0026-895X/03/6405-1085–1091$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 64:1085–1091, 2003

Vol. 64, No. 5
2649/1105148
Printed in U.S.A.

Interaction of G Protein β Subunit with Inward Rectifier K^+ Channel Kir3

QI ZHAO, TAKEHARU KAWANO, HIROKO NAKATA, YASUKO NAKAJIMA, SHIGEHIRO NAKAJIMA, and TOHRU KOZASA

Departments of Pharmacology (Q.Z., H.N, S.N., T.Ko.) and Anatomy and Cell Biology, College of Medicine, University of Illinois at Chicago, Illinois 60612 (T.Ka., Y.N.)

Received May 12, 2003; accepted August 8, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

G protein $\beta\gamma$ subunits bind and activate G protein-coupled inward rectifier K^+ (GIRK) channels. This protein-protein interaction is crucial for slow hyperpolarizations of cardiac myocytes and neurons. The crystal structure of Gβ shows a seven-bladed propeller with four β strands in each blade. The Gβ/Gα interacting surface contains sites for activating GIRK channels. Furthermore, our recent investigation using chimeras between Gβ_1 and yeast β (STE4) suggested that the outer strands of blades 1 and 2 of Gβ1 could be an interaction area between Gβ1 and GIRK. In this study, we made point mutations on suspected residues on these outer strands and investigated their ability to activate GIRK1/GIRK2 channels. Mutations at Thr-86, Thr-87, and Gly-131, all located on the loops between β-strands, substantially reduced GIRK channel activation, suggesting that these residues are Gβ/GIRK interaction sites. These mutations did not affect the expression of Gβ1 or its ability to stimulate PLCβ2. These residues are surface-accessible and located outside Gβ/Gα interaction sites. These results suggest that the residues on the outer surface of blades 1 and 2 are involved in the interaction of G$\beta\gamma$ with GIRK channels. Our study suggests a mechanism by which different effectors use different blades to achieve divergence of signaling. We also observed that substitution of alanine for Trp-332 of Gβ1 impaired the functional interaction of Gβ1 with GIRK, in agreement with the data on native neuronal GIRK channels. Trp-332 plays a critical role in the interaction of Gβ1 with Gα as well as all effectors so far tested.

The family of G protein-coupled inward rectifier K^+ channels (GIRK; Kir3) plays important roles in physiological functions; the activity of the GIRK channels is a major determinant of excitability in many kinds of cells, including cardiac myocytes and neurons (Sakmann et al., 1983; Dascal et al., 1993; Kubo et al., 1993; Wickman and Clapham, 1995; Stanfield et al., 2002). The activity of GIRK channels, in turn, is regulated by G proteins (Breitwieser and Szabo, 1985; Pfaffinger et al., 1985; Kurachi et al., 1986). Furthermore, the $\beta\gamma$ subunits (G$\beta\gamma$) but not the α subunit (Gα) of G proteins were shown to activate the GIRK channels (Logothetis et al., 1987; Ito et al., 1992, Reuveny et al., 1994; Huang et al., 1995; Krapivinsky et al., 1995). Thus, the interaction between G$\beta\gamma$ and GIRK is a crucial process for the physiological functions of the brain and the heart and, as such, is likely to be one of the sites for pharmacological interventions. Despite its importance, the molecular mechanism of this protein-protein interaction is poorly understood. One approach to clarify this interaction is to investigate the events that take place on the side of the K^+ channel. Another is to elucidate the processes occurring on the G$\beta\gamma$ molecules.

The aim of this study was to further clarify the residues of the Gβ molecule that interact with GIRK. X-ray crystallography has revealed that Gβ is a seven-bladed propeller structure with four antiparallel β strands for each blade. Gα interacts with Gβ through the top surface of the propeller. The outer β-strands of each blade form the outer surface of the torus-like structure (Wall et al., 1995; Lambright et al., 1996; Sondek et al., 1996). Because Gβ is capable of interacting with downstream effectors only if Gα is detached from Gβ, it has been assumed that the interaction sites of Gβ with Gα may also be the sites that interact with various effectors. Indeed, mutagenesis studies have identified various Gα-contacting residues of Gβ that are crucial for the interaction between G$\beta\gamma$ and various effectors, such as phospholipase Cβs (PLCβs), adenylyl cyclases, and GIRK channels (Ford et al., 1998; Li et al., 1998).

The Gα-interacting surface of Gβ may not be the only region to bind the effectors. Albsoul-Younes et al. (2001) constructed a set of Gβ1 chimera proteins by replacing a sequence on or near the outermost strand of each of the blades with the corresponding sequence in yeast Gβ (STE4).

This work was supported by National Institutes of Health grants MH57837 (to S.N.), AG06093 (to Y.N.), GM61454, and NS/GM41441, and by American Heart Association Grant (to T.K.). T.K. is an established investigator of American Heart Association.

ABBREVIATIONS: GIRK, G protein-coupled inward rectifier K^+ channels; PLC, phospholipase C; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium.

These proteins were tested for their ability to activate native GIRKs in brain neurons. The results indicated that the chimeras on the outer segments of blades 1 and 2 (called D1 and CD2) significantly impaired the GIRK activation (Albsoul-Younes et al., 2001), suggesting that these locations are important for the interaction between Gβ and GIRKs. These locations do not belong to the interaction sites between Gβ and Gα.

Because we know that the outer segments of blades 1 and 2 are crucial for the Gβ/GIRK interaction, the next step is to precisely identify the amino acid residues in these regions that are crucial for the GIRK activation. In the present experiments, we introduced point mutations on each of the nonconserved (between Gβ1 and STE4) residues on D1 and CD2 to its corresponding residue in STE4 (Albsoul-Younes et al., 2001). Each of these mutant Gβ1 cDNAs was expressed in human embryonic kidney (HEK) 293 cells and was tested for the ability to produce inwardly rectifying K^+ currents. In this way, we have identified three $G\beta_1$ residues located on the outer strands of blade1 and blade 2; mutations of these residues impaired the GIRK activation and therefore may play a crucial role in GIRK activation. The results suggest that in addition to the Gα-interacting surface, these residues on the outer surface of blades 1 and 2 are specifically involved in the interaction of Gβ with GIRK channels. Recently, Mirshahi et al. (2002) also identified 2 residues on blades 1 and 2 that could be the interaction sites with GIRK but not with Gα. Altogether, therefore, 5 residues are putative sites at which Gβ/GIRK interaction would take place outside the Gβ/Gα interface. An abstract of the present work has appeared (Zhao et al., 2002).

Materials and Methods

Molecular Biology. GIRK1 and GIRK2 cDNAs were from the rat. Gβ1 cDNA was from the mouse and Gγ2 cDNAs was from the bovine. All cDNAs used were subcloned into pCMV5 vector (Andersson et al., 1989). Point mutations were introduced by using the QuikChange site-directed mutagenesis kit (Stratagene; La Jolla, CA). The mutations were verified by the sequence facility at the University of Chicago.

Cell Culture and Transfection. HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C with 10% CO_2. Transient transfections of HEK293 cells were performed with Effectene (QIAGEN, Valencia, CA) according to the manufacturer's protocol. The total amount of cDNAs for the same experiment was kept constant by adding empty vectors.

Electrophysiology. HEK293 cells in 60-mm dishes were transfected with cDNAs of wild-type or mutant Gβ1, Gγ2, GIRK1, GIRK2, and GFP. The amount of each cDNA was 0.3 μg, except 0.1 μg for GFP. Twenty-four hours later, the cells were replated onto 35-mm dishes. The dish has a small well (about 1.2 cm in diameter) that was coated with rat-tail collagen (Roche Molecular Biochemicals, Indianapolis, IN). Forty-eight to 56 h after transfection, the whole-cell patch clamp was performed on the stage of a fluorescence inverted microscope. We did experiments on isolated cells with GFP fluorescence. The bathing 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, pH 7.4. The pipette solution contained 151 mM K gluconate, 5 mM HEPES-KOH, 0.5 mM EGTA-KOH, 0.1 mM $CaCl_2$, 4 mM $MgCl_2$, 3 mM Na_2-ATP, and 0.2 mM GDP, pH 7.2. Membrane potentials were corrected for the liquid junction potential between the pipette solution and the bathing solution (the bathing solution side was 7 mV positive). Cell capacitance was determined by cancellation of the transient responses to square-wave voltage inputs as directed in the user's manual for the EPC-7 patch clamp (List Electronic, Darmstadt, Germany). Series resistance was compensated electronically, usually by about 60%, and the remaining resistance was compensated mathematically by a linear approximation. The pCLAMP programs (version 6.03; Axon Instruments, Inc.) were used for the acquisition and analysis of the data. Recordings were performed at room temperature. Statistical results were expressed as mean ± S.E.M.

Western Blot Analysis. HEK293 cells in 35-mm dishes were transfected with 0.75 μg of Gβ1 and 0.75 μg of FLAG-Gγ2. Forty-eight h after transfection, the cells were harvested and lysed on ice for 20 min in 200 μl of lysis buffer (20 mM HEPES-NaOH at pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 25 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 0.5% Triton X-100, and 10% glycerol). Lysates were centrifuged at 15,000*g* for 20 min at 4°C. The supernatants were heated at 100°C for 5 min in a sample buffer (50 mM Tris-HCl, pH 6.8, 8% glycerol, 1% SDS, 2% 2-mercaptoethanol, and 0.008% bromphenol blue). Aliquots (20 μl) of the samples were loaded on 5% stacking, 15% separating SDS-PAGE gel. After electrophoresis, the proteins were transferred to a BA81 nitrocellulose membrane and blocked with blocking buffer (50 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 5% skim milk, 0.2% Nonidet P-40, and 0.02% NaN_3). The proteins were detected by Gβ (T-20) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-FLAG M2 monoclonal antibody (Sigma, Saint Louis, MO). The bound antibodies were visualized by an enhanced chemiluminescence detection system, using horseradish peroxidase-conjugated anti-rabbit or anti-mouse Ig secondary antibodies (Amersham Bioscience, Arlington Heights, IL).

PLCβ Assay in HEK293 Cells. HEK293 cells at 60 to 70% confluence in 24-well plates were transfected with PLCβ2, Gβ1 (wild type or mutant), Gγ2, and $G\alpha_o$. The total amount of cDNA was 0.8 μg per well. One day after transfection, the cells were labeled with 2 μCi/ml [^{3}H]inositol in 0.5 ml of inositol-free DMEM with 1% fetal bovine serum for 24 h. The next day, the cells were washed for 10 min and then stimulated for 50 min at 37°C with 0.5 ml of inositol-free DMEM supplemented with 25 mM HEPES, pH 7.4, and 10 mM LiCl. The reactions were terminated by 20 mM formic acid (0.75 ml), and the cells were incubated at 4°C for 30 min. The samples were neutralized to pH 7 to 8 by 100 μl of 3% ammonium hydroxide, collected, and centrifuged at 15,000 rpm for 5 min. The supernatants were loaded on 1 ml of Dowex AG 1-X8 columns pre-equilibrated with 10 ml of 4 M ammonium formate and 0.2 M formic acid followed by 5 ml of 0.18% ammonium hydroxide solutions. The flow-through of the sample and of the 1 ml of 0.18% ammonium hydroxide solution loaded after the sample was collected as the inositol fraction. The columns were washed with 4 ml of 40 mM ammonium formate and 0.1 M formic acid. The inositol phosphates were eluted with 1 ml of 4 M ammonium formate and 0.2 M formic acid. The inositol fraction and the inositol phosphate fraction were measured by liquid scintillation counting.

Results

Activation of GIRK Currents by Gβ1γ2 Cotransfection in HEK293 Cells. We used HEK293 cells as a heterologous expression system to test the ability of wild-type and mutant Gβs to activate GIRK channels. GIRK1/GIRK2 channel subunits alone or GIRK1/GIRK2 together with Gβ1γ2 were expressed. Whole-cell currents were evoked by a series of voltage steps from −127 to −27 mV at a 10-mV increment. Holding potential was −77 mV. Figure 1, A and B, shows that cells transfected with GIRK1/GIRK2 and Gβ1γ2 cDNAs produced large currents. The currents showed inward rectification and the reversal potential (−65.4 ± 0.8 mV; n = 28)

corresponded to the K^+ equilibrium potential (E_K) (−68.4 mV).

The current amplitudes at −97 mV were chosen to represent the capability of various $G\beta_1$ subunits to activate the GIRK current (Fig. 1C). Transfection with wild-type $G\beta1\gamma2$ plus GIRK1/GIRK2 evoked large currents, whereas cells transfected with GIRK1/GIRK2 (without $G\beta1\gamma2$) produced smaller currents. These small currents would have been derived from the transfected GIRK1/GIRK2 activated by the endogenous $G\beta\gamma$. If neither GIRK1/GIRK2 nor $G\beta\gamma$ was transfected, the current was very small (about one third of the current with GIRK1/GIRK2 only) (Kawano et al., 1999).

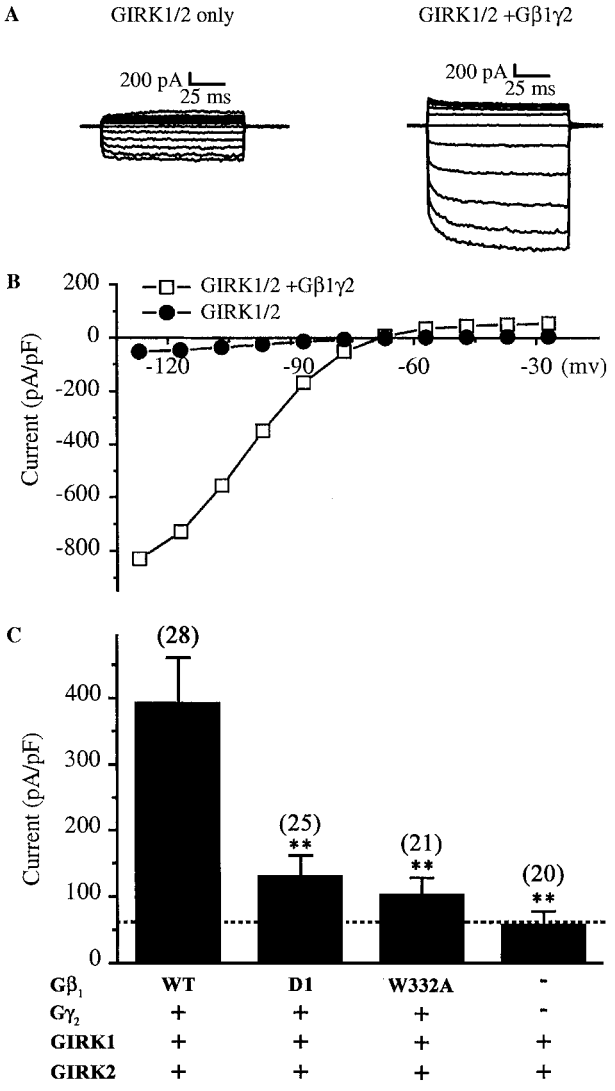

Fig. 1. Activation of GIRK channels by $G\beta1\gamma2$ in HEK293 cells. Cells were transfected with wild-type or mutant Gβ1 together with Gγ2, GIRK1, and GIRK2. Whole-cell currents were evoked by voltage steps from −127 to −27 mV at a 10-mV increment. Holding potential was −77 mV. A, samples of current traces recorded from cells transfected with GIRK1/GIRK2, either with or without $G\beta1\gamma2$. B, the current-voltage relation derived from the current traces shown in A. Currents were expressed in reference to the cell capacitance. □, current from a cell transfected with GIRK1/GIRK2 together with $G\beta1\gamma2$; ●, current from a cell transfected with GIRK1/GIRK2 only. C, Gβ1 mutants D1 and W332A are less effective in activating the GIRK channel. Whole-cell currents at −97 mV were used for statistics. **, $p < 0.01$ (analysis of variance and Bonferroni post tests). The dashed line indicates the current level produced by endogenous $\beta\gamma$.

This small current would have originated from unknown channels endogenous in HEK293 cells (Kawano et al., 1999).

In addition, Fig. 1C shows GIRK1/GIRK2 currents induced by two Gβ1 mutants, the chimera D1 and a point mutation W332A. The D1 mutant, the same as used by Albsoul-Younes et al. (2001), was generated by replacing the Gβ1 sequence on and near the outer strand of blade 1 (S84 to P94) with the corresponding yeast β (STE4) sequences (Fig. 2). W332A is a point mutant on the Gα-contacting surface of Gβ1 (Albsoul-Younes et al., 2001). Both the chimera and W332A produced substantially smaller GIRK current than the wild-type $G\beta1\gamma2$. These data on the genetically identified GIRK1/GIRK2 channels coincide well with the previous data on native GIRK channels in locus ceruleus neurons (Albsoul-Younes et al., 2001).

Identifying the Critical Residues in the D1 Region for Gβ-GIRK Interaction. We mutated each nonconservative Gβ1 residue over the D1 mutation region (i.e., from Ser84 to Pro94) to its corresponding STE4 residue (Fig. 2A);

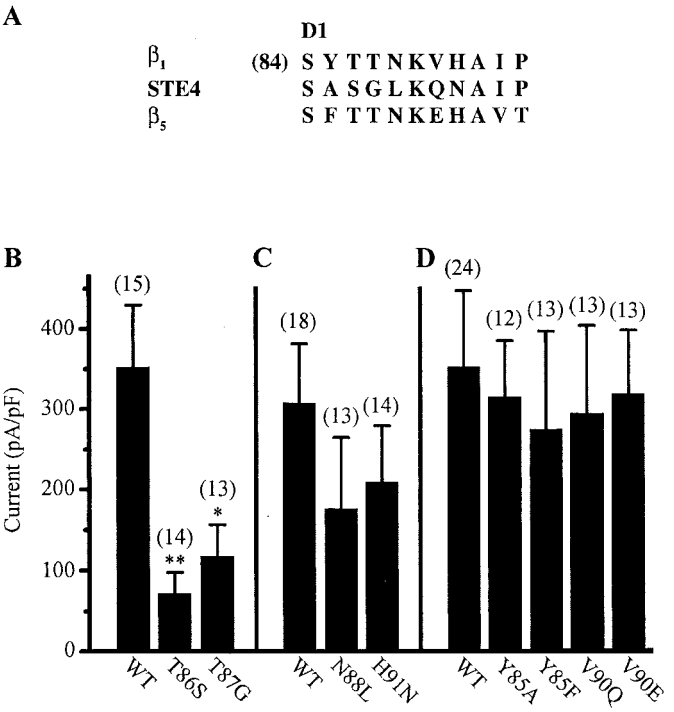

Fig. 2. A, sequence alignment of Gβ1, yeast Gβ (STE4), and Gβ5 in the D1 regions. B to D, effects of single amino acid mutations in the D1 region on the activation of GIRK channels. The mutants were made by replacing a wild-type Gβ1 residue with the corresponding yeast Gβ residue, except for Y85F and V90E, in which the mutants were made by replacing the Gβ1 residue with the corresponding Gβ5 residue. Before the start of the experiments, we had planned to divide these eight mutants into three groups (see below for the categorization), and for each group we compared the mutants with wild type. B, in this group, T86S and T87G were compared with wild-type Gβ1. These two mutants comprise the first two, in which the STE4 residue is different from the corresponding residue of wild-type Gβ1, yet the Gβ5 residue is the same as in Gβ1. T86S and T87G showed less ability to stimulate GIRK currents than the wild type. *, $p < 0.05$; **, $p < 0.01$, respectively (analysis of variance and Bonferroni post tests). C, the Gβ1 mutants, N88L and H91N, are the next two mutants, in which the STE 4 residue is different from the corresponding wild-type Gβ1 residue, yet the Gβ5 residue is the same as in Gβ1. These mutants produced GIRK current not significantly different from that of the wild-type Gβ1. D, in these Gβ1 mutants, Y85A, Y85F, V90Q, and V90E, the corresponding residues for STE4 as well as for Gβ5 are different from those of Gβ1. All these mutants produced GIRK current activation not significantly different from that of wild-type Gβ1.

these are Y85A, T86S, T87G, N88L, V90Q, and H91N. We noticed that two residues, Tyr85 and Val90, are nonconservative not only between β1 and STE4 but also between β1 and β5. The subunit Gβ5 is a distinct member of the Gβ family. Instead of activating GIRK channels, Gβ5 inhibits GIRK currents (Lei et al., 2000). We therefore made two additional mutants, Y85F and V90E, by replacing the Gβ1 residue with the corresponding residue of Gβ5.

Each of these mutant Gβ1 cDNAs was cotransfected with GIRK1/GIRK2 and Gγ2. Figure 2, B, C, and D, summarizes the GIRK current stimulation by these Gβ1 mutants in D1 region. Compared with wild-type Gβ1γ2, mutants T86S and T87G were much less effective in activating GIRK currents ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 2B). No other mutants showed any significant differences from the wild-type β1 in their ability to enhance the GIRK current (Fig. 2, C and D).

Identifying the Critical Residues in the CD2 Region for Gβ-GIRK Interaction. The result obtained by Albsoul-Younes et al. (2001) indicates that the Gβ1 mutant CD2 is less capable of activating GIRK channels in locus ceruleus neurons. In the CD2 mutant, the Gβ1 sequence (Leu126-Asn132) on the loop connecting the two outer strands of blade 2 was replaced with its counterpart in STE4 (Fig. 3A). We generated five Gβ1 mutants, L126V, T128S, R129K, G131V, and N132A, by mutating each of the nonconservative Gβ1 residues over the CD2 region to its corresponding STE4 residue. We also made one deletion mutation, K127Δ (Fig. 3A). These mutants were tested for their ability to stimulate GIRK channels coexpressed in HEK293 cells. As shown in Fig. 3B, Gβ1 mutant G131V showed a substantially lower ability to activate the GIRK current compared with wild type. All other mutants in the CD2 region were able to enhance GIRK current in a manner not significantly different from that of the wild type (Fig. 3, B and C).

Basal Currents or Agonist-Induced Currents. Mirshahi et al. (2002), by using the oocyte heterologous system, identified two Gβ residues, Ser67 and Thr128. Both of these residues are located outside the Gβ/Gα contact region and are likely to be interaction sites between Gβ and GIRK channels. They observed that mutations of these residues inhibited the GIRK "basal currents", which are presumably activated by the endogenous Gβγ. One might ask whether our mutants impair the generation of the GIRK basal current.

In HEK293 cells, unlike the oocyte system, the "basal GIRK current" is relatively small compared with the current induced by exogenous Gβ1γ2 (compare the first column with the fourth column in Fig. 1C). This makes it difficult to determine whether our mutations (T86S, T87G, and G131V) impaired the "basal GIRK current". From our results, therefore, we can conclude only that our mutants impaired the GIRK current stimulated by exogenous Gβ1γ2.

Expression of Gβ1 Mutants in HEK293 Cells. To examine the expression levels of the Gβ1 mutants, HEK293 cells expressing Gβ1 and FLAG-Gγ2 were subjected to the Western blot analysis. An antibody raised against a peptide at the carboxyl terminus of Gβ1 (T-20, Santa Cruz Biotechnology) was used for immunoblotting. Figure 4 shows representative Western blots from cells expressing the Gβ1 mutants. Low levels of Gβ (endogenous Gβ) (the leftmost lane) could be detected in cells transfected with vectors alone (Fig. 4, A and B). All three mutant Gβ1s that had a low ability to activate GIRK channels—T86S, T87G, and G131V—were expressed at a level comparable with that of the wild-type Gβ1 (Fig. 4A); thus, the functional impairment is not caused by the differences in the expression level. All other Gβ1 mutants that can activate the channels as effectively as wild type were also expressed at a level similar to that of the wild-type β1 (Fig. 4B).

It is noted that expression of Gγ2 in the Western blot is fairly variable in the example of Fig. 4. However, inspection

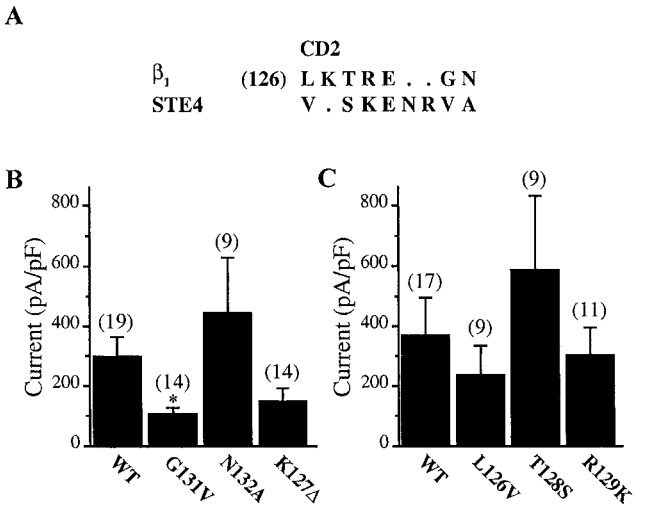

Fig. 3. A, sequence alignment of Gβ1 and yeast Gβ (STE4) in the CD2 region. B and C, effects of single amino acid mutations in the CD2 region on GIRK activation. Each nonconservative Gβ1 residue was mutated to its counterpart in STE4. Before the start of the experiments, we had planned to divide the mutants into 2 groups; for each group, we measured both the response to the wild type and to the mutants. B, G131V produced a much smaller GIRK current ($p < 0.05$), whereas N132A and K127Δ produced currents that were not significantly different from wild-type Gβ1. C, mutants L126V, T128S, and R129K produced currents not significantly different from wild-type Gβ1.

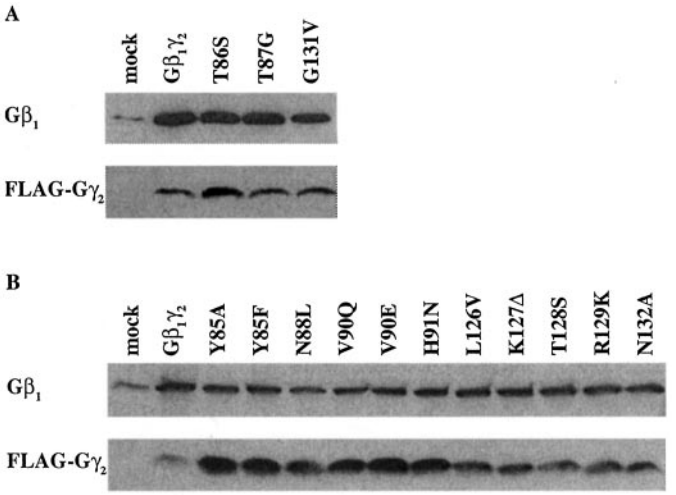

Fig. 4. Point mutations did not affect the expression level of Gβ1 in HEK293 cells. Cells were transfected with Gβ1 and FLAG-Gγ2. The first lanes (mock) show blots from cells transfected with empty vectors. A small amount of endogenous Gβ was detected in these cells. A, despite their reduced effectiveness in activating channels, mutants T86S, T87G, and G131V were expressed at a level comparable with that of the wild-type Gβ1. B, all other Gβ1 mutants in the D1 and CD2 regions, which did not significantly differ from wild type in current stimulation, were expressed as efficiently as wild type.

of all Western blots obtained in this experiment (five runs for Fig. 4A and six runs for Fig. 4B) indicated that there seemed to be no correlation between the Western blot expression of Gγ2 and the functional ability of various mutants and the wild type. It is thus unlikely that the functional defect of the mutants is caused by differences in the Gγ2 expression.

Activation of PLCβ2 by Gβ1 Mutants. Gβγ regulates not only GIRKs but also several other effectors including PLCβs. To test whether the Gβ1 mutants retain their ability to activate PLCβ, we transfected HEK293 cells with PLCβ2, Gβ1, and Gγ2 in either the absence or the presence of $G\alpha_o$. For each sample, the amount of inositol phosphates produced by the lithium treatment was normalized to the total amount of free inositol and inositol phosphates. As shown in Fig. 5, transfection of PLCβ2 alone resulted in the production of a small amount of inositol phosphates. The wild-type Gβ1γ2 caused a 7-fold increase in inositol phosphate production, which could be completely suppressed to the basal level by cotransfection of $G\alpha_o$. The bottom panel shows immunoblots of the samples from the same experiment. Without Gβ1γ2 sequestration by Gαo, the expression level of Gβ1 was more or less proportional to the amount of inositol phosphate produced. Taken together, the elevation of the inositol phosphate production represented the activation of PLCβ2 by Gβ1γ2 coexpressed in the cells. In conclusion, this experiment shows that despite their inefficiency at activating the K^+ channels, the Gβ1 mutants T86S, T87G, and G131V were as effective as wild type in stimulating PLCβ2 activity. In addition, the inhibitory effect of coexpression of $G\alpha_o$ on PLCβ2 activation by these mutants suggests that they can interact with $G\alpha_o$ in a way similar to wild-type Gβ1.

Albsoul-Younes et al. (2001) observed that the CD2 mutant, in which the Gβ1 segment near the outer strand of blade 2 was replaced with the corresponding STE4 sequence, was less potent in activating locus ceruleus GIRK channels and in stimulating the PLCβ2 activity. However, the present study showed that a CD2 region mutant, G131V, which was less effective in activating GIRK channels (Fig. 3B), was able to activate PLCβ2 as effectively as the wild-type Gβ1. This may mean that residues in the CD2 region other than Gly131 is involved with PLCβ2 activation.

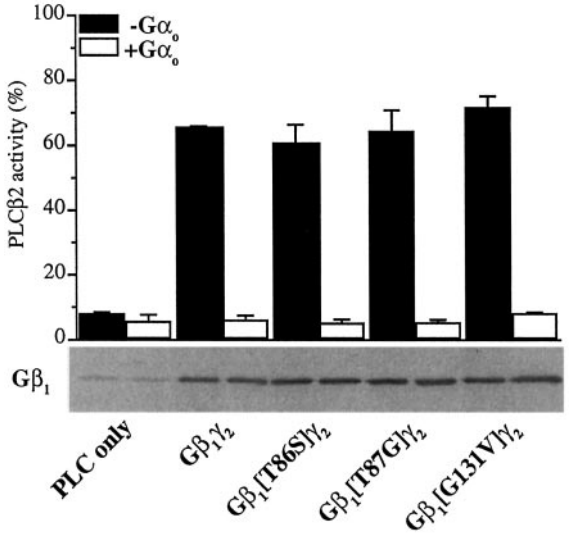

Fig. 5. Activation of PLCβ2 by Gβ1 mutants. HEK293 cells in 24-well plates were transfected with 0.2 μg of each kind of cDNA. In all cases, vector DNA was added to give a final DNA concentration of 0.8 μg/well. The activity of PLCβ2 was expressed as the ratio of the amount of inositol phosphates to the total amount of free inositol and inositol phosphates. The data shown are representatives of three experiments. The error bars indicate the S.E.M. of duplicate assays. ■, without $G\alpha_o$; □, with $G\alpha_o$. Bottom, immunoblots of triplicate samples in the same experiment.

Discussion

Interaction among Gα, Gβ, and GIRK. The signals initiated by Gβγ can be transmitted to downstream effectors (including GIRK channels) only if Gα is dissociated from Gβγ. This feature suggested that the interaction domain between Gβ and Gα is shared with the interaction domain between Gβ and effectors (Ford et al., 1998; Li et al., 1998). As to the interaction between Gβ and GIRK1/GIRK4, the results by Ford et al. (1998) indicate that six Gβ1 residues located on the Gβ/Gα interface could be the interaction sites between Gβ and GIRK (Fig. 6A, ○). Furthermore, Albsoul-Younes et al. (2001), using GIRK channel in brain neurons (presumably GIRK1/GIRK2), found that two more residues on Gβ1, Trp332, and Asp246, belong to this group (Fig. 6A, □).

Gβ5 is a particular type of Gβ subunit. Gβ5 *inhibits* the GIRK channel, whereas all other Gβs activate it (Lei et al., 2000). Mirshahi et al. (2002) have recently determined the Gβ residues that are responsible for this difference; these residues may be the interaction sites between Gβ and GIRK. Their results indicate that Ser67 and Thr128 of Gβ (Fig. 6A, ●) may be the location of Gβ/GIRK interaction. These sites are located outside the Gβ/Gα interaction domain.

Albsoul-Younes et al. (2001) used STE4 to search for the Gβ/GIRK interaction sites. STE4, being the member of the Gβ family that is most distantly related to mammalian Gβs, does not activate GIRK (Peng et al., 2000). Albsoul-Younes et al. (2001) constructed a set of chimera DNAs, in which the sequence at or near the outer β strand of each blade was replaced by the corresponding sequence of STE4. The mutant Gβ1γ2 proteins were purified and were tested on native GIRK channels in locus ceruleus neurons. Substitution of STE4 outer strands of blades 1 and 2 for the wild type resulted in impairment of GIRK channel activation, suggesting that some residues on the outermost sheets of blades 1 and blade 2 are the Gβ/GIRK interaction sites.

In the present study, we used genetically identified GIRK channels (GIRK1/GIRK2) instead of native GIRK channels in locus ceruleus neurons. By creating point mutations on the regions identified previously (Albsoul-Younes et al., 2001), we have localized the Gβ residues that are responsible for the functional impairment of the chimera between STE4 and Gβ1. Our results indicate that three residues on Gβ1 (Thr86, Thr87, and Gly131; Fig. 6A, ■) are putative interaction sites between Gβ1 and GIRK. It is noted that all three residues are located *outside* the Gβ/Gα contact points and they are all located at sites accessible from outside (Fig. 6B). Together with the work by Mirshahi et al. (2002), there are now five residues of this category (Fig. 6A, filled symbols), suggesting that the functional roles of these residues are not trivial. Interestingly, all five residues are located on the loops connecting the β strands; this arrangement is consistent with the notion that active sites of protein-protein interactions are often located on loops between β-strands.

In summary (Ford et al., 1998; Albsoul-Younes et al., 2001;

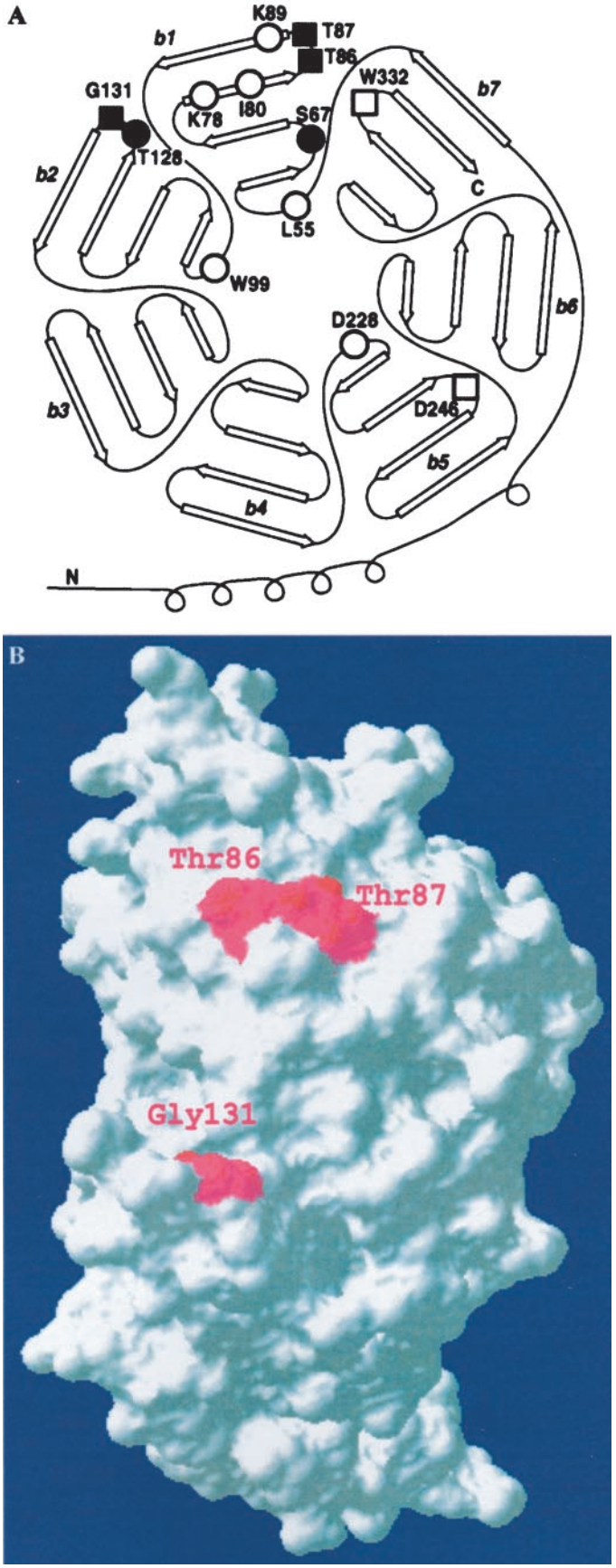

Fig. 6. Interaction sites on Gβ1 for GIRK channels. A, a diagram of Gβ1γ2 based on the published structures by Wall et al. (1995) and Lambright et al. (1996). The seven blades of the Gβ1 propeller were labeled as b1 to b7, following the designation of Wall et al. (1995). ○, eight Gβ1 residues belonging to both the Gβ/Gα interaction sites and the Gβ/GIRK interaction sites (Leu55, Lys78, Ile80, Lys89, Trp99, and Asp228), identified by Ford et al. (1998). □, Asp246 and Trp332 also belong to both the Gβ/Gα interaction sites and the Gβ/GIRK interaction site, described by Albsoul-Younes et al. (2001). Filled symbols belong to the Gβ/GIRK interaction sites outside Gβ/Gα interaction sites: Ser67 and Thr128 (●) were identified by Mirshahi et al. (2002), and Thr86, Thr87 and Gly131 (■) were identified in the present study. B, three-dimensional surface of Gβγ complex. The three Gβ1 residues (Thr86, Thr87, and Gly131; pink), which are putative interaction sites between Gβ1 and GIRK1/2, reside on the surface of the Gβ subunit. The figure was generated with the program Deepview/Swiss-pdb Viewer (Glaxo SmithKline R and D; version 3.7) using published coordinates of $G_{t\beta\gamma}$ (Sondek et al., 1996).

Mirshahi et al., 2002; present study), there are three domains that are involved in the interaction among Gβ, Gα, and GIRK: 1) a Gβ/GIRK interaction area outside the Gβ/Gα interaction site (Fig. 6A, filled symbols), 2) an area belonging both to the Gβ/Gα interaction sites and to the Gβ/GIRK interaction sites (Fig. 6A, open symbols), and 3) an area belonging to the Gβ/Gα interaction region but outside the Gβ/GIRK interaction sites (not shown). In other words, the Gβ/Gα interaction area and the Gβ/GIRK interaction area are not identical, but the two areas intersect.

Protein-Protein Interactions Involving Gβγ. Various signaling pathways from many receptors converge on G proteins. Convergence is caused by the simple fact that the same G proteins couple to many different receptors. The signals then diverge from the G proteins to multiple effectors such as GIRK, calcium channels, PLCβs, and adenylyl cyclases. The results from this study and others have revealed that these effectors (or any interacting proteins) associate with Gβγ through both the Gα-interacting surface and part of the outer-surface of the blade structure. It has also become clear that each of these proteins is using a specific part of the outer surface of Gβ. Certain mutations of the outer strands of blades 2, 6, and 7 impair PLCβ2 activation but not the regulation of adenylyl cyclases (Panchenko et al., 1998; Albsoul-Younes et al., 2001). Mutations of certain residues on the outer surface of blades 1 and 2 affected GIRK activation but not PLCβ2 or adenylyl cyclase type II activation (Albsoul-Younes et al., 2001; present study). Phosducin binds Gβ not only on the Gβ/Gα interaction site but also on the outer strands of blades 1 and 7. However, phosducin does not bind to the three putative Gβ/GIRK interaction sites described in this study (Thr86, Thr87, and Gly131; Fig. 6A) (Gaudet et al., 1996). It seems that Gβ is using its blade structure for the regulation of different nonhomologous effector molecules. This could be one of the mechanisms for achieving divergence of signaling.

On the other hand, certain residues on Gβ seem to play a more universal role for the protein-protein interaction. One of them is Trp332. Alanine mutation of the Trp332 residue in Gβ1 impaired the subunit's ability to activate GIRK1/GIRK2 (Fig. 1C) as well as the native GIRK channel in locus ceruleus (Albsoul-Younes et al., 2001). Recently, Nishida and MacKinnon (2002), based on their crystallographic study of N- and C-termini of GIRK and in analogy with the interaction sites between phosducin and Gβ (Gaudet et al., 1996), proposed the idea that the α-helix near the end of the GIRK C terminus may interact with Gβ at Trp332. It is known that not only is Trp332 an interaction site between Gβ and GIRK and between Gβ and phosducin but also between Gβ and Gα (Lam-

bright et al., 1996). In addition, Trp332 interacts with several effectors such as PLCβ2, PLCβ3, and adenylyl cyclase 2 (Ford et al., 1998; Li et al., 1998). In this respect, Trp332 seems to be a universally key residue for the protein-protein interactions involving Gβ.

One of the marked differences between Gβ/Gα association and Gβ/phosducin association is that the former association does not entail conformational changes of Gβ (Lambright et al., 1996), whereas the latter does (Gaudet et al., 1996; Loew et al., 1998). Because of certain similarities between the Gβ/phosducin association and the Gβ/GIRK association, the question arises of whether conformational changes of Gβ occur accompanying the Gβ/GIRK interaction. Crystallographic studies will answer this.

Acknowledgments

The cDNA of W332A was originally constructed in the laboratory of the late Dr. Eva Neer. Thanks are owed to Moritz Bünemann for suggestions on the culturing technique of HEK293 cells. We thank Tohru Yamada, Constance J. Jeffery, and Diana Arsenieva for help in generating the three-dimensional surface of Gβγ complex and John M Collins for suggestions in the writing.

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Address correspondence to: Tohru Kozasa, Department of Pharmacology, College of Medicine, University of Illinois at Chicago, 835 S. Wolcott Ave., Chicago, IL 60612. E-mail: tkozas@uic.edu