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Interaction of $G\alpha_q$ and Kir3, G Protein-Coupled Inwardly Rectifying Potassium Channels

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ABSTRACT

Activation of substance P receptors, which are coupled to $G\alpha_q$, inhibits the Kir3.1/3.2 channels, resulting in neuronal excitation. We have shown previously that this channel inactivation is not caused by reduction of the phosphatidylinositol 4,5-bisphosphate level in membrane. Moreover, $G\alpha_q$ immunoprecipitates with Kir3.2 (*J Physiol* **564:**489–500, 2005), suggesting that $G\alpha_q$ interacts with Kir3.2. Positive immunoprecipitation, however, does not necessarily indicate direct interaction between the two proteins. Here, the glutathione transferase pull-down assay was used to investigate interaction between $G\alpha_q$ and the K^+ channels. We found that $G\alpha_q$ interacted with N termini of Kir3.1, Kir3.2, and Kir3.4. However, $G\alpha_q$ did not interact with the C terminus of any Kir3 or with the C or N terminus of Kir2.1. TRPC6 is regulated by the signal initiated by $G\alpha_q$. Immunoprecipitation, however, showed that $G\alpha_q$ did not interact with TRPC6. Thus, the interaction between $G\alpha_q$ and the Kir3 N terminus is quite specific. This interaction occurred in the presence of GDP or GDP-AlF_4^-. The $G\alpha_q$ binding could take place somewhere between residues 51 to 90 of Kir3.2; perhaps the segment between 81 to 90 residues is crucial. $G\beta\gamma$, which is known to bind to N terminus of Kir3, did not compete with $G\alpha_q$ for the binding, suggesting that these two binding regions are different. These findings agree with the hypothesis (*J Physiol* **564:**489–500, 2005) that the signal to inactivate the Kir3 channel could be mainly transmitted directly from $G\alpha_q$ to Kir3.

Substance P (SP), an undecapeptide of the tachykinin family discovered by Chang and Leeman (1970), has been shown to excite various neurons. We have been investigating SP as a model excitatory transmitter. One of the mechanisms for neuronal excitation is inactivation of inward rectifier K^+ channels (Stanfield et al., 1985; Velimirovic et al., 1995), including G protein-coupled inwardly rectifying K^+ channels (GIRK, Kir3) (Dascal et al., 1993; Kubo et al., 1993).

The mechanism of the Kir3 activation, which leads to neuronal inhibition, is well clarified. Logothetis et al. (1987) discovered that the Kir3 channel is activated by $G\beta\gamma$ subunits. Later, several groups of investigators (Huang et al., 1995; Inanobe et al., 1995; Krapivinsky et al., 1995b; Kunkel and Peralta, 1995) found that $G\beta\gamma$ binds directly to Kir3. Furthermore, Slesinger et al. (1995) determined that the activation signal is transmitted through this direct interaction of $G\beta\gamma$ and Kir3.

Contrary to the situation of Kir3 activation, the signal transduction mechanism of Kir3 inactivation is poorly understood. It has been shown that $G\alpha_q$ is involved in the signaling of Kir3 inactivation (Leaney et al., 2001; Lei et al., 2001; Koike-Tani et al., 2005). Beyond this, not much is clarified. A prevailing theory attributes the Kir3 inactivation to lowering of the PIP_2 level in the membrane (Kobrinsky et al., 2000; Cho et al., 2001; Lei et al., 2001) or to the PKC-induced phosphorylation (Sharon et al., 1997; Mao et al., 2004). However, in a previous article (Koike-Tani et al., 2005), we presented evidence that the SP-induced inactivation of Kir3 channels cannot be explained by decline of the PIP_2 level. Increasing the Kir3 affinity to PIP_2 by mutation did not change the SP-induced Kir3 inactivation. Moreover, unlike the results using *Xenopus laevis* oocytes (Sharon et al., 1997; Mao et al., 2004), the role of PKC in the Kir3 inactivation is not considerable in mammalian heterologous systems (Lei et al., 2001; Koike-Tani et al., 2005).

One possible mechanism of signal transduction from $G\alpha_q$ to

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ABBREVIATIONS: SP, substance P; GIRK, G protein-coupled inwardly rectifying K^+ channels; GST, glutathione transferase; HEK, human embryonic kidney; PIP_2, phosphatidylinositol 4,5-bisphosphate; RGS, regulator of G protein signaling; GRK2-RH, RGS homology domain of G protein-coupled receptor kinase 2.

the Kir3 inactivation is the direct interaction between $G\alpha_q$ and Kir3. Previously, Koike-Tani et al. (2005) have shown that $G\alpha_q$ coprecipitates with Kir3.2, but not with Kir2.1 or Kir2.2. However, coimmunopreciptation cannot determine whether $G\alpha_q$ and Kir3 interact directly without intermediate elements, because the lysates include various cell components. In the present experiments, we used glutathione transferase (GST) pull-down assays to observe direct interaction between $G\alpha_q$ and Kir3 and to localize the sites of interaction.

Materials and Methods

Culture and Transfection of HEK293A Cells. Human embryonic kidney 293A (HEK293A) cells were purchased from Qbiogene (Irvine, CA). They were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C with 10% CO_2. Transfection was conducted with Effectene Transfection Reagent (QIAGEN, Valencia, CA). Cells on a 6-cm dish were transfected with the following cDNAs: 0.15 μg of Kir3.1, 0.15 μg of Kir3.2 (or Kir3.4), 0.2 μg of SP receptor, 0.3 μg of $G\beta_1$, 0.3 μg of $G\gamma_2$ and 0.1 μg of green fluorescent protein (pEGFP-N1; Clontech, Mountain View, CA). Kir3.1, Kir3.2, and Kir3.4 cDNAs were from the rat, and $G\beta_1$ and $G\gamma_2$ cDNAs were of bovine origin. SP receptor cDNA (Takeda et al., 1991) was human. The total amount of cDNAs was kept at 1.6 μg by adjusting the quantity of the empty vector. All cDNAs used were subcloned into pCMV5 (Andersson et al., 1989). One day after the transfection, cultures were replated on 3.5-cm dishes. Each of the dishes has a center well (~1.2 cm in diameter) coated with rat-tail collagen (Roche Molecular Biochemicals, Indianapolis, IN). Electrophysiological experiments were performed 2 days after the re-plating.

Electrophysiology. The whole-cell version of patch clamp was used. The external solution contained 141 mM NaCl, 10 mM KCl, 2.4 mM $CaCl_2$, 1.3 mM $MgCl_2$, 11 mM D-glucose, 5 mM HEPES-NaOH, and 0.0005 mM tetrodotoxin, pH 7.4. Patch pipette solution contained 141 mM potassium *d*-gluconate, 10 mM NaCl, 5 mM HEPES-KOH, 0.5 mM EGTA-KOH, 0.1 mM $CaCl_2$, 4 mM $MgCl_2$, 3 mM Na_2ATP, and 0.2 mM GTP, pH 7.2. SP (0.5 μM; Peptide International, Louisville, KY) was applied through a sewer pipe system (ALA Scientific Instruments, Westbury, NY). Holding potential was −79 mV. Experiments were performed at room temperature. Statistical values are expressed as mean ± S.E.M.

Protein Purification. To express GST-fusion proteins of the N and C termini of Kirs, each of the Kir cDNAs was constructed in pGEX-2T vector (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The Kir cDNAs used were from the rat, and GRK2 cDNA was of bovine origin. *Escherichia coli* strain JM109 or BL21 was transformed by pGEX-2T vector harboring cDNA interested. To enhance the solubility of some GST-fusion proteins, BL21(DE3) strain was cotransformed by pT-Trx (Yasukawa et al., 1995). Production of protein was induced by 0.1 to 0.3 mM isopropyl β-D-thiogalactoside at A_{600} of 0.6, and then the bacteria were cultured for another 45 min to 16 h at 26–30°C. The bacterial pellet was suspended in 1/20 volume of lysis buffer with the following composition: 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 20 mM 2-melcaptoethanol, 0.2 mg/ml lysozyme, and proteinase inhibitors (16 μg/ml phenylmethylsulfonyl fluoride, 16 μg/ml N-*p*-Tosyl-L-phenylalanine chloromethyl ketone, 16 μg/ml N_α-tosyl-L-lysine chloromethyl ketone, 3.2 μg/ml leupeptin, and 3.2 μg/ml lima bean trypsin inhibitor). The pellet was disrupted by sonication. After centrifugation at 100,000*g* for 30 min after incubation for 20 min at 4°C, the supernatant was loaded onto a glutathione Sepharose 4B (GE Healthcare) column. The column was washed with wash buffer N (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 20 mM 2-melcaptoethanol, and 400 mM NaCl) and then with wash buffer G (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 20 mM 2-mercaptoethanol, and 10% glycerol). GST-fusion protein was eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM 2-mercaptoethanol, 140 mM NaCl, 10% glycerol, and 5–100 mM glutathione). Peak fractions were exchanged with stock buffer (50 mM HEPES-NaOH, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, and 10% glycerol). The recombinant proteins of $G\alpha_q$, $G\beta_1$, and hexahistidine-tagged $G\gamma_2$ expressed by the Sf9-bacurovirus system were purified as described before (Kozasa, 2004).

GST Pull-Down Assays. To perform the pull-down assay with $G\beta_1\gamma_2$, 1 μg of GST-fusion protein (80–200 nM) and 40 nM $G\beta\gamma$ were incubated with glutathione Sepharose 4B at 4°C for 1 h in 200 μl of binding buffer A (PBS containing 2 mM EDTA, 5 mM 2-mercaptoethanol, 0.02% $C_{12}E_{10}$). After incubation, the resin was washed three times with 1 ml of binding buffer A, boiled with sample buffer, and then subjected to Western blotting. For the assay with $G\alpha_q$, 0.5 μg of GST-fusion proteins were incubated with 50 nM $G\alpha_q$ in binding buffer B (PBS containing 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.02% $C_{12}E_{10}$, and 10 μM GDP with or without AlF_4^- [30 μM $AlCl_3$ and 5 mM NaF]). In competitive pull-down assays, binding buffer B with 1 mM $MgCl_2$ was used.

Precipitated proteins were boiled in a sample buffer (50 mM Tris-HCl at pH 6.8, 1% SDS, 2% 2-mercaptoethanol, 0.008% bromphenol blue, and 8% glycerol) and subjected to Western blotting using antibodies described below. The antibodies bound to proteins of interest on nitrocellulose membranes were visualized by the enhanced chemiluminescence detection system (Pierce, Rockford, IL) using anti-mouse or anti-rabbit Ig antibodies conjugated with horseradish peroxidase as secondary antibodies (GE Healthcare). Anti-Gβ (T-20) and anti $G\alpha_{q/11}$ (C-19) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunoprecipitation. HEK293A cells were transfected with plasmid cDNAs of $G\alpha_q$ and TRPC6A or Myc-Kir3.2 by *Trans* IT-LT1 (Invitrogen). The amount of cDNA used for transfection was adjusted by adding pCMV5 to 20 μg total per 10-cm dish of the cells. After 48 h of the transfection, the cells were harvested from the dish and lysed in 550 μl of lysis buffer on ice for 20 min. The lysis buffer consisted of 20 mM HEPES-NaOH at pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 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-X100, 10% glycerol, and 10 μM GDP either with or without AlF_4^- (30 μM $AlCl_3$ and 5 mM NaF). The samples were centrifuged at 15,000*g* for 20 min at 4°C. The supernatants were subjected to immunoprecipitation. The cell lysates (150 μl) were incubated at 4°C for 1 h with antibody (0.3–0.5 μg) and protein A-Sepharose (Amersham Biosciences). Precipitated immune complexes with protein A-Sepharose were washed three times with a wash buffer (20 mM HEPES-NaOH at pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton-X100, and 10 μM GDP), either with or without AlF_4^-. Immunoprecipitated proteins were boiled in sample buffer and subjected to Western blotting. Mouse monoclonal antibody 9E10 against c-Myc epitope was purchased from Covance Research Products (Berkeley, CA). Anti-TRPC6 antibody was from Alomone Labs (Jerusalem, Israel).

Results

SP Inactivates the Kir3.1/3.4 Channels. Activation of the SP receptor inhibits the Kir3.1/3.2 channels with a half-time of 10 to 15 s (Koike-Tani et al., 2005). The Kir3.1/3.2 channels are abundant in brain neurons (Karschin et al., 1996; Liao et al., 1996; Kawano et al., 2004). In contrast, the Kir3.1/3.4 channels are predominantly expressed in cardiac myocytes and play a critical role in regulating heart rate (Krapivinsky et al., 1995a).

We investigated whether the Kir3.1/3.4 channels are inhibited by SP with a time course similar to that for the Kir3.1/3.2 channels. The SP receptor, Kir3.1, Kir3.4 (or Kir3.2), $G\beta_1$, $G\gamma_2$, and GFP were coexpressed in HEK293A

cells. In those cells, because of the expression of $G\beta_1\gamma_2$, the Kir3 channels were active from the beginning.

Application of SP to cells expressing Kir3.1/3.2 (Fig. 1A) as well as to cells expressing Kir3.1/3.4 (Fig. 1B) produced channel inactivation. The half-time ($T_{0.5}$) of the inactivation was ~16 s for Kir3.1/3.2 and ~19 s for Kir3.1/3.4 channels (no significant difference). These results indicate that both Kir3.1/3.2 and Kir3.1/3.4 respond to the SP application in a similar manner (Fig. 1C).

$G\alpha_q$ Binds to the N terminus of Kir3. The N and C termini of Kir 2.1, Kir3.1, Kir3.2, and Kir3.4 were purified as GST-fusion proteins (Fig. 2D). $G\alpha_q$ and $G\beta_1\gamma_2$ proteins were obtained using the Sf9-bacurovirus system as described previously (Kozasa, 2004). The pull-down assays showed that $G\alpha_q$ coprecipitated with the N termini of Kir3.1, Kir3.2, and Kir3.4 but not with the C termini of any Kir3 channels tested (Fig. 2A). (Note: residues 2–96 of the N terminus would virtually correspond to the whole Kir3.2 N terminus. We therefore presented this domain as N-Kir3.2 in Figs. 2 and 5). The $G\alpha_q$ binding to the N termini of Kir3.1, Kir3.2, or Kir3.4 occurred in the presence of GDP as well as in the presence of GDP-AlF_4^-. $G\alpha_q$ did not bind to either the N or the C terminus of Kir2.1. The experiments in Fig. 2A were performed using $G\alpha_q$ at 50 nM. We performed additional assays at lower $G\alpha_q$ concentrations (2–20 nM) to examine the possible affinity difference in the presence or absence of AlF_4^- toward the Kir3.2 N terminus. As shown in Fig. 2B, $G\alpha_q$, at these low concentrations, interacted with the Kir3.2 N terminus, and the interaction was still independent of the presence of AlF_4^-.

As a positive control, the RGS homology domain (residues 1–178) of G protein-coupled receptor kinase 2 (GRK2-RH) was used (Fig. 2, A and B). The binding between GRK2-RH and $G\alpha_q$ occurred in the presence of AlF_4^-, in confirmation of Carman et al. (1999).

We also performed pull-down assays of $G\beta_1\gamma_2$ with these GST-fusion proteins. $G\beta_1\gamma_2$ bound to both N and C termini of Kir3.1, 3.2, and 3.4. However, practically no binding was observed between $G\beta_1\gamma_2$ and the N or C terminus of Kir2.1 (Fig. 2C). These results agree with the data of previous publications (Huang et al., 1995; Inanobe et al., 1995; Krapivinsky et al., 1995b; Kunkel and Peralta, 1995), suggesting that our fusion proteins function as expected.

$G\alpha_q$ Does Not Interact with TRPC6A. The experiment of Fig. 2 shows that $G\alpha_q$ interacts with the N terminus of Kir3s but not with Kir2. The Kir2 channel, being constitutively active and not regulated by $G\alpha_q$, served as a negative control. We, however, wished to perform another type of control experiment by using a channel that is regulated by $G\alpha_q$. For this purpose, we examined a transient receptor potential canonical type channel; this is a family of nonselective cation channels and is suggested to function downstream of $G\alpha_q$ (Clapham et al., 2001).

HEK293A cells were transfected with cDNAs for $G\alpha_q$ and TRPC6A, and coimmunoprecipitation was carried out using anti-$G\alpha_{q/11}$ antibody. The results showed that TRPC6A did not coprecipitate with $G\alpha_q$ in either GDP or GDP-AlF_4^- containing solution, whereas Kir3.2 did coprecipitate with $G\alpha_q$ (Fig. 3). We did not perform a further GST-pulldown assay between $G\alpha_q$ and termini of the TRPC6 channel, because the absence of coprecipitation between $G\alpha_q$ and TRPC6A clearly indicated the absence of interaction between the two types of proteins.

$G\alpha_q$ Binding Region of Kir3. To locate the $G\alpha_q$ binding region within the Kir3.2 N terminus, various GST-fusion proteins, each consisting of part of the Kir3.2 N terminus, were prepared (Fig. 4). Because $G\alpha_q$ is likely to be anchored to the cell membrane through the palmitoyl moiety, we suspected that $G\alpha_q$ might bind to part of the Kir3 N terminus that is close to the cell membrane. We, therefore, constructed GST-fusion proteins having the following Kir3.2 N terminus residues that either lack or retain membrane proximal sequences: 1 to 50, 1 to 60, 1 to 70, 1 to 80, 1 to 90, and 51 to 90 (Fig. 4C). Each of these partial domains of the Kir3.2 N

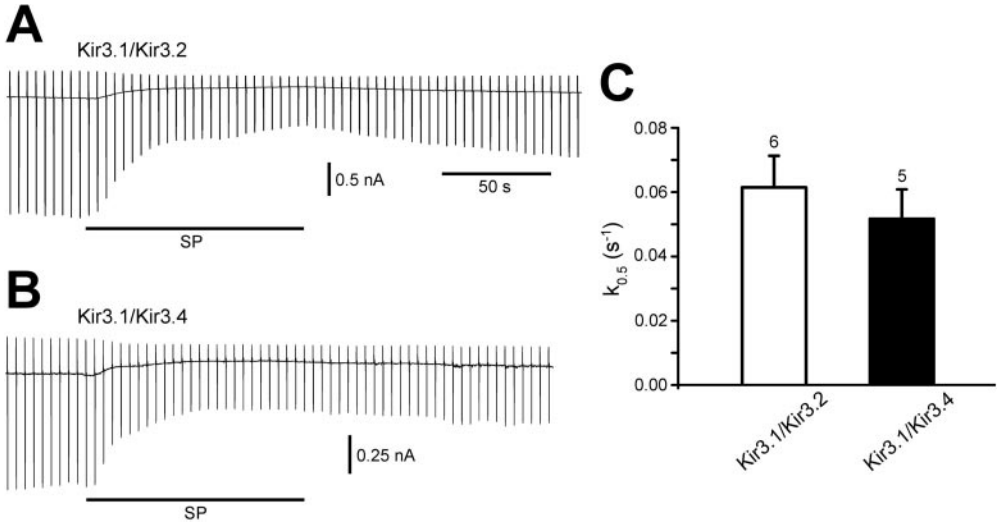

Fig. 1. Inactivation of Kir3.1/3.2 and Kir3.1/3.4 channels by SP. HEK293A cells were transfected with cDNAs for SP receptor, Kir3.1, Kir3.2 (or Kir3.4), $G\beta_1$, $G\gamma_2$, and GFP. Application of SP to a cell expressing Kir3.1/3.2 (A) or to a cell expressing Kir3.1/3.4 (B) inactivated the Kir3 current. The whole-cell recordings were used in both A and B. Holding potential was −79 mV; over this steady holding potential, square-shape depolarizing pulses (20 mV/100 ms; upward swinging line), and square-shape hyperpolarizing pulses (50 mV/100 ms; downward swinging line) were periodically (once per 4 s) superimposed. In this way, changes of currents at −59, −79, and −129 mV could be followed by one experimental run. SP application produces a marked decrease in current amplitudes at −59 mV as well as at −129 mV, indicating that inward rectifying currents were reduced. In both A and B, the SP-induced inactivation recovered partially: for Kir3.1/3.2, the recovery (at ~300 s) was 35 ± 14% (mean ± S.E.M., n = 6), ranging from 0 to 83%. For Kir3.1/3.4, recovery was 38 ± 13% (at ~290 s), ranging from 12.5 to 89%. C, the speed of the SP-induced inactivation ($k_{0.5}$) was plotted ($k_{0.5} = 1/T_{0.5}$, in which $T_{0.5}$ is half-time). Difference was not statistically significant.

terminus was tested for its capability to bind to $G\alpha_q$. As shown in Fig. 4A, GST constructs with the residues 1 to 90, 51 to 90, and 2 to 96 bound to $G\alpha_q$, but those with the residues 1 to 80, 1 to 70, 1 to 60, and 1 to 50 did not. The shortest domain that binds to $G\alpha_q$ was the residues 51 to 90, whereas the residues 1 to 80 did not bind to $G\alpha_q$, suggesting that the residues 81 to 90 are critically involved in binding to $G\alpha_q$.

We also investigated the $G\beta_1\gamma_2$ binding to these GST fusion constructs of the Kir3.2 N terminus (Fig, 4B). $G\beta_1\gamma_2$ bound to the 2 to 96 residues, whereas it did not bind to the 1–90 residues, suggesting that the residues 91 to 96 N-terminal domain may be critical for $G\beta_1\gamma_2$ binding.

$G\alpha_q$ Does Not Compete with $G\beta\gamma$ for Binding to the Kir3.2 N Terminus. The results of the previous section

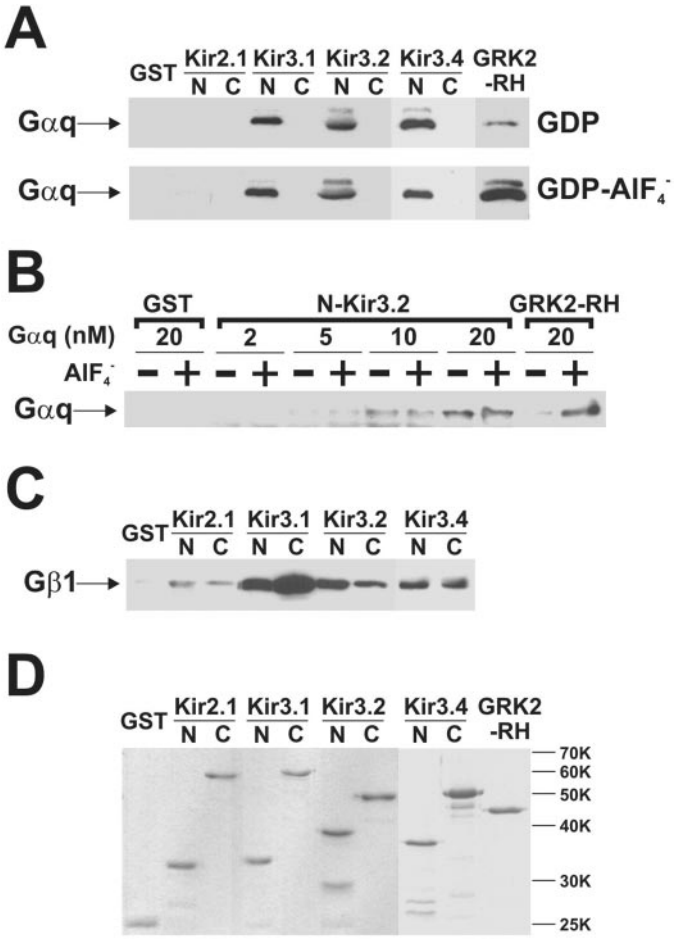

Fig. 2. $G\alpha_q$ binds to the N terminus, but not to the C terminus of Kir3s. A, representative data of GST pull-down assays between $G\alpha_q$ and N- or C-terminal cytoplasmic domain of Kir2, Kir3, and GRK2-RH. Purified $G\alpha_q$ protein (50 nM) was incubated with 0.5 μg of GST-fusion proteins (40–100 nM) in the presence of GDP or GDP-AlF_4^-. Both GDP and GDP-AlF_4^- forms of $G\alpha_q$ bound to the N-terminal domain but not to the C-terminal domains of Kir3.1, Kir3.2, and Kir3.4. However, $G\alpha_q$, in either form, did not bind to the N or C terminus of Kir2.1. B, pull-down assays were conducted at various low concentrations of $G\alpha_q$ to examine the affinity of $G\alpha_q$ to the N terminus of Kir3.2 in the presence or absence of AlF_4^-. In this experiment, binding buffer B contained $MgCl_2$ at 1 mM instead of 10 mM. C, binding assays were performed between $G\beta_1\gamma_2$ and the C terminus, as well as between $G\beta_1\gamma_2$ and the N terminus of Kir3 and Kir2.1. Purified $G\beta_1\gamma_2$ protein (40 nM) was incubated with 1 μg of GST-fusion proteins of the N or C terminus of various Kirs. $G\beta_1\gamma_2$ was bound to the N- and C-terminal domains of Kir3, but not to those of Kir2.1. D, GST-fusion proteins used for the pull-down assays were visualized with the Coomassie Brilliant Blue (CBB) staining. GST-fusion proteins examined are the N terminus (residues 1–86) and the C terminus (179–428) of Kir2.1, the N terminus (1–86) and the C terminus (180–501) of Kir3.1, the N terminus (2–96) and the C terminus (191–414) of Kir3.2, and the N terminus (1–91) and the C terminus (185–419) of Kir3.4 and RH domain (1–178) of GRK2.

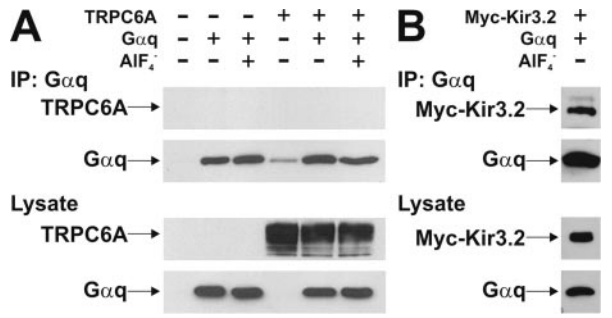

Fig. 3. $G\alpha_q$ does not interact with TRPC6A. HEK293A cells were transfected with $G\alpha_q$ and TRPC6A (A) or Myc-Kir3.2 (B). Immunoprecipitation was performed using anti-$G\alpha_{q/11}$ antibody as described under *Materials and Methods*. Precipitated proteins were subjected to SDS-PAGE and immunoblotting with anti-$G\alpha_{q/11}$, anti-Myc, and anti-TRPC6 antibodies. A, TRPC6A was not coprecipitated with $G\alpha_q$ either in the presence or absence of AlF_4^-. B, Myc-Kir3.2 was coprecipitated with $G\alpha_q$ either in the presence or absence of AlF_4^-, in agreement with the result of Koike-Tani et al. (2005). Cell lysates were subjected to Western blotting to confirm protein expression (A and B, bottom blots).

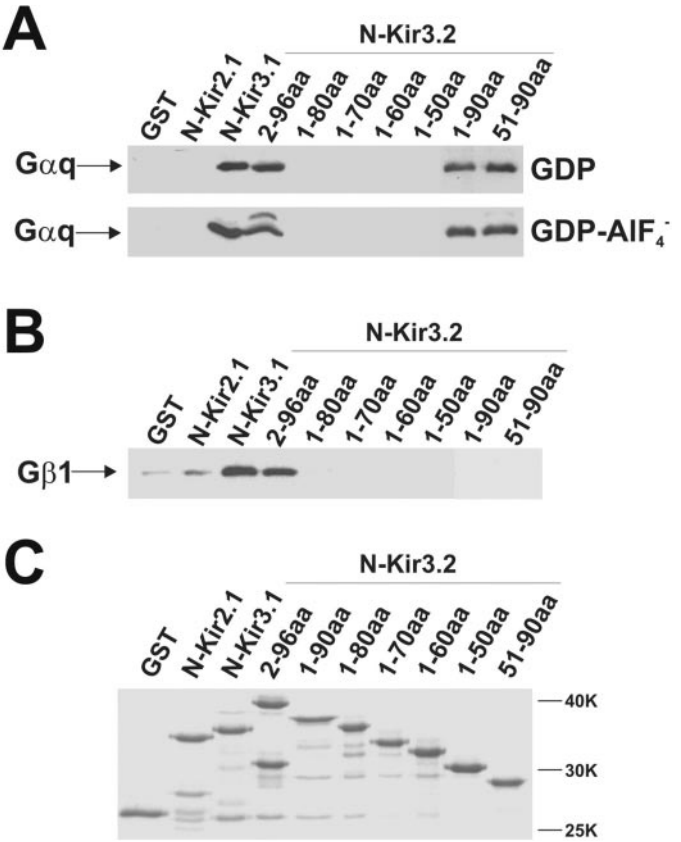

Fig. 4. Mapping of the $G\alpha_q$ binding site and the $G\beta_1\gamma_2$ binding site on the Kir3.2 N terminus. A, $G\alpha_q$ binding to various parts of the Kir3.2 N terminus as well as to the whole N terminus of Kir2.1 and the N terminus of Kir3.1 were examined. $G\alpha_q$ was incubated with GST-fusion proteins in the presence of GDP or GDP-AlF_4^-. B, $G\beta_1\gamma_2$ binding was examined on each of the seven different domains of the Kir3.2 N terminus as well as on the whole N terminus region of Kir2.1 and Kir3.1. C, GST-fusion proteins that were used for the pull-down assays were visualized with Coomassie Brilliant Blue (CBB) staining.

suggest that on the Kir3.2 N terminus, the domain where $G\alpha_q$ attaches and the domain where $G\beta_1\gamma_2$ attaches are in close proximity. This could result in the situation, in which $G\alpha_q$ and $G\beta_1\gamma_2$ compete with each other for the binding site. To examine this possibility, we performed pull-down assays between $G\alpha_q$ (50 nM) and GST-Kir3.2 N terminus (residues 2–96) at various concentrations of $G\beta_1\gamma_2$. The tests were done in the presence of AlF_4^- to keep $G\alpha_q$ and $G\beta_1\gamma_2$ dissociated.

As shown in Fig. 5, $G\alpha_q$ and $G\beta_1\gamma_2$, when incubated together (Fig. 5, lanes 2 to 4 from the left), interacted with GST-N-Kir3.2, essentially to the same extent as in the case of $G\alpha_q$ alone (lane 1) or $G\beta\gamma$ alone (lane 5). Indeed, $G\beta_1\gamma_2$, even at 20 times the concentration of $G\alpha_q$ (1 μM; lane 4), did not interfere with the binding of $G\alpha_q$ to the N terminus. These results suggest that each of $G\alpha_q$ and $G\beta\gamma$, in the presence of AlF_4^-, binds to the Kir3.2 N terminus separately, but not as a $G\alpha_q\beta\gamma$ heterotrimer. These data also suggest that $G\alpha_q$ and $G\beta_1\gamma_2$ each binds to close but separate domains of the N terminus.

Discussion

The Role of $G\alpha_q$/Kir3 Interaction. The focus of this article is the interaction between $G\alpha_q$ and Kir3s. In the beginning, we showed an example in which activation of SP receptor, coupled to $G\alpha_q$, inhibits Kir3 channels in a heterologously expressed system (Fig. 1). This type of channel inhibition through the $G\alpha_q$-Kir3 interaction may be in operation in many types of endogenous signaling. Activation of SP receptor is known to inhibit the endogenous Kir3 channels in locus ceruleus neurons (Velimirovic et al., 1995). Activation of the α_1-adrenoceptors (Braun et al., 1992; Cho et al., 2001) and thyrotropin-releasing hormone receptor (Lei et al., 2001) inhibits Kir3 channels probably through $G\alpha_q$. Likewise, activation of orexin receptor (Hoang et al., 2003, 2004) or ghrelin receptor (Bajic et al., 2004) has been shown to inhibit Kir channels. All of these receptors are probably coupled to $G\alpha_q$ (Offermanns, 2003). Thus, the central theme of this article would be the $G\alpha_q$-Kir3 interaction rather than the SP receptor and the Kir3.

$G\alpha_q$ Binds to Kir3. In the present experiments, we used the GST pull-down assay to study the interaction between $G\alpha_q$ and Kir3. Our data show, for the first time, that $G\alpha_q$ binds directly and specifically to the N terminus of Kir3s. The binding was **direct** without intervening proteins, because the binding was examined with the GST pull-down assay using purified proteins. The binding was **specific** in that $G\alpha_q$ interacted with the N terminus of the Kir3s (Kir3.1, -3.2, and -3.4), whereas $G\alpha_q$ did not interact with the C terminus of any of the Kir3s tested, and did not $G\alpha_q$ interact with the N or C terminus of Kir2.1. Moreover, $G\alpha_q$, which interacts with Kir3, did not interact with TRPC6A, although the signal transduction cascades toward TRPC6 activation and toward Kir3 inhibition are both initiated by $G\alpha_q$.

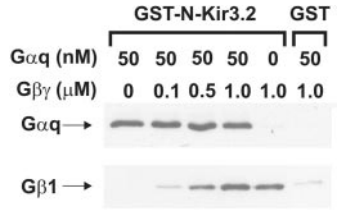

Fig. 5. $G\alpha_q$ and $G\beta_1\gamma_2$ do not compete with each other for the binding to the N terminus of Kir3.2. To examine possible competition between $G\alpha_q$ and $G\beta_1\gamma_2$ for the binding site on the N terminus of Kir3.2, pull-down assays were performed at different concentrations of $G\beta_1\gamma_2$. In the presence of GDP-AlF_4^-, 0.5 μg of GST or GST-N-Kir3.2 (residues 2–96) was incubated with $G\alpha_q$ and $G\beta_1\gamma_2$ at various concentrations.

Our results differ from those of Clancy et al. (2005), who recently reported that $G\alpha_q$ binds to the N terminus of both Kir2.1 and Kir3.2. Part of the discrepancy could have arisen from different conditions used. Clancy et al. (2005) used high concentrations of $G\alpha_q$ (350 nM) and GST-fusion proteins (400 nM), whereas we used lower concentrations of these (50 nM for $G\alpha_q$ and 70 nM for GST-fusion proteins). The concentration of $G\alpha_q$ in our present experiments (50 nM) is similar to those used for functional examinations of $G\alpha_q$ on phospholipase C (Hepler et al., 1996).

The present pull-down assay indicated that $G\alpha_q$, both in GDP-bound form and in GDP-AlF_4^--bound form, interacted with the N terminus of Kir3s. This type of interaction seems to be peculiar for a signal transduction involving a G protein. However, similar examples of signaling have been recently reported: $G\alpha$, independent of its conformation, can interact with regulator of G protein signaling (RGS) inside the signal complex consisting of the receptor, G protein and RGS (Benians et al., 2005, Abramov-Newerly et al., 2006). It is possible that inside these types of microspheres consisting of the receptor, $G\alpha_q$, and the channel, the concentration of $G\alpha_q$ could become quite high, and only a high-concentration $G\alpha_q$ could accomplish the channel inhibition. This type of mechanism could send information more accurately, because a low concentration of stray $G\alpha_q$ molecules would become ineffectual. At present, this is a mere speculation.

How then can this mode of signaling achieve an on-off switching mechanism? Switching can be accomplished by the fact that the GDP form of $G\alpha_q$ has a high affinity to $G\beta\gamma$, resulting in the formation of the heterotrimeric $G\alpha\beta\gamma$ protein, terminating the functional interaction between $G\alpha_q$ and the K^+ channel.

Kir3 Domains That Interact with $G\alpha_q$ and $G\beta\gamma$. In addition to the above primary conclusion, the present experiments have shed light on the location of $G\alpha_q$ binding versus the location of $G\beta\gamma$ binding on Kir3s. We showed that the domain encompassing the residues 51 to 90 of the Kir3.2 N terminus was capable of interacting with $G\alpha_q$, whereas the domain from the residues 1 to 80 was not. This suggests, although preliminarily, that the residues from 81 to 90 are critically involved in the interaction with $G\alpha_q$. Nevertheless, for now, it is safe to say that $G\alpha_q$ can interact with the residues encompassing 51 to 90 of the Kir3.2. Our results also indicated that $G\beta_1\gamma_2$ binds to residues 2 to 96 but not to residues 1 to 90, of the Kir3.2 N terminus, suggesting that residues 91 to 96 are critical for $G\beta_1\gamma_2$ binding. Finally, the occupancy of $G\alpha_q$ and that of $G\beta_1\gamma_2$ on the N terminus of Kir3.2 were independent of each other (Fig. 5), suggesting that the $G\alpha_q$-interacting surface and the $G\beta_1\gamma_2$-interacting surface have no overlap on Kir3.2.

The N-terminal residues 51 to 96 of Kir3.2, where our results suggest that $G\alpha_q$ and $G\beta_1\gamma_2$ attach, contain a domain designated as "slide helix" by Kuo et al. (2003). Based on the crystal structure of prokaryotic Kir (KirBac1.1), Kuo et al. (2003) propose that "slide helix" plays a role in channel gating: the negativity of the helix dipole, being located near the positive charge of the pore helices, may control the movement of the pore helices, thereby controlling the channel gating. Indeed,

the attachment sites of $G\alpha_q$ and $G\beta\gamma$ seem to be located at strategically crucial spots for the functional roles.

$G\alpha_q$-Kir3 Interaction versus $G\alpha_i$-Kir3 Interaction. According to Dascal and his coworkers (Peleg et al., 2002; Ivanina et al., 2004), $G\alpha_i$, apart from its $G\beta\gamma$ sequestering effect, shows inhibitory effects on the basal Kir3 activity. Under ordinary conditions, this capability of $G\alpha_i$ is overshadowed by the channel activating effect of $G\beta\gamma$. On the other hand, $G\alpha_q$, having a strong suppressing effect on the channel, could suppress the activating effect of $G\beta\gamma$. This situation could be related to the physiological data that SP effect to inhibit Kir3 channel overshadows the somatostatin effect to activate Kir3 (Velimirovic et al., 1995). In the present experiment, we observed that $G\alpha_q$ binds to Kir3 channels only at the N terminus, not at the C terminus of all Kir3 channels tested (Kir3.1, Kir3.2, and Kir3.4). In contrast, $G\alpha_i$ binds both N and C termini of Kir3 (Ivanina et al., 2004). This difference could suggest that the mode of $G\alpha_q$ action might be basically different from that of $G\alpha_i$.

Kir3.4 Behaves Similarly to Kir3.2. In the previous study (Koike-Tani et al., 2005), we investigated the properties of Kir3.1 and -3.2, as well as Kir2. In the present study, we have additionally observed the behavior of Kir3.4. Electrophysiological manifestation (Fig. 1) of Kir3.4 and the property of $G\alpha_q$ binding were similar to that of Kir3.2. Thus, the cardiac type of GIRK (Kir3.1/3.4) does not seem to behave differently from the brain type (Kir3.1/3.2).

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