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Gβγ Binds Directly to the G Protein-gated K^+ Channel, I_{KACh}*

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The cardiac G protein-gated K^+ channel, I_{KACh}, is activated by application of purified and recombinant β and γ subunits (Gβγ) of heterotrimeric G proteins to excised inside-out patches from atrial membranes (Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E., and Clapham, D. E. (1987) *Nature* 325, 321–326; Wickman, K., Iniguez-Lluhi, J., Davenport, P., Taussig, R. A., Krapivinsky, G. B., Linder, M. E., Gilman, A., and Clapham, D. E. (1994) *Nature* 368, 255–257). Cardiac I_{KACh} is composed of two inward rectifier K^+ channel subunits, GIRK1 and CIR (Krapivinsky, G., Gordon, E., Wickman, K., Velimirovic, B., Krapivinsky, L., and Clapham, D. E. (1995) *Nature* 374, 135–141). We show that Gβγ directly binds to immunoprecipitated cardiac I_{KACh} as well as to recombinant CIR and GIRK1 subunits, with dissociation constants (K_d) of 55, 50, and 125 nM, respectively. In each case, binding appeared specific as judged by competition of unlabeled Gβγ with radiolabeled Gβγ and inhibition of binding by antigenic peptide or Gα-GDP, but not Gα-GTPγS (guanosine 5′-3-*O*-(thio)triphosphate). In contrast, Gα (GTPγS- or GDP-bound) did not bind to the native channel. We conclude that Gβγ binds directly and specifically to I_{KACh} via interactions with both CIR and GIRK1 subunits to gate the channel.

Acetylcholine (ACh)[1] secreted from the vagus nerve binds cardiac muscarinic receptors, initiating a sequence of events leading to slowing of the heart rate. I_{KACh}, an inwardly rectifying, potassium-selective channel stimulated by the β and γ subunit (Gβγ) of pertussis toxin-sensitive heterotrimeric G proteins, mediates part of this process by hyperpolarizing pacemaker cells in sinoatrial and atrioventricular nodes of the heart (4–7). All evidence indicates that the critical components involved in I_{KACh} activation are confined to the membrane. However, it is unclear whether Gβγ binds directly to I_{KACh} to elicit the stimulatory effect or acts via an unknown intermediate step(s) (4, 5).

Cardiac I_{KACh} is a heteromultimer of two homologous inward rectifier K^+ channel subunits, GIRK1 (8, 9) and CIR (3). Recent evidence also suggests that a similar complex comprised of GIRK1 and GIRK2 (10), a structural and functional CIR homolog, forms a neuronal G protein-gated K^+ channel (11). We tested whether Gβγ binds directly to cardiac I_{KACh} and, if so, to which subunit(s). By immunoprecipitation with subunit-specific antibodies, we were able to effectively purify whole cardiac I_{KACh} channel and individual recombinant subunits and study binding of ^{125}I-labeled Gβγ to these immunoprecipitated species. Here we show that Gβγ binds directly and specifically to the whole channel and to each I_{KACh} subunit.

MATERIALS AND METHODS

Electrophysiological experiments with atrial myocytes were performed as described (2). The pipette and bath solutions were identical (in mM): 140 K^+, 144 Cl^-, 5 EGTA, 2 Mg^{2+}, 10 HEPES, pH 7.2. Holding potential was −80 mV. Spaces in the *upper trace* (see Fig. 1*A*) represent only the time required to add the indicated substance to the bath. The *lower, expanded trace* in Fig. 1*A* was filtered (8-pole Bessel) at 2.5 kHz. The concentration-response analysis in Fig. 1*B* was performed as described (2). The data were fit to the sigmoid function $f(x) = (a - d)/(1 + (x/c)^{-b}) + d$ using the Marquardt-Levenberg least squares curve-fitting algorithm (a and d represent the asymptotic maximum and minimum, respectively; c is K_{act}, the value of x at the inflection point; b is the Hill coefficient).

G-proteins were isolated from bovine brain, separated into Gα and Gβγ subunits as described (12), and additionally purified by affinity chromatography over immobilized Gα (13) or Gβγ (14). Bovine atrial plasma membranes were isolated as described (15). Membranes were solubilized in 1.0% CHAPS-HEDN buffer (in mM: 10 HEPES, 1 EDTA, 1 dithiothreitol, and 100 NaCl) containing protease inhibitors. Two different antipeptide affinity-purified antibodies were used for immunoprecipitation experiments: anti-CIR (aCIRN2, amino acids 19–32, 0.5 μg/assay) and anti-GIRK1 (aCsh, amino acids 356–501, 0.3 μg/assay of atrial membrane protein and 0.7 μg/assay of Sf9 membrane protein). aCIRN2 did not immunoprecipitate *in vitro* translated GIRK1, and aCsh did not immunoprecipitate *in vitro* translated CIR (3).[2] Proteins were immunoprecipitated for 1.5 h at 4 °C with corresponding antibody and PrA FF-Sepharose (Pharmacia Biotech Inc.). Immunoprecipitates were washed four times in the same buffer, followed by two washes with 0.1% CHAPS-HEDN. Anti-Gβ antibody was purchased from Calbiochem.

For radiolabeling of G protein subunits, 20 μg of purified protein was labeled with ^{125}I using 250 μCi of ^{125}I-Bolton-Hunter reagent (DuPont-NEN) yielding ~1 mol of ^{125}I/3 mol of G-protein subunit. ^{125}I-Bolton-Hunter reagent, at this stoichiometry, does not prevent formation of a functional heterotrimer by labeled subunits. Both labeled Gα and Gβγ were able to bind their unlabeled immobilized counterparts, and incubation with AlF_4^- led to their dissociation (data not shown). For each binding assay, immunoprecipitates were obtained from 50 μg of atrial membrane protein and 50 or 500 μg of Sf9 membrane protein containing rCIR or rGIRK1, respectively. Immunoprecipitated proteins were incubated with 1.25 nM ^{125}I-Gβγ (~10^5 cpm) and unlabeled competitors in 0.1% CHAPS-HEDN and rotated for 15 min at room temperature (75 μl, total volume). Subsequently, the Immunobeads were washed four times by centrifugation, each time using 0.5 ml of the same ice-cold buffer. Total washing time was 7 min. In control experiments, the amount of bound Gβγ did not increase after 15 min at room temperature, and $t_{½}$ for dissociation of bound Gβγ was ~90 min at 4 °C. Bound Gβγ was counted using a γ counter. Data were fit to a competition equation with a single binding site (17).

Recombinant baculoviruses containing the 5′-untranslated region and coding region of GIRK1 and the coding region of CIR (3) were

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[1] The abbreviations used are: ACh, acetylcholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate).

[2] Krapivinsky, G., Krapivinsky, L., Velimirovic, B., Wickman, K., Navarro, B., and Clapham, D. E. (1995) *J. Biol. Chem.* **270,** 28777–28779.

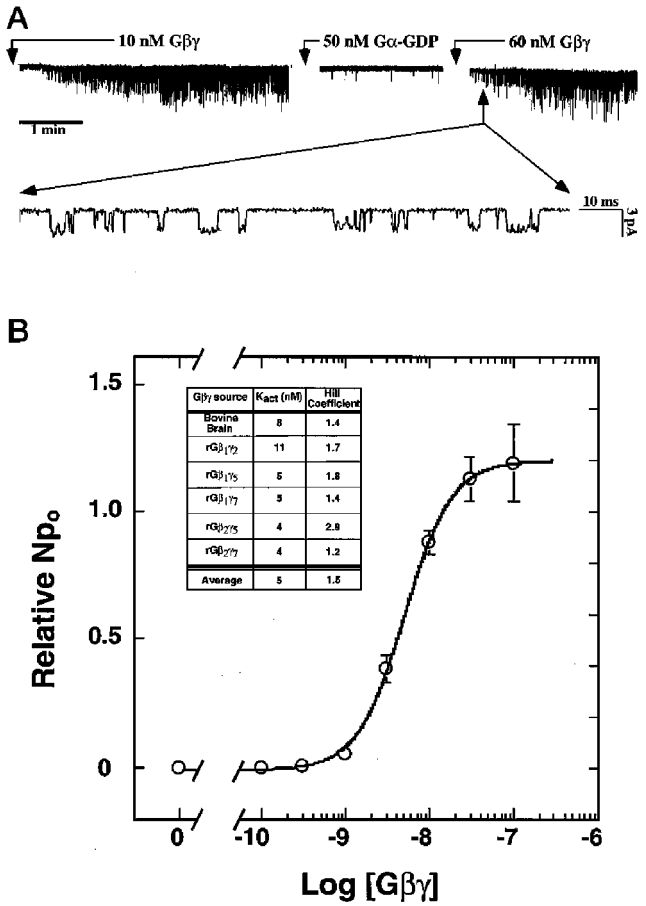

FIG. 1. **Gβγ activates I_{KACh} in inside-out patches from rat atrial myocytes.** *A*, 10 nM bovine brain Gβγ consistently activated I_{KACh}. Subsequent addition of 30–100 nM bovine brain Gα-GDP completely inhibited I_{KACh} activity elicited by Gβγ. Channel activity was restored by the addition of excess Gβγ. *B*, dependence of I_{KACh} activity (relative Np_o) on Gβγ concentration. The cumulative K_{act} and the Hill coefficient determined by averaging data obtained for bovine brain Gβγ and five recombinant Gβγ complexes (includes data reported in Ref. 2) were 5 nM and 1.5, respectively. The *table inset* shows the relevant parameters determined for each type of Gβγ preparation. Np_o values are normalized to subsequent GTPγS stimulation ($Np_o = 1$).

produced using the non-fusion baculovirus transfer vector pBlueBac III. The viruses were generated, isolated, and amplified as described (MaxBac, Invitrogen). Five days after infection, cells were harvested and homogenized in a hypotonic buffer. Membranes were then collected, solubilized, and immunoprecipitated as described for atrial membranes.

RESULTS

The functional interaction between Gβγ and I_{KACh} has been well studied in inside-out membrane patches from atrial myocytes. Bovine brain Gβγ reproducibly activates I_{KACh}, and as expected for a Gβγ-dependent process, channel activity is inhibited by excess Gα-GDP (Fig. 1*A*; see also Refs. 1, 2, 6, and 7). Inhibition by Gα-GDP is overcome by excess Gβγ (Fig. 1*A*; see also Ref. 2). Because there was no statistically significant difference between the potency of bovine brain Gβγ and the potencies of all Gβγ recombinant subunits tested previously (except transducin Gβ1γ1; Ref. 2), these data were averaged to generate the cumulative concentration-response relation shown in Fig. 1*B*. The resultant K_{act} and the Hill coefficient, as determined by the best fit of the cumulative data, were 5 nM and 1.5, respectively.

This type of functional study cannot address whether there is a direct interaction between Gβγ and I_{KACh}. To examine this issue, we studied Gβγ binding to the channel. An anti-peptide antibody (aCIRN2) directed against a unique amino-terminal domain of the CIR subunit immunoprecipitated CIR and coimmunoprecipitated GIRK1 from bovine atrial membranes.[2] Endogenous cardiac Gβγ bound the native channel (Fig. 2*A*). Significantly, native cardiac Gβγ associated with the aCIRN2-immunoprecipitated channel complex only when atrial sarcolemmal membranes were treated with GTPγS to activate endogenous G proteins. This suggests that prior to activation, G protein heterotrimers do not complex with I_{KACh}. Although the association of native cardiac Gβγ with the channel was clear, the signal was inadequate for accurate quantitation of binding.

To quantify binding of Gβγ to the channel, we measured the binding of ^{125}I-labeled, purified bovine brain Gβγ to the immunoprecipitated atrial GIRK1-CIR complex (I_{KACh}; Fig. 2*B*). The observed binding was due to an interaction between Gβγ and the channel as determined by competition with antigenic peptide. As shown in Fig. 2*B*, the presence of this peptide resulted in a significant decrease in Gβγ binding. Unlabeled Gβγ similarly decreased the level of binding of labeled Gβγ, demonstrating specificity of ^{125}I-Gβγ binding in the concentration range under study. Finally, consistent with results from electrophysiological experiments, the presence of excess Gα-GDP, but not Gα-GTPγS, prevented an interaction between Gβγ and I_{KACh} (Fig. 2*B*). We conclude that Gβγ binds directly and specifically to native cardiac I_{KACh}. Given the previous reports of I_{KACh} stimulation by Gα-GTPγS (18), we tested whether ^{125}I-Gα (GDP- or GTPγS-bound) interacted physically with immunoprecipitated I_{KACh}. The amount of ^{125}I-Gα that bound to immunoprecipitates was insignificant (~100-fold less) in relation to the amount of bound Gβγ (data not shown).

The binding constant for the interaction between Gβγ and I_{KACh} was determined by competition between unlabeled and labeled Gβγ for channel binding sites (17). The binding data were most simply and adequately fit by a model with a single type of binding site. The Gβγ binding constant to cardiac I_{KACh} immunoprecipitated by aCIRN2 was 55 nM (Fig. 2*C*). Thus, the affinity of the channel for Gβγ determined in this binding assay was ~10-fold lower than that suggested by results from electrophysiological experiments (Fig. 1*B*). Since this discrepancy could potentially be explained by antibody interference with Gβγ binding, we also determined the Gβγ binding constant to the channel using an immunoprecipitating antibody targeting the carboxyl terminus of the GIRK1 subunit of I_{KACh} instead of the CIR subunit (aCsh (3)). The Gβγ binding affinity to aCsh and aCIRN2 immunoprecipitates were virtually identical (Fig. 2*C*), indicating that Gβγ binding was not affected by the immunoprecipitating antibody. Note that Lubrol PX, even at low concentrations (0.1%), significantly inhibited Gβγ binding, consistent with the inhibition by Lubrol PX of Gβγ-induced I_{KACh} activation observed in patch-clamp experiments (19).

Given the results of the binding studies between Gβγ and native cardiac I_{KACh}, the next logical step was to determine whether GIRK1, CIR, or both subunits interacted with Gβγ. Since the subunit-specific antibodies did not affect the binding of Gβγ to cardiac I_{KACh}, they were used to study binding of Gβγ to GIRK1 and CIR subunits expressed and isolated from Sf9 cells. The control experiments used to confirm specificity of Gβγ binding to cardiac I_{KACh} (see Fig. 2*B*) were also performed for the individually expressed subunits to assess the significance of any observed interactions. Both recombinant GIRK1 and CIR subunits demonstrated specific binding to Gβγ (Fig. 3, *A* and *B*). There was no evidence for cooperativity in the binding of Gβγ to either recombinant subunit; the data were well fit to a model with a single type of binding site. The apparent affinity of Gβγ for CIR was almost identical to that for cardiac

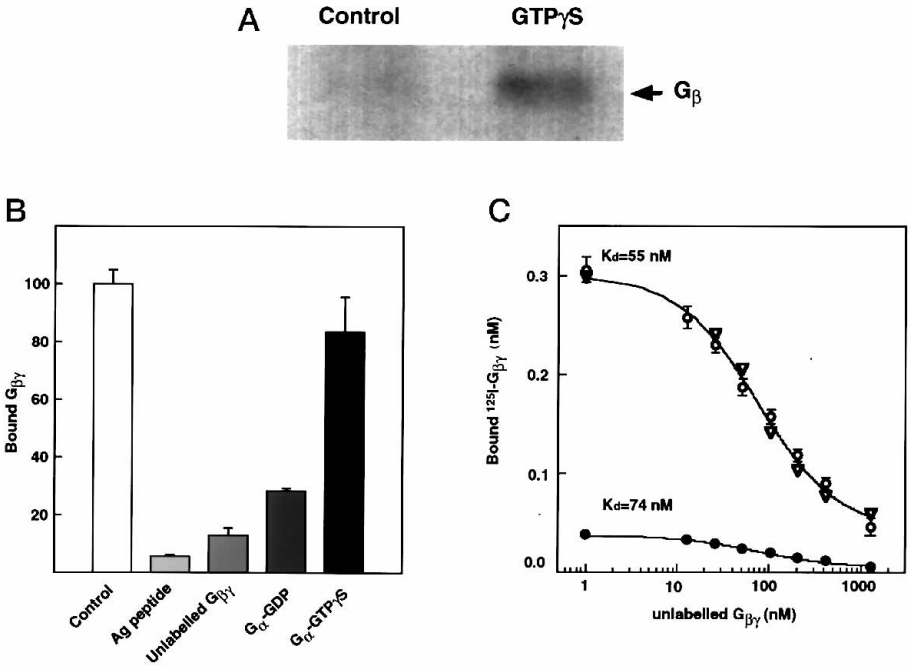

FIG. 2. **Gβγ binds to native atrial I_{KACh}.** *A*, Gβγ binds to I_{KACh} only after dissociation of endogenous heterotrimeric G proteins by GTPγS. Bovine atrial plasma membranes (1 mg) were treated with 100 μM GTPγS, solubilized in 1.0% CHAPS-HEDN, and immunoprecipitated by aCIRN2. Immunoprecipitated proteins were Western blotted with an anti-Gβ antibody. *B*, ^{125}I-Gβγ binds to cardiac I_{KACh} immunoprecipitated by the anti-CIR antibody, aCIRN2. Binding of 1.25 nM ^{125}I-Gβγ was inhibited by 200 μM CIRN2 antigenic (*Ag*) peptide, 1.3 μM unlabeled Gβγ, and 125 nM Gα-GDP. In contrast, virtually no inhibition was observed with 125 nM Gα-GTPγS. For this and Fig. 3, *A* and *B*, 100 on the *y* axis refers to full binding of ^{125}I-Gβγ in the absence of competing proteins. *C*, competition of unlabeled Gβγ and ^{125}I-Gβγ for binding sites on I_{KACh} was used to evaluate the equilibrium binding constant. Binding of ^{125}I-Gβγ to aCIRN2-precipitated I_{KACh} in 0.1% CHAPS (○) or 0.1% Lubrol PX (●) and binding of ^{125}I-Gβγ to aCsh-precipitated I_{KACh} in 0.1% CHAPS ( ) is shown.. All data points represent the average of three separate experiments. The data were fit well to a model consisting of a single type of binding site.

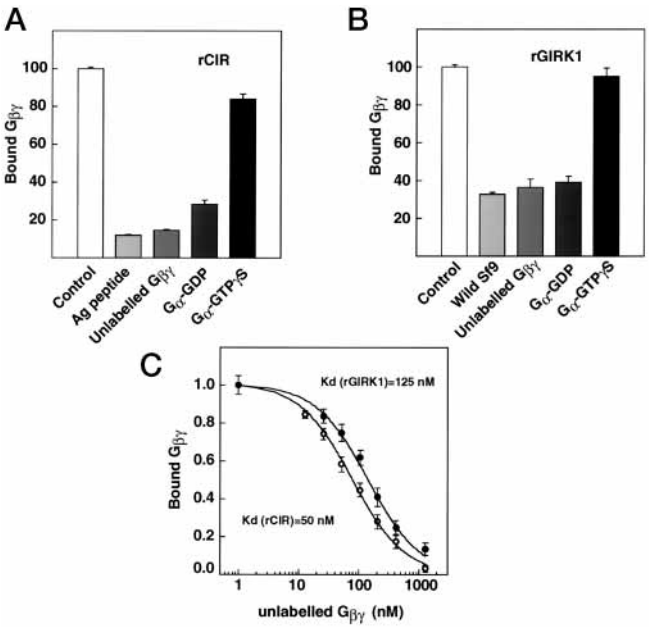

FIG. 3. **Gβγ binding to recombinant CIR and GIRK1 subunits.** *A* and *B* demonstrate the specificity of Gβγ binding to recombinant CIR (*rCIR*) or to recombinant GIRK1 (*rGIRK1*). Recombinant CIR and recombinant GIRK1 were immunoprecipitated with aCIRN2 and aCsh, respectively. Conditions were identical to those described in the legend to Fig. 2. Nonspecific binding for aCsh immunoprecipitates was determined using 500 μg of wild type Sf9 cell membranes. *C*, binding affinity of Gβγ to recombinant CIR and recombinant GIRK1. Nonspecific binding was subtracted, and each *point* was normalized to maximal binding in absence of competitor.

I_{KACh} (K_d = 50 nM; Fig. 3*C*) and slightly lower for GIRK1 (K_d = 125 nM).

DISCUSSION

Despite the widespread electrophysiological evidence for membrane-delimited G protein activation of ion channels (20), no biochemical evidence has been presented for a direct interaction between G protein subunits and channel proteins for two major reasons. First, in comparison to other G protein effectors such as adenylyl cyclase, cGMP phosphodiesterase, and phospholipase Cβ, channel proteins are of lower abundance in cells. Ion channels of specific subtypes number only a few thousand per cell. Second, the most dramatic example of a G protein-regulated ion channel is I_{KACh}, a member of a class of ion channels only recently cloned (8, 21). The generation of immunoprecipitating antibodies to the I_{KACh} channel subunits, GIRK1 and CIR, enabled us to develop an assay for Gβγ binding. The present work shows that Gβγ binds CIR, GIRK1, and the native cardiac channel with similar affinities.

There are two findings in the current study that are discrepant with the electrophysiological data. The concentration of Gβγ eliciting half-maximal I_{KACh} activity was ~5 nM in inside-out atrial patches, while the calculated Gβγ binding constant for the solubilized channel was ~50 nM. This difference could be simply due to the presence of the higher concentration of CHAPS in the binding reaction than in the electrophysiological experiments (1.6 *versus* 0.13 mM). On the other hand, Gβγ is hydrophobic and when added to an inside-out patch might concentrate in the membrane, giving a higher Gβγ concentration in the vicinity of the channel compared with the bath concentration. Thus, such functional studies might overestimate the real affinity of Gβγ for I_{KACh}. Our electrophysiologic data were fit with a Hill coefficient of 1.5, suggesting mild cooperativity (but see Ref. 7). However, we did not observe cooperativity in Gβγ binding to the channel or to its individual

subunits. It is possible that detergent solubilization of the channel could influence interactions between Gβγ binding sites on the channel. Our current hypothesis is that one Gβγ binds each GIRK1 or CIR subunit of the I_{KACh} heteromultimer to activate the channel.

We have shown that I_{KACh} is gated by Gβγ, not Gα (1, 2), that I_{KACh} is a heteromultimer of GIRK1 and CIR inward rectifier K^+ channel subunits (3), and that Gβγ, not Gα, directly binds both subunits of cardiac I_{KACh}. We have not localized the binding sites for Gβγ on the individual GIRK1 or CIR subunits, but Gβγ has been shown to associate with a fusion protein containing the full carboxyl-terminal residues of GIRK1 (16). Interestingly, our studies indicate that the antibodies raised against this entire region do not interfere with the observed binding of Gβγ to cardiac I_{KACh}. Initially, the obvious candidate region for Gβγ binding in GIRK1 was the extreme ~150 carboxyl-terminal amino acids, since this domain was not present in the G protein-insensitive IRK (Kir 2.0) or ROMK (Kir 1.0) families. However, CIR, which binds Gβγ even better than GIRK1, has no corresponding region. Comparisons of the GIRK (Kir 3.0) family (including CIR) carboxyl-terminal regions do not reveal any unique GIRK family similarities, which might suggest a functional Gβγ-binding domain. In contrast, the amino termini of the GIRK family contain scattered conserved amino acids not found in ROMK and IRK subfamilies. Additional structure/function studies will address these issues.

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