < .05$ by ANOVA followed by Dunnett's, using IC peptide as reference). For comparison, the reported IC_{50} for βARK1 peptide inhibition of $G_{\beta\gamma}$-stimulated βARK1 activity is 76 μM (Koch et al., 1993).

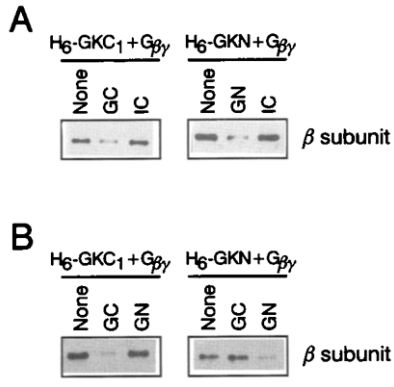

Figure 6. Inhibition of $G_{\beta\gamma}$ Binding to Fusion Proteins by Synthetic Peptides Derived from GIRK1

$G_{\beta\gamma}$ (40 nM) binding to H_6-GKC_1 (300 nM) is inhibited by 100 μM peptide GC but not by peptide IC (A, left) or GN (B, left). Conversely, $G_{\beta\gamma}$ (40 nM) binding to H_6-GKN (300 nM) is inhibited by 100 μM peptide GN but not by peptide IC (A, right) or GC (B, right). The duration of autoradiographic exposure of immunoblot was ~12 times longer for the experiment with H_6-GKN than for the experiment with H_6-GKC_1 (1 min versus 5 s).

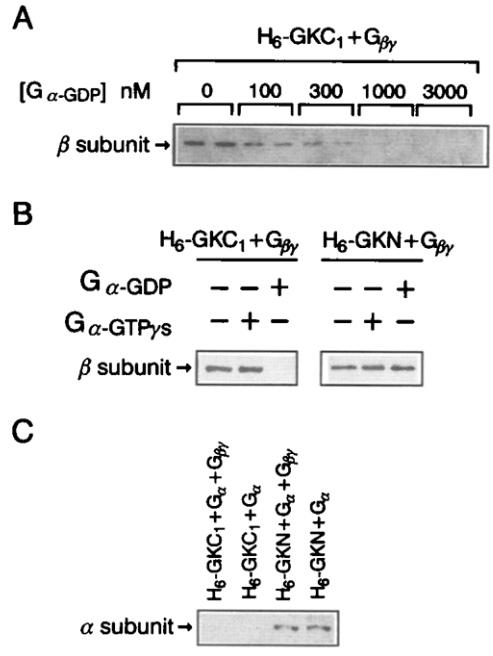

Figure 7. Effect of G_α-GDP on $G_{\beta\gamma}$ Binding to GIRK1 Fusion Proteins

(A) Dose-dependent inhibition of $G_{\beta\gamma}$ binding to H_6-GKC_1 by G_α-GDP. $G_{\beta\gamma}$ (40 nM) was preincubated with G_α-GDP at the indicated concentrations at 4°C for 30 min before addition of H_6-GKC_1 (200 nM) and H_6 affinity beads.

(B) Specific inhibition of $G_{\beta\gamma}$ association with H_6-GKC_1, but not H_6-GKN, by G_α-GDP. $G_{\beta\gamma}$ (40 nM) was preincubated with 1 μM G_α-GDP or G_α-GTPγs before addition of 200 nM H_6-GKC_1 or H_6-GKN and H_6 affinity beads. G_α-GDP was converted into G_α-GTPγS as described (Thomason et al., 1994). The length of autoradiographic exposure of immunoblot was ~12 times longer for the experiment with H_6-GKN than for the experiment with H_6-GKC_1.

(C) Coprecipitation of G_α-GDP with H_6-GKN but not with H_6-GKC_1. G_α-GDP (500 nM) was incubated with either 40 nM $G_{\beta\gamma}$ (lanes 1 and 3 from left) or buffer alone (lanes 2 and 4) before the addition of 200 nM H_6-GKN (lanes 3 and 4) or H_6-GKC_1 (lanes 1 and 2). The reaction buffer is the same as in Experimental Procedures, except for the addition of 5 mM $MgCl_2$ and 10 μM GDP in experiments involving G_α-GDP or addition of 5 mM $MgSO_4$ and 0.5 mM GTPγS in experiments involving G_α-GTPγS. The precipitated proteins were analyzed by immunoblotting with antibodies against the β subunit (A and B) or α subunit (C) of G protein (Casey et al., 1990). The concentration of G_α-GDP required to eliminate $G_{\beta\gamma}$ binding to H_6-GKC_1 is found for several batches of G_α-GDP to be consistently higher than that required for blockade of channel activation by membrane-associated $G_{\beta\gamma}$ (~100 nM; Wickman et al., 1994). It is possible that the large amount of H_6-GKC_1 in solution allows for more effective competition with G_α-GDP for $G_{\beta\gamma}$ binding than the low density of muscarinic K^+ channel complexes found in the excised membrane patch. It is also possible that G_α-GDP may prevent channel activation by $G_{\beta\gamma}$ without totally abolishing $G_{\beta\gamma}$ binding to the channel, given that the Hill coefficient for channel activation is >3 (Ito et al., 1992).

gle $G_{\beta\gamma}$ binds to both the N- and C-terminal domains and, if so, whether the $G_{\beta\gamma}$-binding site is composed of the N- and C-terminal domains of the same subunit or two adjacent subunits of the multimeric GIRK1 channel.

Our study strongly indicates that direct contact of $G_{\beta\gamma}$ with both the N- and C-terminal hydrophilic domains of GIRK1 is important for activation of the GIRK1 channel by $G_{\beta\gamma}$, because peptides that disrupt either interaction also reduce GIRK1 channel activation by $G_{\beta\gamma}$. This finding is also consistent with our observation that chimeras of GIRK1 and IRK1 show $G_{\beta\gamma}$ sensitivity if they contain either the N- or the C-terminal domain of GIRK1 (Slesinger et al., 1995 [this issue of *Neuron*]). The exact amino acid residues of GIRK1 involved in binding $G_{\beta\gamma}$, however, remain to be elucidated. The results of experiments using peptides GN and GC suggest that these peptides contain residues important for interaction with $G_{\beta\gamma}$. However, we have not shown that such residues are in direct contact with $G_{\beta\gamma}$. Other amino acids of GIRK1 may also be involved in $G_{\beta\gamma}$ interaction, since a 100 μM concentration of both peptides did not completely suppress channel activation by $G_{\beta\gamma}$ (see Figure 5F). It may be of interest to note that a region of adenylyl cyclase II containing the Gln-X-X-Glu-Arg motif is critical for adenylyl cyclase regulation by $G_{\beta\gamma}$ (Chen et al., 1995). Two similar motifs, amino acids 378–382 (Asn-X-X-Glu-Arg) and 446–450 (Asn-X-X-Glu-Lys) (included in the GC peptide sequence) are found in the $G_{\beta\gamma}$-binding region of the C-terminal domain of GIRK1. Further studies are necessary to examine the points of contact between $G_{\beta\gamma}$ and GIRK1, as well as the possibility of direct interaction between $G_{\beta\gamma}$ and CIR, a likely subunit of heteromeric $I_{K(ACh)}$ channels in the heart (Krapivinsky et al., 1995), and GIRK2 or GIRK3, potential subunits of heteromeric G protein–activated K^+ channels in the brain (Kofuji et al., 1995).

Potential Implication of Direct Interaction between the GIRK1 N-Terminal Domain and the Trimeric G Protein

One unresolved question concerning the activation of an effector such as the muscarinic K^+ channel by the $G_{\beta\gamma}$ subunit of the activated G protein concerns the specificity of transmitter or hormone action. In the atrium, activation of the m2 muscarinic receptor by the parasympathetic

transmitter acetylcholine and activation of the α_1-adrenergic receptor by phenylephrine both lead to activation of the muscarinic K^+ channel. Unlike the former, the latter is blocked by lipoxygenase inhibitors and is therefore not due to direct action of $G_{\beta\gamma}$ on the channel (Kurachi et al., 1989a). Furthermore, the lack of effect of β-adrenergic activation on muscarinic K^+ channels indicates that $G_{\beta\gamma}$ released by β-adrenergic receptor activation does not activate muscarinic K^+ channels (Hille, 1992). Similarly, in central neurons from the locus coeruleus, somatostatin and metenkephalin activate an inwardly rectifying K^+ channel, probably via a membrane-delimited pathway, whereas substance P suppresses the activities of these channels via mechanisms that involve diffusible second messengers (Takano et al., 1995; Velimirovic et al., 1995).

How can different G protein–coupled receptors exert different effects on an ion channel if they all activate G proteins and release $G_{\beta\gamma}$ and if the channel can be activated by $G_{\beta\gamma}$? Different recombinant $G_{\beta\gamma}$ subunits have been shown to be effective in activating the cardiac muscarinic K^+ channel. The affinities of these different $G_{\beta\gamma}$ subunits do not differ by more than a factor of 10 (Wickman et al., 1994). Thus, it seems unlikely that association of different $G_{\beta\gamma}$ isoforms with different receptors can explain specific activation of the muscarinic K^+ channel by some, but not other, receptors. One possible mechanism for receptors to exert specific control of effectors is compartmentalization of the receptors and their respective effectors (Braun and Levitzki, 1979; Nakamura and Rodbell, 1991; Neubig, 1994; Kwon et al., 1994). The physical association between the trimeric G protein and the N-terminal domain of the GIRK1 channel protein, as we observed in this study, is consistent with the possibility that the receptor, the G protein, and the channel may be compartmentalized together to allow specific activation of the channel by the receptor.

Membrane-Delimited Actions by G Protein–Coupled Receptors

Direct activation of an ion channel by G protein subunits allows acetylcholine released from the vagal nerve to exert its calming effect on the heart rate within a tenth of a second (Hartzell, 1980; Nargeot et al., 1983; Yatani and Brown, 1989). Indeed, if the muscarinic K^+ channel, the G protein, and the muscarinic receptor were physically segregated together, they would appear to be functionally equivalent to a slow ligand-gated ion channel (Sakmann et al., 1983; Soejima and Noma, 1984). Unlike ligand-gated ion channels, however, different G protein–coupled receptors for different transmitters or hormones may be coupled to the same type of ion channels, as found for a wide range of inhibitory transmitters that activate inwardly rectifying K^+ channels (for review, see North, 1989; Nicoll et al., 1990). It is also possible for one type of G protein–coupled receptor to exert relatively fast actions on multiple ion channels via membrane-delimited pathways. For example, cholinergic activation of the muscarinic receptor can lead to both activation of the ATP-sensitive K^+ channel by $G_{i\alpha}$-GTP and activation of the muscarinic K^+ channel by $G_{\beta\gamma}$ in the same cell (Ito et al., 1992). Further variations of the G protein action may range from a direct action of the G protein on the channel, as found in our study, to the involvement of other membrane-associated second messengers. The versatility of such systems is evident from the many examples of membrane-delimited actions of G proteins on Ca^{2+}, Na^+, and K^+ channels in the animal kingdom (Brown and Birnbaumer, 1990; Nicoll et al., 1990) and the recent demonstration of membrane-delimited G protein regulation of K^+ channels in the guard cells of a plant (Wu and Assmann, 1994). It would be of interest to determine which subset of these membrane-delimited pathways of channel regulation by G proteins involve direct binding of G protein subunits to the channel protein. The study reported here provides one example for direct actions of G protein on an ion channel and presents an opportunity for mechanistic analysis of the control of channel activity by protein–protein interactions between the channel protein and subunits of the G protein. Given the wide distribution of *GIRK1* mRNA in the mammalian brain (Dascal et al., 1993; Kubo et al., 1993b; DePaoli et al., 1994; Karschin et al., 1994), biochemical and electrophysiological analysis of GIRK1 channel activation by $G_{\beta\gamma}$ is of potential significance in our understanding of transmitter actions in the central nervous system as well as the heart.

Experimental Procedures

Expression and Purification of Channel Proteins and G Protein Subunits

The pVL1393 vector (Invitrogen) was used for baculovirus-mediated expression of proteins in Sf9 cells. Nucleotide sequence containing ATG, an in-frame H_6 tag, and an enterokinase cleavage site was inserted into the BamHI site of the multiple cloning sites of the vector. cDNAs of GIRK1 (Kubo et al., 1993b) and IRK1 (Kubo et al., 1993a) were then subcloned in-frame into the vector immediately 3′ to the inserted oligonucleotide. This engineering results in addition of 15 amino acids, $MG(H)_6(D)_4KGS$, N-terminal to the wild-type channel proteins. Proteins were expressed in baculovirus-Sf9 cells as described (O'Reilly et al., 1992). Recombinant viruses containing G protein β1 and γ2 subunits were from Dr. A. G. Gilman (Iñiguez-Lluhi et al., 1992). Membranes of Sf9 cells expressing H_6-tagged GIRK1, IRK1, or wild-type G protein β1γ2 subunit were homogenized, fractionated, and solubilized by 1% Lubrol at a protein concentration of ~4 mg/ml as described (Iñiguez-Lluhi et al., 1992). The level of protein expressed was ~0.1% of total membrane proteins for H_6-GIRK1 and H_6-IRK1 and ~1% for $G_{i\gamma}$, as estimated by comparing the intensity of the band on the gel to that of the known amount of molecular weight standards visualized by silver or Coomassie blue staining.

Proteins containing K^+ channel fragments were expressed as H_6-tagged fusion proteins in DE3 strain of E. coli using pRset vector (Invitrogen) or as GST-tagged fusion proteins in TG1 cells using pGEX-2T vector (Pharmacia). cDNAs encoding fragments of channel protein were generated by polymerase chain reaction, sequenced, and subcloned in-frame into pRset or pGEX-2T vector. Expression of H_6-tagged fusion proteins was induced by 1 mM IPTG at 37°C for 4 hr; GST-tagged fusion proteins by 0.1 mM IPTG for 3 hr. H_6-tagged fusion proteins were purified by H_6 affinity nickel column under denaturing conditions according to the product manual, renatured on the nickel column by subjection to a 8 to 0 M urea gradient, and then eluted from the column using 0.5 M immidazole. They were further purified to apparent homogeneity using the fast protein liquid chromatography gel filtration column, Superose 6 (Pharmacia). GST-tagged fusion proteins were first purified using glutathione–Sepharose beads (Pharmacia) as described (Koch et al., 1993) and further purified to homogeneity using fast protein liquid chromatography ion exchange column, mono Q (Pharmacia). G_o (mixture of $G_{o\alpha}$ and $G_{o\gamma}$) and $G_{\beta\gamma}$ were

purified from bovine brain as previously described (Casey et al., 1989). Purity and integrity of protein were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining before use.

Coaffinity Precipitation and Western Blotting

Solubilized membrane proteins from Sf9 cells expressing H_6-tagged GIRK1 or IRK1 were incubated with solubilized membrane proteins of $G_{\beta\gamma}$ at 4°C for 30 min in 0.5 ml of reaction buffer containing 150 mM KCl, 50 mM HEPES (pH 7.5), 1 mM β-mercaptoethanol, 100 mM immidazole, and 0.2 % Lubrol. Immidazole, a histidine analog, was used to reduce nonspecific binding of $G_{\beta\gamma}$ to the H_6 affinity beads. H_6 affinity beads (20 μl; imminodiactic acid coupled to Sepharose beads and charged with nickel, suspended in reaction buffer at 1:1 to Sepharose beads) were then added to the reaction mixture and incubated further for 30 min. For the binding of $G_{\beta\gamma}$ to H_6-GKN and H_6-GKC, purified bacterial fusion proteins were incubated with purified bovine brain $G_{\beta\gamma}$ and then processed for coprecipitation in 50 μl of the above reaction buffer. Binding of $G_{\beta\gamma}$ to the GST fusion proteins (GST-IKC, GST-GKC_2, GST-GKC_7, and GST) was examined by mixing purified GST fusion proteins with $G_{\beta\gamma}$ in 50 μl of PBS containing 2 mM EDTA and 5 mM β-mercaptoethanol, followed by the addition of 20 μl of glutathione-Sepharose 4B and precipitation of GST fusion proteins and any associated proteins (Koch et al., 1993). Beads were pelleted by centrifugation for 30 s in a microfuge. Precipitated beads were washed 3 times in the same buffer by repeated resuspension and centrifugation. The final precipitates were denatured by heating to 65°C for 10 min in 20 μl of SDS-PAGE loading buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane for Western blotting and detection by the ECL system (Amersham). The amount of fusion protein bound by affinity beads was estimated as follows: serial dilutions of bound fusion proteins (in the pellet) or free fusion proteins that remain in the supernatant were run on the same gel, and the Western blot was probed with antibodies (see below) and developed for short periods of time to avoid saturation of signal. Once we determined equivalent intensities of Western blot signal for a specific fraction of the bound fusion protein (1/x) and the specific fraction of free fusion protein (1/y), we could calculate the percentage of fusion protein bound by affinity beads as $x/(x + y)$. By this method, we estimate that 20 μl of H_6 affinity beads can bind >97% of 2 μM H_6-tagged fusion proteins in 50 μl of solution (amount of H_6-tagged fusion protein in pellet is >30 times that in supernatant); and 20 μl of glutathione-Sepharose beads can bind >95% of 1 μM GST-tagged fusion proteins in 50 μl of solution (amount of GST fusion protein in pellet is >20 times that in supernatant).

Polyclonal antibodies against GKN, GKC, or IKC were raised from rabbits against synthetic peptide corresponding to amino acids 6-42 or 346-375 of GIRK1 or 376-404 of IRK1, respectively, and were affinity purified. Antibodies were used at 0.73 μg/ml (GKN), 0.55 μg/ml (GKC), or 0.52 μg/ml (IKC). Incubation at room temperature for 2 hr with these amounts of antibodies allowed detection of ~1 ng of GKN (equivalent to 66 fmol of full-lengh GIRK1), ~2 ng of GKC_7 (44 fmol of GIRK1), and ~2.5 ng of IKC (61 fmol of IRK1) proteins. Antisera for $G_{i\alpha}$ (B-600 at 1:1000 dilution) and G_{γ} (X-263 at 1:100 dilution) were from Drs. A. G. Gilman and S. M. Mumby, and P. C. Sternweis, respectively (Linder et al., 1993; Muntz et al., 1992).

Electrophysiology

Oocytes were removed from Xenopus laevis under anesthesia, washed in Ca^{2+}-free ND96 (96 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM HEPES; pH 7.6), treated with collagenase (2 mg/ml) in Ca^{2+}-free ND96 for ~2 hr to remove follicular cells, and washed extensively with ND96 (96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM HEPES; pH 7.6). In vitro mCAPcRNA transcripts were made as described previously (Kubo et al., 1993a). Oocytes were injected with a 46 nl solution containing cRNA for GIRK1 (~5 ng), β1 (~8 ng), and γ2 (~8 ng) or IRK1 alone (~5 ng) and incubated in ND96 for 3-6 days at 18°C. In some experiments, ~1 ng of cRNA for CIR was also injected. Single-channel activity was recorded at 22°C-25°C from inside-out patches with a LIST EPC-7 amplifier. Single-channel currents were stored continuously on VCR tape (Instrutech), transferred at 2.8-5.6 kHz to disk, and filtered at 0.6-1 kHz for analysis. External recording solution contained 75 mM K_2SO_4, 15 mM KCl, 2 mM $MgSO_4$, 10 μM $GdCl_3$, 5 mM KOH, and 10 mM HEPES (pH 7.4). Internal recording solution contained 72.5 mM K_2SO_4, 15 mM KCl, 4.4 mM $MgSO_4$, 0.2 mM β-mercaptoethanol, 2.5 mM K_2ATP, 5 mM KOH, and 10 mM HEPES (pH 7.4). Peptides GN (amino acids 1-38) and GC (amino acids 434-462) of GIRK1 and IC (amino acids 376-404 preceded by a cysteine at the N-terminal end) of IRK1 were synthesized by Dr. Chris Turck at the Howard Hughes Medical Institute. Stock solutions of peptides were made by dissolving peptides in the intracellular solution plus 2 mM dithiothreitol (pH 7.4). Excised patches were perfused continuously with peptides delivered through polyethylene tubing (inside diameter, 0.58 mm). NP_o plots were made as described previously (Reuveny et al., 1994). For statistical analysis, an ANOVA followed by Dunnett's test using peptide IC as reference was performed on the percentage inhibition calculated by dividing the NP_o measured in 1-2 min segments of recording after peptide application by the NP_o measured in 1-2 min segments just before peptide application. $G_{\beta\gamma}$ was prepared and applied as described previously (Reuveny et al, 1994). Macropatch IRK1 currents were recorded using the giant-patch technique (Collins et al., 1992). To minimize rundown, patches were perfused with a Mg^+-free intracellular solution containing 150 mM KCl, 10 mM EDTA, 1 mM EGTA, 0.2 mM β-mercaptoethanol, 5 mM KOH, and 10 mM HEPES (pH 7.4). Extracellular solution contained 150 mM KCl, 2 mM $MgCl_2$, 5 mM KOH, and 10 mM HEPES (pH 7.4).

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Note Added in Proof

During review of this manuscript, Inanobe et al. reported that $G_{\beta\gamma}$ binds to the carboxyl terminus of GIRK1 (Biochem. Biophys. Res. Commun., *212*, 1022–1028, 1995).