Interaction Sites of the G Protein β Subunit with Brain G Protein-coupled Inward Rectifier K+ Channel*

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G protein-coupled inward rectifier K+ channels (GIRK channels) are activated directly by the G protein βγ subunit. The crystal structure of the G protein βγ subunits reveals that the β subunit consists of an N-terminal α helix followed by a symmetrical seven-bladed propeller structure. Each blade is made up of four antiparallel β strands. The top surface of the propeller structure interacts with the Gα subunit. The outer surface of the βγ torus is largely made from outer β strands of the propeller. We analyzed the interaction between the β subunit and brain GIRK channels by mutating the outer surface of the βγ torus. Mutants of the outer surface of the β subunit were generated by replacing the sequences at the outer β strands of each blade with corresponding sequences of the yeast β subunit, STE4. The mutant β1γ2 subunits were expressed in and purified from Sf9 cells. They were applied to inside-out patches of cultured locus coeruleus neurons. The wild type β1γ2 induced robust GIRK channel activity with an EC50 of about 4 nM. Among the eight outer surface mutants tested, blade 1 and blade 2 mutants (D1 and CD2) were far less active than the wild type in stimulating GIRK channels. However, the ability of D1 and CD2 to regulate type I and type II adenyl cyclases was not very different from that of the wild type β1γ2. As to the activities to stimulate phospholipase C/β2, D1 was more potent and CD2 was less potent than the wild type β1γ2. Additionally we tested four β1 mutants in which mutated residues are located in the top Gαβ interacting surface. Among them, mutant W332A showed far less ability than the wild type to activate GIRK channels. These results suggest that the outer surface of blade 1 and blade 2 of the β subunit might specifically interact with GIRK and that the β subunit interacts with GIRK both over the outer surface and over the top Gα interacting surface.

Heterotrimeric G proteins transduce a variety of regulatory signals from a large number of heptahelical receptors to effectors such as adenyl cyclases, phosphodiesterases, phospholipases, and ion channels (1). Each G protein oligomer contains a guanine nucleotide binding α subunit and a high affinity dimer of β and γ subunits. The agonist-bound receptor activates the G proteins and generates GTP-bound α subunits and free βγ subunits. Both GTP α and βγ can regulate downstream effectors. The hydrolysis of GTP to GDP on α subunits leads to the reassociation of α and βγ subunits to form inactive heterotrimers.

The crystal structure of the βγ subunit reveals that the β subunit consists of an N-terminal α helix followed by a symmetrical seven-bladed propeller structure based on WD repeat sequences, repeating motif of about 40 amino acids (2, 3). Each blade consists of four antiparallel β sheets. The top surface of the propeller structure interacts with the α subunit. The bottom surface of the torus is the major site for interaction with the γ subunit. The outer surface of the torus is largely made up of the outer β strands of seven blades. βγ subunits directly regulate various effectors, including phospholipase Cβs (PLCβs), adenyl cyclases, and ion channels. Experiments using mutated β subunits have demonstrated that some of the amino acid residues, which are located at the interaction sites between Gα and -β subunits, are crucial for the interaction between βγ and the effectors, and each effector demonstrates its specific domain of interaction on the β subunit (4, 5). Further mutational analysis showed that the activation of PLCβ2 also involves residues in the outer strands of blades 2, 6, and 7. However, mutations of β subunits that affected PLCβ2 activity did not influence the interaction between βγ and type I or type II adenyl cyclase (6). These results suggest that βγ interacts with effectors through both the Gα binding surface and the outer surface of the propeller structure and that each effector is interacting with βγ using different regions of its outer surface.

The present study was undertaken to investigate the interaction sites of β1 with brain GIRK channels. We focused our investigation on the outer surface of the β1 subunit. We made sets of β1 mutants in which the amino acid residues located on or near the outermost β strand of each blade were replaced with the corresponding residues of the yeast β subunit (STE4), a most distantly related member of β from mammalian β subunits. In this study we show that mutating residues located on or near the outer strands of blade 1 and blade 2 disrupt GIRK activation by β1γ2. In contrast, the same mutations did not substantially affect the ability of β1γ2 to regulate adenyl cyclases, indicating that no global disruption of β1 occurred because of the mutations. It is possible that the sites of the β1

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†† The abbreviations used are: PLCβ, phospholipase Cβ; GIRK, G protein-coupled inward rectifier K+; ANOVA, analysis of variance; GTPγS, guanosine 5′-O-thiotriphosphate.
subunit in blade 1 and blade 2 could specifically be involved in brain GIRK activation.

**EXPERIMENTAL PROCEDURES**

**SF9 Cell Culture and Construction of Recombinant Baculoviruses—**SF9 cells were cultured in suspension in IPL-41 medium containing 1% Pluronic F68 and 10% heat-inactivated fetal bovine serum at 27 °C with constant shaking (150 rpm). The site-directed mutagenesis of βi cDNA was performed using the Mutagen Gene in vitro mutagenesis kit (Bio-Rad). The amino acid sequence of each mutation is shown in Fig. 3. The mutations were confirmed by sequencing the mutating region. Mutated βi cDNAs were subcloned into the pVL1392 transfer vector, and the resulting plasmids were cotransfected into SF9 cells with BacPac6 viral extract using an nickel-nitrilotriacetic acid column as described (8). The elute fractions from the nickel-nitrilotriacetic acid column were concentrated, and the buffer was exchanged into 20 mM NaHEPES (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, and 0.5% octyl glucoside with Centricron-30 (Amicon). Recombinant Gαg was purified from SF9 cells as described previously (8).

**Purification of G Protein Subunits from SF9 Cells—**SF9 cells (1.5 × 10^6/ml) were coinfectected with amplified recombinant baculoviruses encoding wild type or mutant βi, γ2, and His6-Gαg. Cells were harvested after 48 h, and recombinant βi protein was purified from the membrane extract using an nickel-nitrilotriacetic acid column as described (8). The elue fractions from the nickel-nitrilotriacetic acid column were concentrated, and the buffer was exchanged into 20 mM NaHEPES (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, and 0.5% octyl glucoside with Centricron-30 (Amicon). Recombinant Gαg was purified from SF9 cells as described previously (8).

**Cultures of Locus Coeruleus Neurons—**Cultured neurons from the locus coeruleus were made from 2–4-day-old postnatal Long-Evans rats (Charles River Breeding Laboratories). The culture methods were described previously (9, 10). Rats were anesthetized with ether, their brains were decapitated, and the brain slices were made from isolated brainstems using a Vibratome (Leica). Locus coeruleus was visually identified under a dissecting microscope and excised out. The excised pieces were incubated in a papain solution, dissociated by trituration, and cultured. The culture medium contained a minimum essential medium with Earle’s salts (Life Technologies, Inc., catalog no. 11430–030), modified by adding t-glucose (5 mg/ml), NaHCO3 (3.7 mg/ml), and t-glutamine (0.292 mg/ml). The medium was supplemented by heat-inactivated rat serum (2 or 5%, prepared in our laboratory), L-ascorbic acid (10 mg/ml). The medium was supplemented by heat-inactivated rat serum (2 or 5%, prepared in our laboratory), and 5 mM HEPES-NaOH (pH 7.4). The GDP-containing cytoplasmic solution (the bathing solution and the patch electrode solution).

**Electrophysiology—**Electrophysiological experiments were performed with the inside-out patch clamp technique (11, 12). The patch pipettes were made from thoroughly washed glass capillaries. The patch pipette solution (external solution) contained 156 mM KCl (or potassium glutamate), 2.4 mM CaCl2, 1.3 mM MgCl2, 0.5 μM tetrodotoxin, and 5 mM HEPES-NaOH (pH 7.4). The GDP-containing cytoplasmic side solution (the bathing solution) contained 141 mM potassium glutamate, 8.7 mM NaCl, 5 mM EGTA-KOH, 1 mM MgCl2, 2 mM Na2ATP, 0.1 mM GDP, 5 mM HEPES-KOH, and ~5.5 mM KOH (pH 7.2). Membrane potential was corrected for the liquid junction potential between the bathing solution and the patch electrode solution.

**RESULTS**

To measure adenylyl cyclase activity, purifi ed βγ subunits were reconstituted with 10 μg of membranes from SF9 cells expressing type I or type II adenylyl cyclase for 3 min at 30 °C in a final volume of 20 μl. Assays were then performed for 7 min at 30 °C in a total volume of 50 μl containing 4 mM MgCl2 and 0.2% octyl glucoside as described (4).

**Activation of GIRK Channels by βγ Subunits in Locus Coeruleus Neurons—**When a gigaseal was formed in the on-cell mode in locus coeruleus neurons, some channel activity was usually observed. Upon making an inside-out patch, this activity started to subside, reaching a low level within a minute (presumably because the intracellular GTP was washed away in exchange of the GDP-containing solution). We then applied solution was recorded for a few minutes. The solution in the bath (−0.1−0.17 ml) was then exchanged with 0.5 ml of various kinds of βγ subunits dissolved in the same GDP-containing bathing solution. The exchange was done manually by using a pipetter wrapped up with a grounded aluminum foil. The degree of solution exchange was tested by measuring the change in channel activity as an indicator; the test showed that this manual method resulted in the replacement of about 86% of the original solution (using the bath volume of 0.15 ml).

When the inside-out patch configuration was established, usually the patch produced a very infrequent activity of GIRK channels (basal GIRK activity) and an activity of channels of small amplitude (of unknown origin) in the GDP-containing bathing solution. In some patches, the occurrence of large flickering channels (about 100 picowatts with [K+], of 156 mm), whose activity was not dependent on GTP, was observed (12). Frequent occurrence of these large background channels hindered the analysis of GIRK channel, and thus such patches were not included in our sample. Occasionally, instead of an inside-out patch, vesicle formation occurred. (The vesicle formation was inferred by the appearance of a current drooping because of the existence of membrane resistance and capacity on the opposite side of the patch.) (11). These patches were not included. Sometimes we observed rather vigorous basal activity of GIRK-like channels in the GDP-containing bathing solution before the application of βγ. This basal activity might have originated from the local presence of overexpressed βγ or from the basal activity of the receptor. Because the purpose is not to analyze the GIRK channel activation induced by the application of exogenous βγ proteins, we did not pursue the investigation of the patches with a high frequency basal activity (more than about once per second).

Stock solutions of βγ proteins were diluted with the GDP-containing bathing solution to a final concentration of 1–100 nM. At 10 nM Gβγ, the solution contained the following buffer/detergent: 0.0033% octyl glucoside, 0.066 mM HEPES, 0.0033 mM EDTA, and 0.24–2.1 μM dithiothreitol. Experiments were done with a bath temperature of ~21 °C.

**Statistical Treatment of Electrophysiological Data—**The distribution pattern of NPo was almost always non-Gaussian; this can be inferred from substantial discrepancies between the mean and the median values (see e.g. 44 and 6). This non-Gaussian distribution suggests the possible existence of more than one type of channel (e.g. solitary channels and aggregates of channels). Because the mean value in our samples is influenced greatly by a small number of patches with a large NPo, the median was a more appropriate parameter to represent the channel activity of a group. Also, statistical comparisons of NPo were done, unless otherwise noted, by using the nonparametric statistics (the Kruskal-Wallis and Mann-Whitney U test, see e.g. 44, 45 and a postest using the Mann-Whitney with Bonferroni adjustment).

We used two different types of wild type β1γ2 with and without hexahistidine tagging at the N terminus of β1. The median value of NPo for the channel activated by the hexahistidine-tagged wild type β1γ2 was 0.109 (n = 47), and the median value of NPo for nontagged β1γ2 was 0.099 (n = 35) (difference not significant; p > 0.5). We will refer to these two types simply as β1γ2.

**In Vitro Assays for Phospholipase C and Adenylyl Cyclase Activity—**Phospholipase C activity was measured using sonicated micelles containing 50 μM phosphatidylinositol 4,5-bisphosphate, 500 μM phosphatidylethanolamine, and linositol-2'-H3phosphatidylinositol 4,5-bisphosphate (PerkinElmer Life Sciences) (2500 cpm/assay) in a solution containing 50 mM NaHEPES (pH 7.5), 0.42 mM EDTA, 3 mM EGTA, 2 mM MgCl2, 1.7 mM CaCl2, 42 mM NaCl, 47 mM KCl, 4 μg GDP, 0.125 mg/ml bovine serum albumin, 1 mM dithiothreitol, and 0.35% octyl glucoside with 0.1 mM PLCβ2 and the indicated amount of βγ subunit. The mixture was incubated at 30 °C for 8 min, and the amount of IP3 generated was quantitated as described (4).

To measure adenylyl cyclase activity, purified βγ subunits were reconstituted with 10 μg of membranes from SF9 cells expressing type I or type II adenylyl cyclase for 3 min at 30 °C in a final volume of 20 μl. Assays were then performed for 7 min at 30 °C in a total volume of 50 μl containing 4 mM MgCl2 and 0.2% octyl glucoside as described (4).
wild type $\beta_1\gamma_2$ (10 nM), which induced, after a latency, vigorous channel activity (Fig. 1A) (13, 14). The channels showed typical GIRK-like characteristics (12, 14) with a chord conductance of 30–35 picosiemens, exhibiting a mixture of short openings, the latter sometimes showing bursts (Fig. 3); 100%) was 0.265. The number of patches was 7 at 30 nM, 2 at 10 nM, 7 at 3 nM, 5 at 1 nM, and 5 at 0.3 nM.

10 nM Is an Almost Saturated Concentration for $\beta_1\gamma_2$—Fig. 2B shows a concentration-response relation in wild type $\beta_1\gamma_2$. Values of $NP_0$ over 5 to 9 min after the introduction of $\beta_1\gamma_2$ were plotted. The wild type $\beta_1\gamma_2$ activated the brain GIRK with an $EC_{50}$ of about 4 nM, and the activation almost saturated with 10 nM. These results are in approximate agreement with previous studies on cardiac and cloned GIRK channels (17–20).

$\beta_2$ Mutations at Outer Blades of K⁺ Channel—Within the $\beta$ subunit family, the STE4 gene product of Saccharomyces cerevisiae is most distantly related to mammalian $\beta$ subunits. So far, no evidence has been presented to suggest the regulation of adenyl cyclase, PLC, or K⁺ channel activity by yeast $\beta$ subunits. Therefore, we suspected that exchanges of the effector-interacting domains of the $\beta$ subunit with the correspond-

FIG. 1. Effects of wild type $\beta_1\gamma_2$ and mutant D1 on GIRK channels from locus coeruleus neurons; single channel recordings using the inside-out mode. A, the time course of GIRK channel activation ($NP_0$) induced by wild type $\beta_1\gamma_2$. Each circle represents the $NP_0$ every 30 s. The thick, solid horizontal line indicates the period when the GDP-containing control solution (Ctr) was exchanged with wild type (Wt) $\beta_1\gamma_2$ (10 nM)-containing solution. About 2 min later, the $NP_0$ started to increase. The upper left panel (a) shows a segment of the record during the control period (Ctr). Openings of GIRK channels were observed very infrequently; the record was taken 120 s after the beginning of the record (see the main graph). The upper right panel (b) shows that the application of wild type $\beta_1\gamma_2$ (Wt) produced robust GIRK channel activity. The record was taken 501 s after the start of the record. The downward direction corresponds to inward currents. B, lack of activation of GIRK channels by the application of D1 (10 nM). The thick solid horizontal line indicates the period when the control cytoplasmic side solution (Ctr) was exchanged with a solution containing 10 nM D1. The upper left panel (a) shows a record segment during the control period (Ctr); the record was taken 159 s after the start of the record. The upper right panel shows a record (b) after the application of D1 (10 nM) (465 s after the start of the record). In both A and B, the holding potential was −101 mV, and the patch pipette contained the 156 mM KCl solution.

FIG. 2. A, current-voltage relation of GIRK channels from locus coeruleus neurons; single channel recordings using the inside-out mode. The channel activity was induced by 10 nM wild type $\beta_1\gamma_2$. The solid line is the fit of the data to a second order polynomial. Five patches (represented by different symbols) were used. The inset records show single channel currents at different membrane potentials. The experiment was done in the 156 mM potassium gluconate pipette solution. B, dose-response relationship of wild type $\beta_1\gamma_2$. For each patch, two or three different concentrations (including the standard concentration, 10 nM) of wild type $\beta_1\gamma_2$ were applied in ascending order. The channel activity was determined by averaging $NP_0$ during the 5–9 min of introducing a new concentration of $\beta_1\gamma_2$ (except that values at zero concentration were determined by $NP_0$ during 1 min before introducing $\beta_1\gamma_2$). For each patch, the activity was normalized to the $NP_0$ value at the standard concentration (10 nM). The mean $NP_0$ at the standard concentration (solid square; 100%) was 0.266. The vertical lines are S.E. The continuous line is drawn by fitting to a logistic equation using $EC_{50} = 3.75$ nM and Hill’s coefficient $= 2.03$. The number of patches was 7 at 30 nM, 22 at 10 nM, 7 at 3 nM, 5 at 1 nM, and 5 at 0.3 nM. 

To test the hypothesis that regions on the side surface of the $\beta\gamma$ complex are important for the regulation of effectors, we mutated residues of an outer strand of each blade into the corresponding sequence of STE4 (Fig. 3). Each $\beta\gamma$ mutant was coexpressed with His₆-Gα₁₄ and γ₂ in Sf9 cells, and mutant $\beta\gamma$ subunits were purified as described under “Experimental Procedures.”

First, we surveyed all these outer blade mutants (eight mutants altogether) for their ability to activate GIRK channels.
We used β1γ2 mutants at a concentration of 10 nm, which is, for the wild type β1γ2, an almost saturating dose for activating the K+ channel (Fig. 2B). The variability of NP, among different patches was quite large (see “Experimental Procedures”) (Fig. 4A). Nevertheless, the mutant D1 clearly showed far less ability to activate the GIRK channel than did the wild type (p < 0.001) (Figs. 1B and 4A). The mutant CD2 was also significantly less effective in activating the channels compared with the wild type (p < 0.05). Mainly because of the large variation of NP, we could not obtain significant differences in NP, between the wild type and each of the other mutants (D2F, D2R, D3, D4, D6, and D7) (Fig. 4A). We, therefore, focused on D1 and CD2 mutants and tested a higher concentration for their ability to activate GIRK channels. Even at 100 nM, both D1 and CD2 mutants significantly less effective in activating the channels compared with the wild type (p < 0.05). Mainly because of the large variation of NP, we could not obtain significant differences in NP, between the wild type and each of the other mutants (D2F, D2R, D3, D4, D6, and D7) (Fig. 4A). We, therefore, focused on D1 and CD2 mutants and tested a higher concentration for their ability to activate GIRK channels. Even at 100 nM, both D1 and CD2 produced only a small amount of channel activity. Comparison of the channel responses by D1 and CD2 with the wild type dose-response curve clearly indicates the impaired ability of these two mutants (CD1 and CD2) to activate the K+ channel (Fig. 4B).

β1 Mutations at Outer Blades: Adenylyl Cyclases and PLCβ2—Despite the impaired ability of D1 and CD2 to activate GIRK channels, these two mutants were as active as the wild type to stimulate type II adenylyl cyclase (Fig. 5A). They could also inhibit type I adenylyl cyclase, similarly to wild type β1γ2 (data not shown). Both D1 and CD2 were capable of stimulating PLCβ2. The mutant D1 was, however, more active and the mutant CD2 was less active than the wild type within the concentration range of the assays (Fig. 5B).

Effects of Detergent—We also tested whether channel activities were affected by the detergent in which the protein was dissolved. We tested the buffer with 0.033% octyl glucoside, 0.66 mM HEPES, 0.033 mM EDTA, and 6–17 μM dithiothreitol, corresponding to those used for 100 nm β1γ2. This buffer alone did not induce channel activity during ~10 min of application (mean NP; 0.000189 before application; 0.000232 after application; n = 5; p > 0.7; paired t test). We also compared the
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**Fig. 5.** Activation of type II adenylyl cyclase and PLCβ2 by purified mutant βγ subunits. A, the indicated amount of each βγ mutant was reconstituted with 10 μg of Sf9 cell membrane expressing type II adenylyl cyclase in the presence of 100 nM GTPγS-Gsa. Adenylyl cyclase activity was measured as described under “Experimental Procedures.” B, the indicated amount of each βγ mutant was reconstituted with 0.1 nM PLCβ2, and the synthesis of IP3 was measured over 8 min at 30 °C as described under “Experimental Procedures.” The data of each panel show the average of duplicate determinations from a single experiment that is representative of three such experiments. The vertical bars indicate S.E.



**Fig. 6.** Effect of mutating some of the Go/β interaction sites on GIRK activity in locus coeruleus neurons. The thick horizontal lines represent the median values, and the heights of the columns represent the mean values of NPo. The vertical lines represent S.E. The number in parentheses indicates the number of patches. For each patch, the channel activity was determined by averaging NPo over 5–9 min after introducing various types of βγγ2 (wild-type and mutants). The mean basal activity (NPo) before applying various types of βγγ2 of this sample was 0.0057 (n = 69). The 156 mM RCI pipette solution was used. Comparison with the wild type: ***, p < 0.001 (nonparametric ANOVA and posttest).

**DISCUSSION**

GIRK channels are activated directly by the G protein βγ subunit. The interaction sites of βγ with GIRK have been investigated on the Go interacting surface of the βγ subunit (5). In the present study, we have demonstrated that regions outside of the Go/β interaction surface of the β subunit also participate in the interaction with GIRK channels. We characterized the interaction of β with brain GIRK by using β1 mutants on the outer strands of the seven-bladed β-propeller structure. Mutations of certain residues on the outer strands of blade 1 (D1) and blade 2 (CD2) resulted in the severe disruption of their ability to activate GIRK channels. However, these mutants could regulate adenylyl cyclases similarly to the wild type, suggesting that the mutations of the D1 and CD2 areas did not produce a global disruption of β1 structure. Thus, the results suggest that the mutated residues on the side of blade 1 and blade 2 of the βγ torus might be specifically involved in the regulation of GIRK channels.

It should be noted that in this study, we have concentrated on analyzing mutations that produced a large functional deterioration in the K+ channel activation. This study does not exclude the possibility that other mutants may have a moderate defect in their ability to interact with K+ channels.

It was previously shown that the activation of PLCβ2 involves the outer strands of blades 2, 6, and 7 (6). Interestingly, CD2 also showed a defect in its ability to activate PLCβ2. The results further support the importance of blade 2 for the interaction with PLCβ2. As shown in Fig. 5, D1 was 2–3-fold more potent than the wild type in its ability to stimulate PLCβ2. The exact reason for this difference is currently unclear.

Our results on β mutations over the Go/β interaction surface indicate that W332A showed far less ability to activate GIRK channels. Although only one concentration (10 nM) of β1γ2 was tested, we observed a very large difference in their activity between W332A and the wild type. Because 10 nM is an almost saturating dose for the wild type, this result suggests that W332A is much less active than the wild type in its ability to stimulate GIRK channels. Thus, β/γ interaction sites partially overlap with the Go/β interaction sites, but they are not identical. This is similar to the case of β subunit interaction with PLCβ2 or adenylyl cyclase (4, 5).

It is known that βγ can interact with effectors only if Go is dissociated from βγ. X-ray crystallographic studies have shown that the β subunit does not undergo conformational changes when it is dissociated from Go (3). It has been demonstrated that GDP-bound Go could sever the βγ-GIRK association quickly (22, 23). Because the interaction sites of GIRK overlap with the Go interaction surface on Gβγ, this effect of Go could be explained by a simple spatial (three-dimensional) competition on the β subunit between Go and GIRK (4). It is also possible that the association of GIRK to βγ induces a conformational change of the β subunit, and this change would favor the binding of βγ and GIRK. Conformational changes of the βγ subunit are demonstrated in the complex of βγ with phosducin (24). The bind-
ing of phosducin to the \( \beta y \) subunit produces a distinct conformational change in blade 6 and blade 7 of the \( \beta \) subunit. The phosphorylation of phosducin on Ser-73 reduces its affinity for \( \beta y \) and the released \( \beta y \) subunit and then switches back to the conformation of free \( \beta y \) or that of the heterotrimer (24). In the case of \( \beta y \) complexed with GIRK, when \( \text{G}_{\alpha i} \) interacts with a certain region of \( \text{G}_{\alpha i} / \beta \) interaction sites, the conformation of \( \beta y \) could return to the resting state (before GIRK was attached), and this could decrease the affinity of GIRK with \( \beta y \). The determination of the structure of \( \beta y \) complexed with GIRK will be necessary to answer this question.

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