Importance of the G protein γ subunit in activating G protein-coupled inward rectifier K^+ channels

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Received 17 October 1999; received in revised form 18 November 1999

Edited by Shozo Yamamoto

Abstract The G protein-coupled inward rectifier K^+ channel (GIRK) is activated by direct interaction with the heterotrimeric GTP-binding protein βγ subunits (Gβγ). However, the precise role of Gβ and Gγ in GIRK activation remains to be elucidated. Using transient expression of GIRK1, GIRK2, Gβ1, and Gγ2 in human embryonic kidney 293 cells, we show that C-terminal mutants of Gβ1, which do not bind to Gγ2, are still able to associate with GIRK, but these mutants are unable to induce activation of GIRK channels. In contrast, other C-terminal mutants of Gβ1 that bind to Gγ2, are capable of activating the GIRK channel. These results suggest that Gγ plays a more important role than that of an anchoring device for the Gβγ-induced GIRK activation.

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Key words: G protein-coupled inward rectifier K^+ channel; G protein; Gβγ; Electrophysiology; Immunoprecipitation; HEK 293 cell

1. Introduction

Inward rectifier K^+ channels (Kirs) are the main ion channels which determine the cellular membrane potential near the resting level. The G protein-coupled inward rectifier K^+ channels (GIRK, Kir3) [1], which make up a subfamily among Kirs [2], are of particular interest, because the activity of GIRKs is regulated by hormones and neurotransmitters through the heterotrimeric GTP-binding proteins (G proteins) [3]. Four types of cDNAs belonging to the GIRK subfamily have been isolated from mammalian tissues [4–7]. The GIRK channel is a heterotetramer, consisting of four GIRK molecules [8,9]. In the atria of the heart, activation of GIRK1/GIRK4 (GIRK1/4) channels by acetylcholine induces hyperpolarization, resulting in slowing the heart rate. In the nervous system, many slow acting transmitters induce neuronal inhibition by activating GIRK1/2 channels. These GIRK channels are activated by direct interaction of the G protein βγ subunits (Gβγ) and the GIRK molecules [10–15]. However, little is understood about the molecular mechanism of GIRK activation by Gβγ.

G protein β subunit (Gβ) and γ subunit (Gγ) form a heterodimer in the physiological condition. Previously, we demonstrated that Gβ mutants, which lack the ability to bind to Gγ, still induce the activation of c-Jun N-terminal kinase in human embryonic kidney 293 cells [16]. In the present study using the Gβ1 mutants, the role of Gβ and Gγ in activating GIRK is investigated. We found that Gβ1 mutants that do not bind to Gγ2, are still capable of attaching to GIRK1 and to GIRK2, but these mutants are not capable of activating the GIRK channels. These results indicate the importance of Gγ for the Gβ subunit to functionally influence the effector.

2. Materials and methods

2.1. Construction of plasmids

Complementary DNAs for GIRK1 and GIRK2 were isolated from a rat brain cDNA library by polymerase chain reaction (PCR), and were subcloned into the mammalian expression vector pCMV5 [17]. HA-GIRK1 and Myc-GIRK2 were constructed by attaching HA and Myc epitope to the N-terminal, respectively. Plasmids of Gβ1 and Gγ2 were constructed as described before [16,18,19].

2.2. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's (DME) medium with 10% fetal calf serum. HEK 293 cells were transfected by the calcium phosphate coprecipitation technique according to the standard protocol. For biochemical experiments, the amount of DNA used for transfection was adjusted, by adding pCMV5, to 40 μg per 10 cm dish of the cells.

For electrophysiological experiments, 1 day before the transfection, HEK 293 cells were subcultured in 3.5 cm dishes coated with rat tail collagen in the center (about 1.2 cm in diameter) [20]. The amount of each kind of plasmid was 0.25 μg per 3.5 cm dish, except that 0.1 μg was used for GFP (plasmid pGreen Lantern-1, Life Technologies, Gibco-BRL). By adding pCMV5, the total amount of plasmids was kept at 1.1 μg per dish. The calcium phosphate solution containing plasmids was added to the rat collagen-coated region.

2.3. Immunoprecipitation

Transfected cells on the 10 cm dish were harvested and solubilized in 500 μl of lysis buffer (20 mM HEPES-NaOH (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 25 mM β-glycerophosphate, 0.5% Triton X-100, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 20 μg/ml aprotinin) on ice for 20 min. The samples were centrifuged at 15 000×*g* for 20 min at 4°C, and the supernatants were subjected to immunoprecipitation. The cell lysates (150 μl) were incubated at 4°C for 1 h with antibody and protein A-Sepharose. Immune complexes with protein A-Sepharose were washed three times with washing buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 20 mM $MgCl_2$, 0.1% Triton X-100). Immunoprecipitated proteins were boiled in sample buffer (50 mM Tris–HCl (pH 6.8), 1% SDS, 1% 2-mercaptoethanol, 0.1% bromophenol blue, and 8% glycerol) and resolved by SDS-PAGE.

2.4. Immunoblotting

After SDS-PAGE, the proteins were transferred to BA81 nitrocellulose membranes (Schleicher and Schuell). The membranes were blocked with blocking buffer (50 mM Tris–HCl (pH 8), 2 mM $CaCl_2$,

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PII: S0014-5793(99)01656-7

80 mM NaCl, 5% non-fat dry milk, 0.2% NP-40, and 0.02% NaN_3), and proteins were immunoblotted with antibodies described below. The bound antibodies were visualized by enhanced chemiluminescence detection system, using anti-mouse or anti-rabbit Ig antibodies conjugated with horseradish peroxidase as secondary antibodies (Amersham Life Science).

2.5. Antibodies

Rabbit polyclonal antibody (06-238) which recognizes Gβ1 and Gβ2 was purchased from Upstate Biotechnology, Inc. Mouse monoclonal antibody M2 against FLAG epitope was from Eastman Kodak Company. Mouse monoclonal antibody 12CA5 and rabbit polyclonal antibody against HA epitope were from Boehringer Mannheim and MBL, respectively. Mouse monoclonal antibody 9E10 and rabbit polyclonal antibody A-14 against c-Myc epitope were from Berkeley Antibody Company and Santa Cruz Biotechnology, Inc., respectively.

2.6. Electrophysiology

Electrophysiological experiments were performed 20 to 28 h after the transfection by using the whole-cell version of patch clamp. Experiments were performed, by using a fluorescence inverted microscope, only on cells that fluoresced and were isolated from other cells. The bathing solution (external solution) contained 146 mM sodium gluconate, 10 mM potassium gluconate, 2.4 mM $CaCl_2$, 1.3 mM $MgCl_2$, 5 mM HEPES-NaOH and 0.5 μM tetrodotoxin. The pipette solution (internal solution) contained 151 mM potassium gluconate, 5 mM HEPES-KOH, 0.5 mM EGTA-KOH, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 3 mM Na_2-ATP and 0.2 mM GDP (pH 7.2). Values of membrane potentials were corrected for the liquid junction potential between the patch pipette solution and the external solution (the external solution side 7 mV positive). The resistance of the filled patch pipette ranged from 3 to 6 MΩ. Series resistance was electronically compensated. In addition, the remaining resistance was mathematically accounted for as described before [21]. $BaCl_2$-containing solutions were applied through a computer-controlled sewer pipe system (ALA Scientific Instruments, New York, USA). The pCLAMP programs (version 6.03; Axon Instruments, Inc.) were used for the acquisition (2 kHz digitization) and analysis of the data. Recordings were performed at ~21°C. Statistical values are expressed in mean ± S.E.M.

3. Results

Several mutants of Gβ1 were expressed transiently in HEK 293 cells, and the association between Gβγ and GIRK was investigated. As shown in Fig. 1A, Gβ1ΔC1 and Gβ1ΔC2 lack one and two amino acid residue(s), respectively, at the C-terminal. Gβ1$(His)_6$ has six additional histidine residues at the C-terminal. Gβ1W339A has a point mutation of the 339th amino acid tryptophan replaced by alanine.

We previously demonstrated that Gβ1ΔC1, Gβ1$(His)_6$, and Gβ1W339A, but not Gβ1ΔC2, are able to bind to Gγ2 [16]. In the present experiments, HA-GIRK1 and FLAG-Gγ2 which are tagged with HA and FLAG epitope at the N-terminal, respectively, were co-transfected with the Gβ1 mutants. When wild-type Gβ1, Gβ1ΔC1, Gβ1$(His)_6$, and Gβ1W339A were co-expressed with HA-GIRK1 and FLAG-Gγ2, HA-GIRK1 was co-precipitated with anti-FLAG antibody (Fig. 2A). In the case of Gβ1ΔC2 and in the absence of exogenously expressed Gβ1 wild-type, only a very small amount of endogenous Gβ and HA-GIRK1 were co-precipitated with FLAG-Gγ2. These results suggest that Gγ2 does not bind directly to GIRK1.

The GIRK1 is known to function as heterotetramer with GIRK2, 3, or 4, but not as homotetramer. In order to simulate this functional condition, Myc-GIRK2 was co-transfected with HA-GIRK1. The co-precipitation pattern of HA-GIRK1 and Myc-GIRK2 using anti-FLAG antibody was similar to that of HA-GIRK1 without Myc-GIRK2 (Fig. 2B).

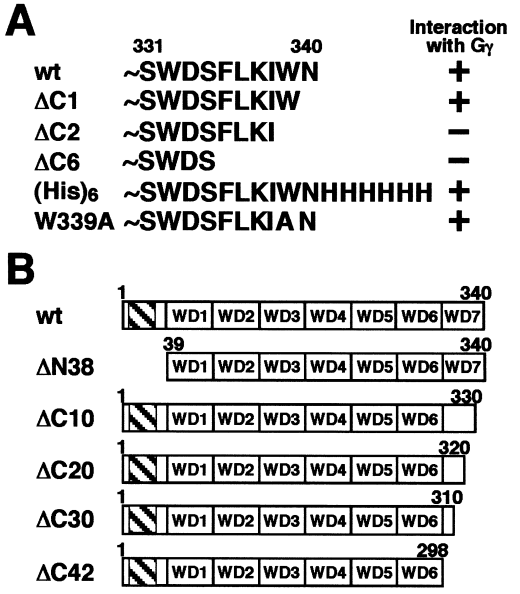

Fig. 1. Structure and sequence of the Gβ1 mutants. A: Comparison of C-terminal amino acid sequences of Gβ1 mutants. The ability to interact with Gγ2 was shown on the right. B: Schematic representation of N- or C-terminal deleted mutants of Gβ1. Hatched boxes indicate α-helix. WD indicates WD motif.

Next, immunoprecipitation of Myc-GIRK2 with anti-Myc antibody was carried out using the same cell lysates prepared in Fig. 2B. HA-GIRK1 was co-precipitated with Myc-GIRK2 (Fig. 2C). Co-precipitation of Gβ1 wild-type, Gβ1ΔC1, and Gβ1$(His)_6$ were observed. Unexpectedly, Gβ1ΔC2, which does not form a dimer with Gγ2, was also co-precipitated with Myc-GIRK2. This result suggests that the binding of Gγ to Gβ is not necessary for the association of Gβ with GIRK, and that GIRK directly binds to Gβ1 in the absence of Gγ. Other deletion mutants, Gβ1ΔN38, Gβ1ΔC6, Gβ1ΔC10, Gβ1ΔC20, Gβ1ΔC30, and Gβ1ΔC42, which lack the ability to bind to Gγ2 [16,19], were examined. These Gβ1 mutants were co-expressed with HA-GIRK1 and Myc-GIRK2, and immunoprecipitation was carried out using anti-Myc antibody (Fig. 2D). All of these deletion mutants were co-precipitated with Myc-GIRK2, indicating that the N-terminal 38 amino acids and C-terminal 42 amino acids of Gβ1 are not essential for attachment to GIRK.

Since the Gβ mutants that lack the ability to bind to Gγ associate with GIRK1/2 as shown above, the function of the GIRK channel was electrophysiologically examined by using the whole-cell mode of patch clamp on HEK 293 cells transfected with GIRK1, GIRK2, Gβ1, and Gγ2. Green fluorescence protein (GFP) was used as a marker. The transfection with wild-type Gβ1, Gγ2, GIRK1 and GIRK2, together with GFP, is designated as the positive control. Fluorescence positive cells exhibited inwardly rectifying currents as shown in Fig. 3A, which reversed at -61.5 ± 1.7 mV ($n = 22$) (the real reversal potential, after correcting for the leak current was −67 mV) coinciding well with the theoretical value of the K equilibrium potential (−69 mV). Cells transfected only with GFP revealed a very small, almost linear current (Fig. 3A). The GIRK channel is known to be blocked by Ba^{2+} ions. Application of 100 μM Ba^{2+} reduced the inwardly rectifying currents by $58.9 \pm 3.2\%$ ($n = 5$) (data not shown).

The amplitudes of the inward currents at −127 mV are

analyzed. Fig. 3B shows that the mean inward current in the positive control was 111 pÅ/pF. Cells transfected with the GIRK1 and GIRK2, but with neither Gβ1 nor Gγ2, showed inward rectifier currents, which were smaller than the positive control, but larger than the current in the absence of GIRK1/2. This result suggests the presence of endogenous Gβγ, which activates the exogenously expressed GIRK1/2 channels in HEK 293 cells. When transfected with Gβ1, GIRK1 and

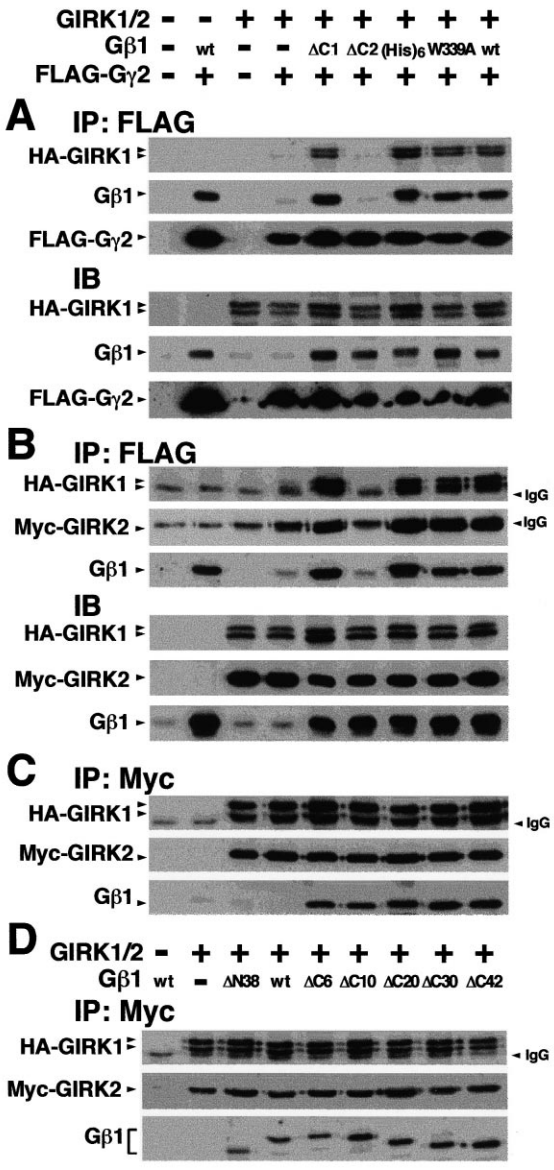

Fig. 2. Expression and co-immunoprecipitation of GIRK and Gβ1 mutants with Gγ2. A: HA-GIRK1 was co-expressed with FLAG-Gγ2 and Gβ1 mutants in HEK 293 cells. Aliquots of the cell lysates were used for immunoprecipitation with anti-FLAG antibody and immunoblotted with anti-FLAG, anti-common Gβ, and anti-HA antibodies (upper). Other aliquots were immunoblotted with the same antibodies (lower). HA-GIRK1 was expressed in two forms, unglycosylated 57 kDa form and glycosylated larger form. B: HA-GIRK1 and Myc-GIRK2 were co-expressed with FLAG-Gγ2 and Gβ1 mutants. Aliquots of the cell lysates were used for immunoprecipitation with anti-FLAG antibody and immunoblotted with anti-common Gβ, anti-HA, and anti-Myc antibodies (upper). Other aliquots were immunoblotted with the same antibodies (lower). IgG indicates heavy chain of IgG antibody used for immunoprecipitation. C: Aliquots described above were used for immunoprecipitation with anti-Myc antibody. D: HA-GIRK1 and Myc-GIRK2 were expressed with N- or C-terminal deleted mutants of Gβ1, and immunoprecipitation was carried out with anti-Myc antibody. Immunoblotting was carried out with anti-common Gβ, anti-HA, and anti-Myc antibodies.

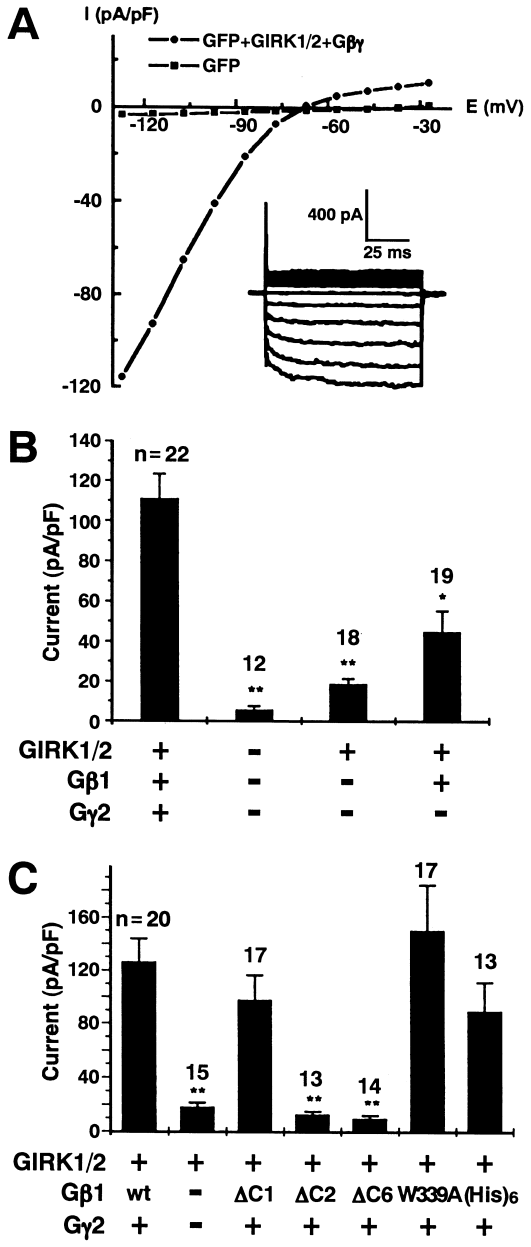

Fig. 3. Whole-cell currents induced by exogenously expressed GIRK channels and Gβγ. A: The I-V relation in two HEK 293 cells. The holding potential was −77 mV. Currents (pA) were expressed in reference to the input capacitance of the cell (pF). In negative control, the cell was transfected with GFP only (squares). In positive control, the cell was transfected with Gβ1, Gγ2, GIRK1, GIRK2 and GFP (circles). In the I-V relation, the series resistance was compensated electronically and mathematically (see Section 2). The inset records show currents from the positive control cell evoked by voltage steps from −127 to −27 mV at a 10 mV interval; the series resistance was compensated only electronically. B: Effects of transfection with various combinations of Gβ, Gγ, and GIRK. C: Effect of C-terminal mutants of Gβ1. The procedures of B and C were as follows: For each cell, the whole-cell current at −127 mV was used for statistics. The vertical lines represent S.E.M. The symbol (*) means $P < 0.01$ and the symbol (**) $P < 0.001$ (ANOVA), both in reference to the value of each positive control.

GIRK2, but without Gγ2, the mean inward rectifier current was about a half of the positive control.

Next, Gβ1 mutants were tested. Gβ1ΔC1, Gβ1W339A, and Gβ1$(His)_6$, all of which bind to Gγ, resulted in inward currents comparable to that of the wild-type Gβ1 (Fig. 3C). In contrast, Gβ1ΔC2 and Gβ1ΔC6, both of which do not bind to Gγ, produced very small inward currents compared to that of Gβ alone.

4. Discussion

It has been demonstrated by the yeast two-hybrid system that N-terminal 100 residues of Gβ1 are sufficient for interaction with the N-terminal domain of GIRK1 [22]. In the present study we demonstrated the interaction of GIRK1/2 with Gβ1ΔN38 or Gβ1ΔC42 (Fig. 2). These results suggest that the region of Gβ essential for interaction with GIRK appears to be between amino acids 39 and 100. The crystal structure of Gβγ has been revealed [23–25]. Gβ has an N-terminal α-helix and a symmetrical seven-bladed propeller structure. Blade 1 and a part of blade 7 are composed of the amino acid 39–100 residues, which appear to participate in interaction with GIRK.

The Gβ1 mutants, which did not bind to Gγ2, were still able to associate with GIRK1/2 (Fig. 2). Thus, Gβ associates with the GIRKs in the absence of Gγ. Furthermore, we found that the Gβ mutants, which lack the ability to bind to Gγ, and GIRK1/2 exist in the membrane fraction (data not shown). On the other hand, electrophysiological experiments indicated that these Gβ1 mutants were incapable of activating GIRK1/2 channels. These results suggest that the binding of Gγ to Gβ may be important for the activation of GIRK1/2 channels.

Over-expression of Gβ alone induced more currents than Gβ1ΔC2, Gβ1ΔC6, or GIRK1/2 without Gβγ (Fig. 3). This result suggests at least two possibilities. One possibility is that there is a small amount of excess Gγ which is able to form a dimer with exogeneous Gβ. The purification of Gγ3 and Gγ11 freed from Gβ was reported previously [26,27]. Another possibility is that wild-type Gβ has the ability to activate GIRK in the absence of Gγ, but to a lesser extent. In order to fully activate GIRK, the presence of Gγ is necessary. Although these possibilities remain to be elucidated, data from the present study indicate the importance of Gγ for the activation of GIRK channel.

Several reports have shown that prenylation of Gγ is important not only for membrane anchoring [28–30], but also for the functional interaction of Gβγ with receptors [31,32]. However, not much attention has been paid on the role of Gγ in the functional interaction with effectors. Recently, Myung et al. [33] have reported that the effectiveness of the Gβγ to activate a phospholipase and an adenylyl cyclase depends both on the type of prenylation of Gγ and on the amino acid sequence of Gγ. They suggested that Gγ plays an important role in determining the functional interaction of Gβγ with the effectors. Although the effectors used (phospholipase and adenylyl cyclase vs. GIRK) and the experimental approaches are different between their experiments and ours, our main conclusion agrees with that of Myung et al. [33].

Previously, we observed that mutant Gβ1$(His)_6$ is incapable of activating a mitogen-activating protein kinase ERK2; however, this mutant is able to activate c-Jun N-terminal kinase JNK1 [16]. In contrast, Gβ1ΔC1 is less capable of activating JNK1. In the present study, the mutants Gβ1$(His)_6$ and Gβ1ΔC1 caused almost the same degree of GIRK activation as that by the wild-type Gβ1. It seems that Gβγ regulates the activity of different effector molecules through a unique contact site of Gβ, in agreement with Ford et al. [34] and Li et al. [35]. Thus, depending on the type of effector, the involvement of Gγ in the activation is different.

Acknowledgements: This work was supported by grants from the Ministry of Education, Science, Sport, and Culture of Japan, from CREST, and from National Institutes of Health, MH57837 (USA). Laboratory at Tokyo Institute of Technology is supported by funding from Schering-Plough Corporation.

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