Signal transduction pathway for the substance P-induced inhibition of rat Kir3 (GIRK) channel

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Certain transmitters inhibit Kir3 (GIRK) channels, resulting in neuronal excitation. We analysed signalling mechanisms for substance P (SP)-induced Kir3 inhibition in relation to the role of phosphatidylinositol 4,5-bisphosphate (PIP_2). SP rapidly – with a half-time of ∼10 s with intracellular GTPγS and ∼14 s with intracellular GTP – inhibits a robustly activated Kir3.1/Kir3.2 current. A mutant Kir3 channel, Kir3.1(M223L)/Kir3.2(I234L), which has a stronger binding to PIP_2 than does the wild type Kir3.1/Kir3.2, is inhibited by SP as rapidly as the wild type Kir3.1/Kir3.2. This result contradicts the idea that Kir3 inhibition originates from the depletion of PIP_2. A Kir2.1 (IRK1) mutant, Kir2.1(R218Q), despite having a weaker binding to PIP_2 than wild type Kir3.1/Kir3.2, shows a SP-induced inhibition slower than the wild type Kir3.1/Kir3.2 channel, again conflicting with the PIP_2 theory of channel inhibition. Co-immunoprecipitation reveals that $G\alpha_q$ binds with Kir3.2, but not with Kir2.2 or Kir2.1. These functional results and co-immunoprecipitation data suggest that G_q activation rapidly inhibits Kir3 (but not Kir2), possibly by direct binding of $G\alpha_q$ to the channel.

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Substance P (SP), an undecapeptide belonging to the tachykinin family (Chang & Leeman, 1970), has been shown to be an excitatory transmitter in the brain and peripheral neurones (Otsuka *et al.* 1972). It is one of the brain transmitters in which the mechanism of slow excitation has been well investigated (Stanfield *et al.* 2002). The SP receptor (NK1) is located not only in the sensory tracts but also in various regions of the brain including the hippocampus, the olfactory bulb, hypothalamic nuclei and the locus coeruleus (Dam & Quirion, 1994) as well as the cholinergic neurones of the nucleus basalis. SP has been shown to inhibit inwardly rectifying K^+ channels (Stanfield *et al.* 1985), the M-current (Nowak & Macdonald, 1982; Adams *et al.* 1983), or the calcium-activated K^+ current (Vanner *et al.* 1993), thereby leading to neuronal excitation.

Activity of G protein-coupled inward rectifier K^+ channels (Kir3; GIRK) (Dascal *et al.* 1993; Kubo *et al.* 1993) is reciprocally regulated in locus coeruleus neurones in the brain stem (Velimirovic *et al.* 1995): the channel is activated by somatostatin, and the same channel is suppressed by the excitatory transmitter SP. The time course of the suppression is fairly quick (a half-time of ∼12 s with intracellular GTPγS) (Velimirovic *et al.* 1995). Kir3 inhibition by neurotensin in brain dopaminergic neurones is also fairly fast (Farkas *et al.* 1997).

The mechanism of Kir3 activation through $G\beta\gamma$ is well clarified (Logothetis *et al.* 1987; Reuveny *et al.* 1994). In contrast, the mechanism of the transmitter-induced Kir3 inhibition is poorly understood (Stanfield *et al.* 2002). The receptor stimulates $G\alpha_q$, which activates phospholipase Cβ (PLCβ). PLCβ hydrolyses PIP_2 into diacylglycerol and inositol 1,4,5-trisphosphate, resulting in a reduction of the PIP_2 level. Recently, evidence has accumulated to indicate that changes in the PIP_2 level regulate the activity of various ion channels (Huang *et al.* 1998) such as: Kir2, Kir3 (Kobrinsky *et al.* 2000; Cho *et al.* 2001; Lei *et al.* 2001; Meyer *et al.* 2001), K_{ATP} (Hilgemann & Ball, 1996; Shyng *et al.* 2000), KCNQ2/3 (Suh & Hille, 2002; Zhang *et al.* 2003), and TRP channels (Runnels *et al.* 2002; Prescott & Julius, 2003; Hardie, 2003).

Here, we investigated the signal transduction mechanism of Kir3 inhibition produced by SP. The main experiments have been done using the HEK293 heterologous system. We constructed mutant Kir3 and Kir2 channels in which the strength of channel binding to PIP_2 was altered. The time courses of SP-induced

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inhibition of these mutant channels, in comparison with the wild type channels, indicate that depletion of PIP_2 does not explain the SP-induced inhibition of Kir3 channels. Co-immunoprecipitation experiments have revealed that $G\alpha_q$ interacts with Kir3.2, but not with Kir2.2 or Kir2.1. These data suggest that G_q activation quickly inhibits Kir3 channels, possibly by direct binding of $G\alpha_q$ to the channel. A preliminary account of this work has been published (Koike *et al.* 2003).

Methods

Primary culture of brain neurones

Neurones of the nucleus basalis were cultured from 3- to 4-day-old postnatal rats as previously described (Nakajima *et al.* 1985; Nakajima & Masuko, 1996). The rats were anaesthetized with ether, and after the rats became completely unconscious, the forebrain was removed, immediately followed by decapitation. This protocol was reviewed and approved by the Animal Care Committee of the University of Illinois at Chicago. The removed brain was sectioned, and the nucleus basalis was isolated from brain slices under a dissection microscope. The nuclei were dissociated with papain, plated on a glial feeder layer, and cultured at 37°C with 10% CO_2. The culture medium was a modified minimum essential medium with Earle's salt (88%; Gibco BRL) containing heat-inactivated rat serum (2%, prepared in our laboratory) and 50 ng ml^{-1} of 2.5 S nerve growth factor. The medium had been kept overnight in glia cultures (conditioned medium) (Baughman *et al.* 1991).

Culture and transfection of HEK293 cells

Human embryonic kidney 293 (HEK293 or HEK293A) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) at 37°C with 10% CO_2. The cells were transfected by using Effectene Transfection Reagent (Qiagen, Valencia, CA, USA). Kir3.1 and Kir3.2 cDNAs were from the rat, and $G\beta_1$ and $G\gamma_2$ cDNAs were of bovine origin. SP receptor cDNA was human (Takeda *et al.* 1991). Mutants were constructed by a mutagenesis kit (QuikChange; Stratagene; La Jolla, CA, USA). Each mutation was verified by the sequence facility at the University of Chicago. Unless otherwise stated, cells were transfected in 6 cm culture dishes with the following cDNAs subcloned into plasmid pCMV5 (Andersson *et al.* 1989). To study the SP-induced effect on Kir3 channels, 0.2 μg SP receptor, 0.15 μg each of Kir3.1 and Kir3.2, 0.3 μg each of $G\beta_1$, and $G\gamma_2$, and 0.1 μg green fluorescent protein (pEGFP-N1; Clontech) cDNAs per dish were used. To investigate the SP effects on Kir2.1 channels, 0.2 μg SP receptor, 0.3 μg Kir2.1 (Wischmeyer *et al.* 1995) and 0.1 μg pEGFP-N1 cDNAs per dish were transfected. The total amount of cDNAs was kept at 1.6 μg by adjusting the quantity of the empty vector. The next day cultures were re-plated on 3.5 cm culture dishes, which have a centre well (~1.2 cm in diameter) coated with rat-tail collagen (Roche Molecular Biochem., Indianapolis, IN, USA). Electrophysiological experiments were performed 2–3 days after the re-plating.

To investigate the effects of anti-PIP_2 antibody on the channels, HEK293A cells were transfected as follows. For experiments on Kir3.1/Kir3.2 (or their mutants), the standard amounts of cDNAs, unless otherwise noted, were: 0.15 μg each of Kir3.1 and Kir3.2 (or their mutants), 0.3 μg each of $G\beta_1$ and $G\gamma_2$, and 0.1 μg of pEGFP-N1 with the total amount of plasmid adjusted to 1.6 μg by empty vector (for each 6 cm culture dish). To investigate Kir2.1 (or its mutants), we used 0.3 μg of Kir2.1, and 0.1 μg of pEGFP-N1, again the total amount of plasmid adjusted to 1.6 μg.

Electrophysiology

The whole-cell version of patch clamp was used. The external solution contained (mM): 141 NaCl, 10 KCl, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 11 D-glucose, 5 Hepes-NaOH, and 0.0005 tetrodotoxin (pH 7.4). The patch pipette solution contained (mM): 141 potassium gluconate, 10 NaCl, 5 Hepes-KOH, 0.5 EGTA-KOH, 0.1 $CaCl_2$, 4 $MgCl_2$, 3 Na_2ATP and 0.2 GTPγS (or 0.2 GTP) (pH 7.2). The external solutions were exchanged with a sewer pipe manifold, except for certain early experiments, where a pressure ejector was used. The half-time of the SP-induced inhibition was obtained in most cells by a curve fitting program (Origin programs) with the Boltzmann or exponential functions.

The binding strength of the channels to PIP_2 was determined by measuring the time courses of inactivation upon sudden application of anti-PIP_2 antibody to the inside-out patch (Huang *et al.* 1998; Zhang *et al.* 1999). The patch pipette contained (mM): 155 KCl, 2.4 $CaCl_2$, 1.3 $MgCl_2$ and 5 Hepes(NaOH), pH 7.4. The bath solutions (bathing the cytoplasmic side) were similar to those described in Huang *et al.* (1998), containing fluoride (F) and vanadate (V) to inhibit the lipid phosphatases. The FV solution contained (mM): 146 KCl, 5 EGTA(KOH), 10 Hepes(KOH), 5 NaF, and 0.1 Na_3VO_4, pH 7.2. The FV-ATP solution contained (mM): 136 KCl, 5 EGTA(KOH), 10 Hepes(KOH), 5 NaF, 0.1 Na_3VO_4, 3 ATP(KOH) and 2 $MgCl_2$ (pH 7.2). Anti-PIP_2 monoclonal antibody was obtained from Assay Designs, Inc (Ann Arbor, MI, USA). The antibody was purified, thanks to the help of Stephen C. Lam and Joseph M. Schober (University of Illinois at Chicago), by protein A–sepharose. IgG_{2b} for the negative control was from Sigma-RBI.

Physiological experiments were conducted at a bath temperature of ~24°C. The statistical values are expressed as mean ± S.E.M.

Immunoprecipitation

HEK293 cells were transfected with plasmid cDNAs of $G\alpha_q$ and either Myc-Kir3.2, T7-Kir2.2 or Myc-Kir2.1 by *Trans* IT-LT1 (Panvera). The amount of cDNA used for transfection was adjusted by adding pCMV5 to 20 μg total per 10 cm dish of the cells. Transfected cells from the 10 cm dish were harvested and solubilized 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^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin A, 0.5% Triton-X 100, 10% glycerol and 10 μM GDP. In some experiments, 30 μM $AlCl_3$ and 5 mM NaF were added to the lysis buffer to activate $G\alpha_q$. 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. 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-X 100 and 10 μM GDP) either with or without 30 μM $AlCl_3$ and 5 mM NaF. Immunoprecipitated proteins were boiled in a sample buffer (50 mM Tris-HCl at pH 6.8, 1% SDS, 2% 2-mercaptoethanol, 0.008% bromophenol blue, and 8% glycerol) and resolved by SDS-PAGE. After SDS-PAGE, the proteins were transferred to nitrocellulose membranes. The membranes were blocked with blocking buffer (50 mM Tris-HCl at pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 5% skim milk, 0.2% NP-40 and 0.02% NaN_3), and proteins were immunoblotted with antibodies described below. The bound antibodies were visualized by the enhanced chemiluminescence detection system (Pierce), using antimouse or antirabbit Ig antibodies conjugated with horseradish peroxidase as secondary antibodies (Amersham). Rabbit polyclonal antibody which recognizes $G\alpha_{q/11}$ was purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody 9E10 against c-Myc epitope was from Covance. Mouse monoclonal antibody against T7 epitope was from Novagen.

Results

Primary cultured brain neurones

It has been shown that sympathetic neurones can be transfected by introducing cDNAs into cell nuclei by a microinjector (Ikeda, 1997). Figure 1*A–C* shows that our primary cultured brain neurones from the nucleus basalis can be transfected using the same microinjection method.

In Fig. 1*D–F*, cultured nucleus basalis neurones were transfected, using the microinjection method, with cDNAs for M2-muscarinic receptor, RGS4 (or empty vector for control), and GFP. We then performed whole-cell recordings using pipettes containing GTPγS (200 μM). About 30 s after the giga-seal was broken, we started recording the conductance. The inward rectifier conductance (measured with 50 mV hyperpolarization) began to increase, reaching a plateau in about 5 min. This increase results from activation of endogenous G_i protein caused by spontaneous exchange of GDP (bound to $G\alpha_i$) with GTPγS; this in turn would result in the liberation of $G\beta\gamma$, which opens the Kir3 channels. In the present experiments, while the conductance was increasing, we activated the M2-muscarinic receptors by muscarine (20 μM), which further accelerated the conductance increase. After reaching a plateau, the conductance often started to decrease.

Application of SP (0.3 μM) to a control neurone after the conductance reached a plateau caused a rapid reduction of the conductance (Fig. 1*D*). SP receptor activation causes a rapid dissociation of $G\alpha_q$ from the $G\beta\gamma$ subunit; $G\alpha_q$ then, through an unknown mechanism, inhibits the Kir3 channels. Because the patch pipette contained GTPγS, the SP application activated the receptor almost maximally without complications from receptor desensitization. The GTPγS-bound $G\alpha$ and the isolated $G\beta\gamma$ remain separated indefinitely.

As shown in Fig. 1*E* and *F*, the rate of the SP-induced inhibition was considerably reduced in neurones in which RGS4, an inhibitor of $G\alpha_q$ and $G\alpha_i$ (Hepler *et al.* 1997), had been additionally expressed. As there are two types of Kir channels that are affected by SP in nucleus basalis neurones (Bajic *et al.* 2002), the interpretation is not straightforward. Nevertheless, the present result is consistent with the notion that $G\alpha_q$ mediates the SP-induced inhibition of *native* Kir3 channels in brain neurones, in agreement with conclusions from heterologous systems by Leaney *et al.* (2001) and Lei *et al.* (2001).

An explanation is needed on the rationale for our experimental protocol in Fig. 1*D–F*. The rationale relies on the difference between $G\alpha_q$ and $G\alpha_i$ in their activation kinetics in the presence of GTPγS. Shortly after the break of the patch, the conductance begins to increase spontaneously (without receptor activation) in the presence of GTPγS; this probably reflects spontaneous dissociation of $G\alpha_i$ from $G\beta\gamma$ caused by an exchange of GDP with GTPγS. At the peak of the conductance, activation of $G\alpha_q$ by SP application produces a large inhibition, suggesting that the spontaneous activation of $G\alpha_q$ by GTPγS is much slower than the spontaneous activation of $G\alpha_i$ by GTPγS. Biochemical data using an *in vitro* preparation indicate that in order to measure the

spontaneous replacement of GDP by GTPγS, a 100 times higher GTPγS concentration is needed for Gα_q than for Gα_i (Linder *et al.* 1990; Hepler *et al.* 1993); this means that the rate of the replacement, at the same GTPγS concentration, would be much slower for Gα_q than for Gα_i.

RGS4 inhibits not only Gα_q, but also Gα_i. Therefore, possible inhibition of Gα_i by RGS4 could affect the initial spontaneous conductance increase in the presence of GTPγS. Our data, however, showed that the conductance increase, which was caused by the combination of spontaneous and muscarine-induced activation, was almost the same in control and in the RGS4-transfected cells. The conductance reached a level of 203 ± 36% during the initial 254 ± 16 s in control cells ($n = 8$), and to a level of 220 ± 30% during the initial 304 ± 31 s in the RGS4-transfected cells ($n = 8$). These results indicate that the initial conductance increase was not much affected by the presence of RGS4. This is expected because RGS inhibits the Gα subunit only after it is split from the G$\beta\gamma$, and the conductance increase of Kir3 is mediated by G$\beta\gamma$ not by Gα. By the time SP was applied, almost all the G_i had probably been fully activated in part by the spontaneous GDP–GTPγS exchange, and in part by the muscarine application. Therefore, it is likely that the SP-induced conductance inhibition is caused by the G_q subunit, not by G_i, for both control and the RGS-transfected cells.

HEK293 cells: role of Gα_q

From this point on, all data were obtained from HEK293 cells. To examine the mechanism of the SP-induced

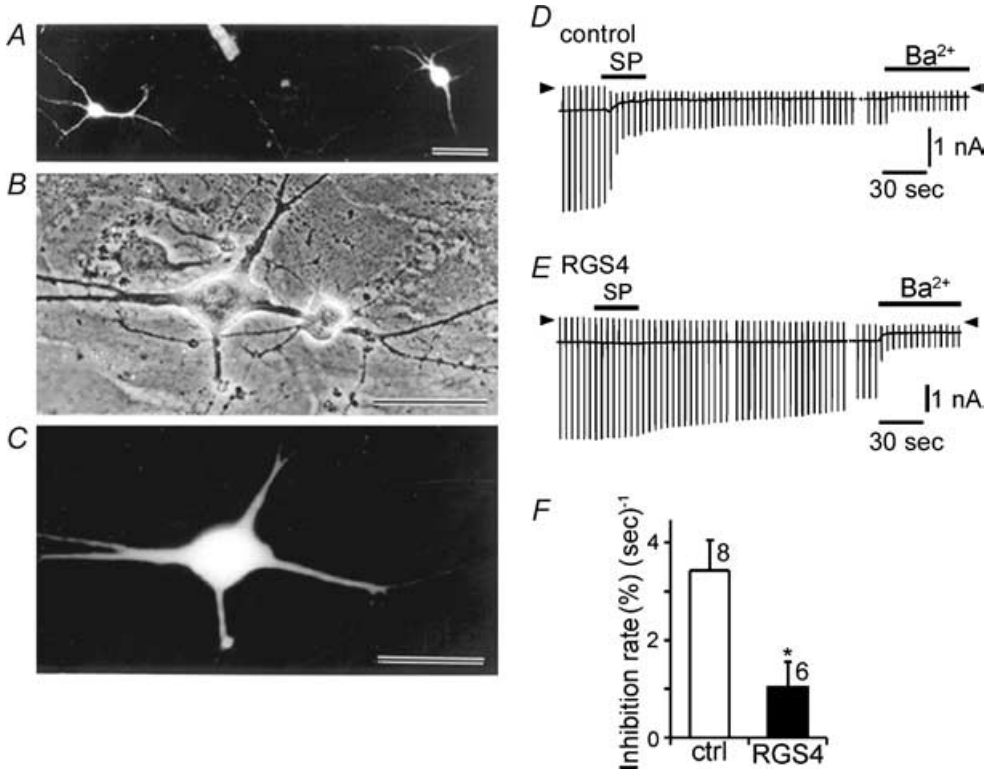

Figure 1. Transfection of cultured neurones from the nucleus basalis and the effect of RGS4
A–C, primary cultured neurones from the nucleus basalis transfected by intranuclear injection of cDNA for GFP, Gβ_1 and Gγ_2. The injection pressure was 30 hPa for 0.1 s. The micrographs were taken without fixation. *A*, fluorescence micrograph. *B* and *C,* microscopic view of a neurone with phase-contrast in *B*, and fluorescence optics in *C*. Calibration bars: 100 μm (in *A*) and 50 μm (in *B* and *C*). *D* and *E*, SP-induced Kir current inhibition in nucleus basalis neurones. Injection of cDNA for RGS4 reduced the rate of SP-induced Kir inhibition. Each neurone received an intranuclear injection of cDNAs for M2-muscarinic receptors (0.3 μg μl^{-1}), RGS4 (or empty vector) (0.5 μg μl^{-1}), and GFP (0.1 μg μl^{-1}). Whole-cell recordings were done 30–50 h after the injection. Holding potential, −79 mV. Command voltages of 20 mV depolarization and 50 mV hyperpolarization (each 100 ms duration) were imposed once every 4 s. The current change upon the 50 mV hyperpolarization minus the Ba^{2+} (100 μM)-resistant current was taken as amplitude of the Kir current. First, muscarine (20 μM) was applied to activate Kir3 channel (not shown in the figures), followed by SP (0.3 μM) application. Control: SP application caused a fast inhibition of the Kir channels (in *D*). A neurone injected with RGS4 cDNA showed a slow inhibition by SP (in *E*). *F*, speed of the SP-induced inhibition of the Kir channel. $^*P < 0.05$. The diameter of the neurones was: control, 29.3 ± 1.3 μm ($n = 8$); RGS4, 29.8 ± 1.6 μm ($n = 6$). These large neurones are most probably cholinergic (Nakajima *et al.* 1985).

Kir3 inhibition, we used HEK293 cells expressing the SP receptor, Kir3.1, Kir3.2, $G\beta_1$, $G\gamma_2$ and GFP. In these cells, the Kir3.1/Kir3.2 channels were active from the beginning because of the co-transfection of $G\beta_1$ and $G\gamma_2$. Application of SP to these cells produced a fairly quick inhibition (Fig. 2*A*). The half-time of the inhibition ($T_{0.5}$) was ∼11 s (Fig. 2*C*), which is about the same as that of the SP-induced inhibition of native Kir3 channels in locus coeruleus neurones (12 s; Velimirovic *et al.* 1995). The N-terminus construct of G protein-coupled receptor kinase 2 (GRK2-nt) specifically interacts with the active form of $G\alpha_q$, but not with $G\alpha_i$ (Carman *et al.* 1999). When HEK293 cells were transfected with GRK2-nt cDNA, in addition to the control set of cDNAs, the rate of the Kir3 inhibition ($k_{0.5}$) became considerably slower (Fig. 2*B* and *C*), suggesting that SP inhibited the Kir3.1/Kir3.2 channels through $G\alpha_q$.

In Fig. 2, as well as in the following figures, we expressed the rate of the inhibition as the reciprocal of half-time, $k_{0.5} = (T_{0.5})^{-1}$. In the present experiments, $k_{0.5}$ was a better behaved variable than half-time. If half-time was used, occasionally one cell with an unusually large half-time resulted in a large arithmetic mean, overshadowing the behaviour of other cells.

Kir3 mutants

The binding strength of a channel to PIP_2 is an important determinant of the channel activity (Hilgemann & Ball, 1996; Huang *et al.* 1998; Zhang *et al.* 1999). A convenient way to assess the strength of interaction is to apply anti-PIP_2 antibody suddenly to the inside of the membrane and measure the rate of the conductance decrease (Huang *et al.* 1998). The rate is related to the 'binding strength of the channel to PIP_2': the stronger the binding, the slower the speed of channel inactivation by the antibody (i.e. smaller value of $k_{0.5}$). Affinity of the channel (reciprocal of the dissociation constant) to PIP_2 can be measured by a dose–response relationship between water-soluble PIP_2 and channel activity (Rohacs *et al.* 2002; Lopes *et al.* 2002; Zhang *et al.* 2003). Under most conditions, the two variables (the binding strength and the affinity to PIP_2) would parallel each other. In fact, Logothetis' group showed that the binding strength of the channel to PIP_2 measured by the antibody in various Kir2.1 mutants parallels the channel activity (Lopes *et al.* 2002), which in turn would represent the 'affinity' of the channel.

According to Zhang *et al.* (1999), the binding strength to PIP_2 (as measured by anti-PIP_2 antibody) is higher in mutant Kir3.4(I229L) than in the wild type Kir3.4. We therefore constructed Kir3.1 and Kir3.2 mutants at the residues corresponding to Kir3.4(I229L) (namely, Kir3.1(M223L) and Kir3.2(I234L)). HEK293 cells were then transfected with these mutant Kir3s. The strength of the binding of the mutant Kir3 channel to PIP_2 was then examined by applying a monoclonal antibody against PIP_2 to inside-out patches. As shown in Fig. 3*A*–*C*, the purified monoclonal antibody against PIP_2 (∼50 nM) eliminated the activity of the wild type Kir3.1/Kir3.2 with a half-time of ∼29 s, while the same antibody treatment hardly affected the activity of Kir3.1(M223L)/Kir3.2(I234L) over several minutes (see also Fig. 3*D*). As a negative control, we used purified IgG_{2b} (120 nM), which did not change the Kir3 activity (4 cells). These data, in agreement with Zhang *et al.* (1999), indicate that Kir3.1(M223L)/Kir3.2(I234L) has a much stronger binding to PIP_2 than wild type Kir3.1/Kir3.2.

Interestingly, the rate of SP-induced inhibition of Kir3.1(M223L)/Kir3.2(I234L) channels was essentially the

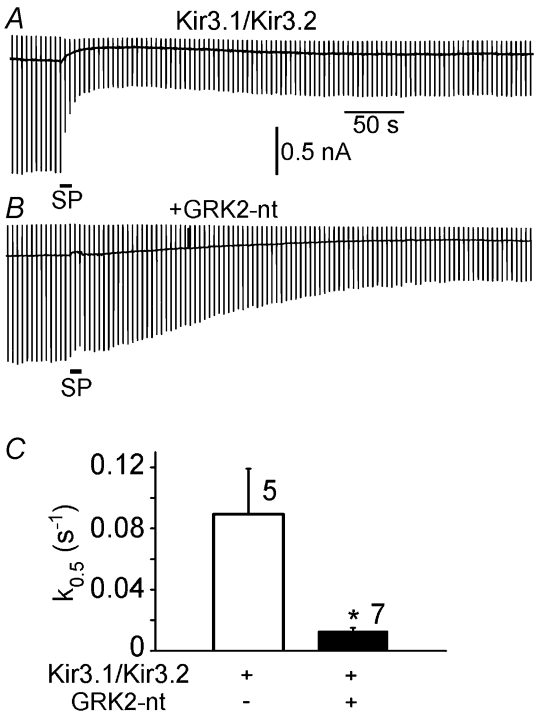

Figure 2. SP induces a quick Kir3.1/Kir3.2 inhibition mediated by $G\alpha_q$
A, HEK293 cells transfected with SP receptor, wild type Kir3.1/Kir3.2 and $G\beta_1\gamma_2$. Application of SP (0.5 μM) inhibited the Kir3 current rapidly. Whole-cell current with the pipette solution containing GTPγS (200 μM). Holding potential, −79 mV. Command voltages of 20 mV depolarization and 50 mV hyperpolarization (each 100 ms duration) were imposed once every 4 s. The amplitude of current change upon the 50 mV hyperpolarization was taken as the magnitude of the Kir current. The above protocol was used as a standard procedure in the following experiments unless otherwise stated. See Kawano *et al.* (1999) and Zhao *et al.* (2003) for the current–voltage relation recorded from HEK293 cells using a similar transfection protocol. *B*, rate of the inhibition ($k_{0.5}$) (see text) was reduced by transfecting the cells with GRK2-nt (Carman *et al.* 1999), which interacts with the active form of $G\alpha_q$. *C*, comparison of the rate of SP-induced inhibition between the control (Kir3.1/Kir3.2) and the test (Kir3.1/Kir3.2 with GRK2-nt). $^*P = 0.0101$. HEK293 cells were transfected with the standard protocol plus either 0.8 μg of empty vector (control) or GRK2-nt cDNA.

same as that of the wild type Kir3.1/Kir3.2 (Fig. 3*E–G*). If anything, the rate of SP-induced inhibition for the mutant channels was slightly higher, in contrast to what the antibody data would suggest (Fig. 3*G*). This result does not agree with the idea that SP-induced Kir3 inhibition originates from depletion of PIP_2 (Kobrinsky *et al.* 2000). If the SP-induced inhibition were caused by PIP_2 depletion only, then Kir3.1(M223L)/Kir3.2(I234L), which binds strongly to PIP_2, would have hardly been inhibited by the SP application.

The above experiments were all performed in the presence of internal GTPγS. In contrast, the experiments in Fig. 3*H–J* were performed under more physiological conditions using a patch pipette solution containing GTP instead of GTPγS. The result shows that the rate of SP-induced inhibition ($k_{0.5}$) of the mutant channel Kir3.1(M223L)/Kir3.2(I234L) was again almost the same as that of the wild type Kir3.1/Kir3.2 (for the experiments with GTP, $T_{0.5}$ was 14 s in control and 12 s in the mutant). We also measured the magnitude of conductance reduction by SP application in experiments with GTP-containing solution. The reduction was essentially the same between the wild type and the mutant; namely, for the wild type it was $51.2 \pm 5.7\%$ ($n = 7$) and for the mutant $49.2 \pm 6.1\%$ ($n = 6$). In conclusion, the results of these experiments show that the SP-induced Kir3 inhibition cannot be attributed to depletion of PIP_2.

Kir2.1 (R218Q)

Kir2.1(R218Q) is a mutant with weak binding to PIP_2 (Zhang *et al.* 1999). The channel activity of this mutant Kir2.1 therefore is readily inhibited by a small decline of the PIP_2 level (Kobrinsky *et al.* 2000). We constructed Kir2.1(R218Q) and examined its responses to anti-PIP_2 antibody and to SP application. In agreement with the result by Zhang *et al.* (1999), the inactivation rate by the antibody was faster in Kir2.1(R218Q) than that of the wild type Kir3.1/Kir3.2, indicating that the mutant Kir2.1(R218Q) binds to PIP_2 less strongly than does the wild type Kir3.1/Kir3.2 (Fig. 4*A* and *B*).

The rates of SP-induced inhibition of these channels, however, contradicted those from PIP_2 reduction. If the SP-induced inhibition was caused by PIP_2 depletion, the

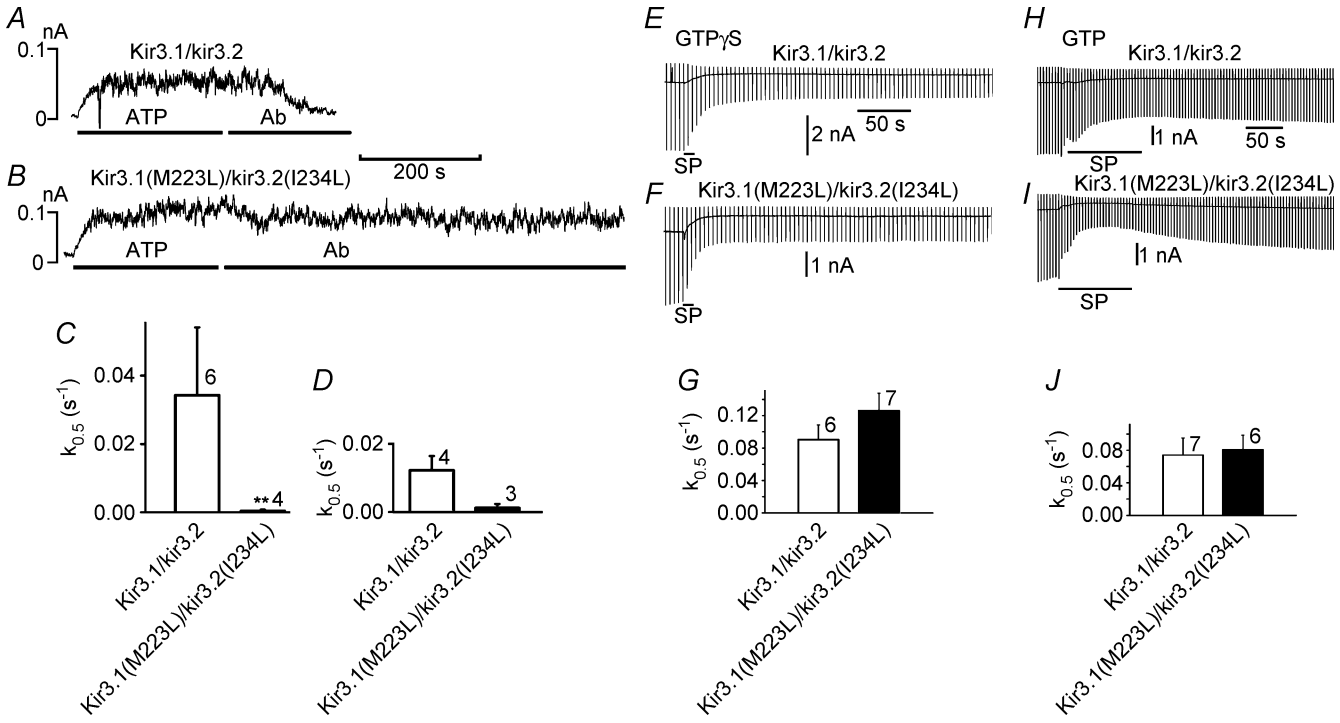

Figure 3. Anti-PIP_2 antibody effects and SP effects on Kir3 channels
A and *B*, an inside-out patch was made from a HEK293A cell transfected with wild type Kir3.1/Kir3.2 plus G$\beta_1\gamma_2$ or Kir3.1(M223L)/Kir3.2(I234L) plus G$\beta_1\gamma_2$. Holding potential, −90 mV. The patch was first perfused with FV solution (Methods), followed by the Mg^{2+}-ATP-containing FV solution (ATP), and finally by anti-PIP_2 antibody-containing FV solution (Ab). *C* and *D*, the antibody produced a very slow inhibition in mutant Kir3.1(M223L)/Kir3.2(I234L). In *C*, purified monoclonal antibody was used at ∼50 nM. $^{**}P < 0.01$. In *D*, a non-purified monoclonal antibody was used at 1 : 50 dilution. $P = 0.06$. *E–G*, SP-induced inhibition in whole-cell recording with pipettes containing GTPγS. *H–J*, SP-induced inhibition with patch-pipettes containing 0.2 mM GTP. The recovery from the SP effect was measured 180 s after the peak of the SP effect: $15.5 \pm 3.3\%$ ($n = 6$) for the wild type and $17.8 \pm 3.3\%$ ($n = 6$) for the mutant. The difference is not significant.

inhibition of Kir2.1(R218Q) should occur faster than that of the wild type Kir3.1/Kir3.2. On the contrary, the time course of SP-induced inhibition of Kir2.1(R218Q) was considerably slower than that of the wild type Kir3.1/Kir3.2 (Fig. 4*C–E*) ($P < 0.001$). The result indicates that SP-induced Kir3 inhibition arises from a mechanism that is independent of PIP_2 depletion.

Kir2.1

According to Zhang *et al.* (1999), the rank order of the binding strength of various Kir2.1 s to PIP_2, as measured by the antibody, is: wild type Kir2.1 > Kir2.1(R228Q) > Kir2.1(R218Q) (Zhang *et al.* 1999). We examined the SP-induced inhibition of Kir2.1 channels with whole-cell recordings using patch pipettes containing GTPγS. In wild type Kir2.1, we usually did not observe a SP-induced inhibition; only in 1 out of 5 cells did SP application result in a very slow inhibition. The mutant Kir2.1(R228Q) responded to SP more readily: 4 out of 7 were clearly inhibited by SP with an approximate value of $k_{0.5}$ between 0.03 and 0.005 s^{-1}. As mentioned in the previous section, the mutant Kir2.1(R218Q), which binds PIP_2 weakly, showed a SP-induced inhibition with a mean $k_{0.5}$ of 0.0178 s^{-1}.

These data, although not amenable to statistical analysis, suggest that the rank order of difficulty of SP-induced channel inhibition is: Kir2.1 > Kir2.1(R228Q) > Kir2.1(R218Q). This rank order is compatible with the known order of binding strength for PIP_2. Thus, as long as we consider only the responses of Kir2.1 and its mutants, the data are compatible with the idea that the SP-induced response originates from PIP_2 depletion.

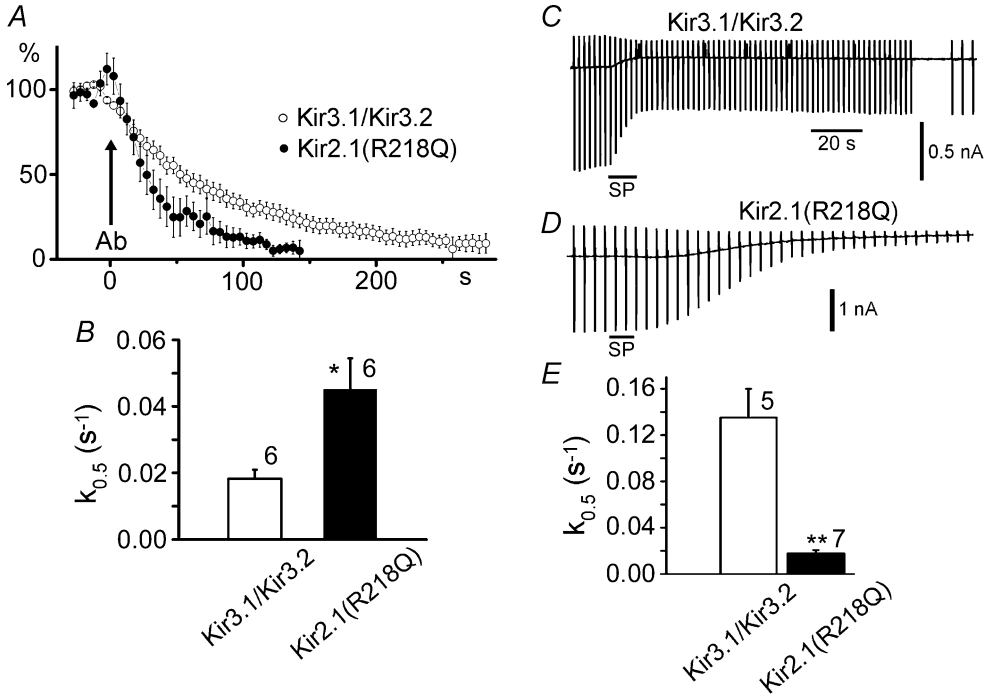

Figure 4. Inactivation of Kir3 and Kir2 activity by anti-PIP_2 antibody and by SP
A and *B*, anti-PIP_2 antibody-induced inactivation of the currents recorded from inside-out patches. Holding potential, −90 mV. After activating the channel with Mg^{2+}-ATP in FV solution, anti-PIP_2 antibody (Ab) in FV solution was applied (time zero). Each symbol on the curves represents normalized mean current amplitude ($n = 6$). A non-purified monoclonal antibody at 1 : 200 dilution was used. $^*P < 0.05$. *C–E*, SP-induced inhibition of the whole-cell current, with the pipette containing 0.2 mM GTPγS. Protocol of DNA transfection was: 0.5 μg SP receptor, 0.2 μg each of Kir3.1, Kir3.2, Gβ_1 and Gγ_2 (or 1.0 μg of Kir2.1(R218Q)), and 0.1 μg of GFP (with total adjusted to 1.6 μg by pCMV5). We have sometimes noticed that SP application produced a small inward current, followed by the conductance decrease (inhibition of Kir channels) in HEK293 cells. Analysis of these transient inward currents in primary cultured neurones indicated that the transient inward current was caused by activation of non-selective cation channels (Farkas *et al.* 1996). We further noticed that an increase in the external divalent cation concentration tended to inhibit the non-selective cation channels. We therefore used a high Ca^{2+} concentration (20 mM) external solution in the hope of diminishing possible activation of non-selective cation channels (both for Kir3.1/Kir3.2 and for Kir2.1(R218Q)) (*C–E*), but the transient inward current still occurred. *E*, summary; $^{**}P < 0.01$. We repeated the same experiment either with the standard conditions (Methods) or with a slight modification (internal Mg^{2+}-ATP was 2 mM, instead of 3 mM). The overall results were: mean of $k_{0.5}$ for wild type Kir3.1/Kir3.2 was 0.103 s^{-1} ($n = 18$) and that for Kir2.1(R218Q) was 0.0228 s^{-1} ($n = 10$).

During this set of experiments, we also recorded the SP-induced inhibition of wild type Kir3.1/Kir3.2 (in the presence of $G\beta\gamma$). The rate for Kir3.1/Kir3.2 was fast ($k_{0.5} = 0.087 \pm 0.011\ s^{-1}$; $n = 8$) and was essentially the same as that for Kir3.1/Kir3.2 in other sets of experiments. Thus, the SP-induced inhibition rate for wild type Kir2.1 as well as that for Kir2.1(R228Q) was much slower than that of Kir3.1/Kir3.2 (in the presence of $G\beta\gamma$).

The conclusion of this section is that (a) if we focus on the behaviour of Kir2.1 and its mutants, the SP-induced inhibition is compatible with the notion that inhibition originates from the depletion of PIP_2, and (b) the SP-induced inhibition of Kir3.1/Kir3.2 is unique and far faster than expected from the depletion of PIP_2.

Role of protein kinase C

The role of protein kinase C (PKC) in the inhibition of Kir3 channels has been a controversial subject. Using the *Xenopus* oocyte expression system, Sharon *et al.* (1997) showed that staurosporine (non-specific protein kinase inhibitor) or bisindolylmaleimide (PKC inhibitor affecting the catalytic domain) almost completely blocks the transmitter-induced inhibition of *basal* Kir3 current. Mao *et al.* (2004) showed that treatment of oocytes with calphostin C (PKC inhibitor acting on the regulatory domain) or chelerythrine (PKC inhibitor acting on the catalytic domain) completely blocks the SP-induced Kir3 inhibition (see also Hill & Peralta, 2001).

However, results obtained using mammalian host cells (HEK293) are different. Leaney *et al.* (2001) report that treatment with bisindolylmaleimide I or Ro-31-8220 (3 μM for at least 5 min) reduces the muscarine-induced inhibition of the *basal activity* of Kir3.1/Kir3.2 channels by ~40–60%. Lei *et al.* (2001) find that application of phorbol ester produces little effect on *activated* (*not basal*) Kir3.1/Kir3.2 currents in HEK293 cells. Nor does the application of bisindolylmaleimide I (5 μM; > 30 min) have any effect on the TRH-induced inhibition of *activated* Kir3 channels.

We tested the effect of bisindolylmaleimide I (3 μM, for ~10–30 min) in the presence of internal GTPγS. As shown in Fig. 5*C* and *D*, application of the inhibitor did not change the rate ($k_{0.5}$) of the SP-induced inhibition of *activated* Kir3.1/Kir3.2 channels (Fig. 5*C*). The magnitude of the SP-induced inhibition became slightly less through the application of bisindolylmaleimide I (but statistically not significant; Fig. 5*D*). These results agree with those of Lei *et al.* (2001). The most conspicuous effect produced by bisindolylmaleimide I was that the Kir3.1/Kir3.2 current itself was reduced considerably (Fig. 5*E*). It is interesting to note that despite the reduced conductance of Kir3.1/3.2 caused by the PKC inhibitor, the mechanism to produce the quick inhibition by SP seems mostly intact. This is in sharp contrast to the effect of the $G\alpha_q$ inhibitor GRK-nt

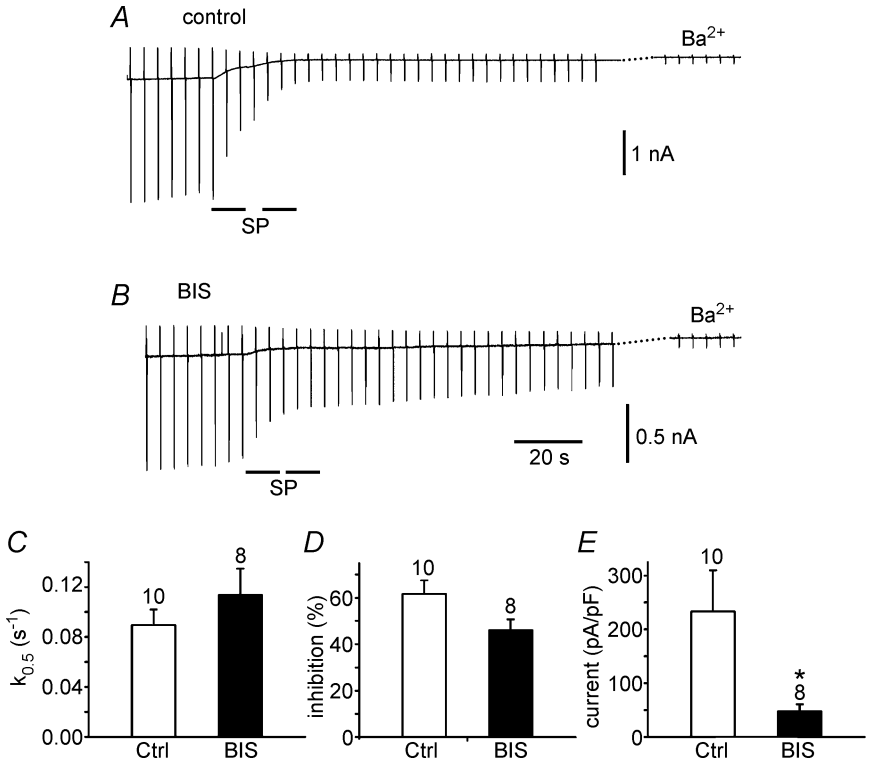

Figure 5. Effects of a PKC inhibitor bisindolylmaleimide I (BIS) on the SP-induced inhibition on the Kir3.1/3.2 current
HEK293 cells were transfected with cDNAs for SP receptor, Kir3.1, Kir3.2, $G\beta_1$ and $G\gamma_2$ (see Methods). *A*, a control cell with 0.1% DMSO in the external solution. See Fig. 2 legend for the sequence of the command voltage pulses. Application of SP (1 μM) inhibited Kir3.1/Kir3.2 current. The current amplitude upon 50 mV hyperpolarization was taken as representing Kir3.1/Kir3.2 current. *B*, BIS-treated cells. *C*, the rate ($k_{0.5}$) of the SP-induced inhibition was not affected by the BIS treatment (3 μM, for 11–36 min). *D*, the magnitude of the SP-induced Kir3.1/Kir3.2 current inhibition relative to control current. *E*, the BIS treatment considerably reduced the Kir3.1/Kir3.2 current (before the SP application). Ordinate: current amplitude produced by a 50 mV hyperpolarization divided by the input capacitance. $^*P < 0.05$.

(Fig. 2), in which the speed of the SP-induced inhibition became slower.

We can summarize the above results from different investigators as follows: when expressed in mammalian cells, the role of PKC in the receptor-mediated inhibition of *strongly activated Kir3 channels* seems to be small, whereas PKC does play a moderate role in inhibiting the *basal activity* of Kir3 channels. On the contrary, PKC does play a substantial role in the inhibition of Kir3 channels expressed in *Xenopus* oocytes.

$G\alpha_q$ physically interacts with Kir3 but not with Kir2

The above results suggest that the SP effect on Kir3.1/Kir3.2 is faster than and of different origin from the SP effect on Kir2. There must be another quicker signalling route for Kir3.1/Kir3.2 inhibition. To elucidate the mechanism of this signalling pathway, we examined the interaction of $G\alpha_q$ with Kir3 or with Kir2 by co-immunoprecipitation. Previously, Simen *et al.* (2001) reported that $G\alpha_q$ and a type of Ca^{2+} channel physically interact with each other.

We found that $G\alpha_q$ co-precipitated with Kir3.2 (Fig. 6*A*), whereas $G\alpha_q$ did not co-precipitate with either Kir2.2 (Fig. 6*B*) or with Kir2.1 (Fig. 6*C*). Both the transition state form (GDP-AlF_4^--bound form) and the GDP-bound form of $G\alpha_q$ were co-precipitated with Kir3.2 (Fig. 6*A*), but not with Kir2.2 or Kir2.1 (Fig. 6*B* and *C*).

Discussion

The present results show that the speed of SP-induced Kir3 inhibition was much faster than predicted from the assumption that Kir3 inhibition originates from PIP_2 reduction. Although the binding strength of Kir3 to PIP_2 was increased considerably (~90 times) by mutation (Kir3.1(M223L)/Kir3.2(I234L)) (Fig. 3), the rate of SP-induced inhibition of the mutant channel was

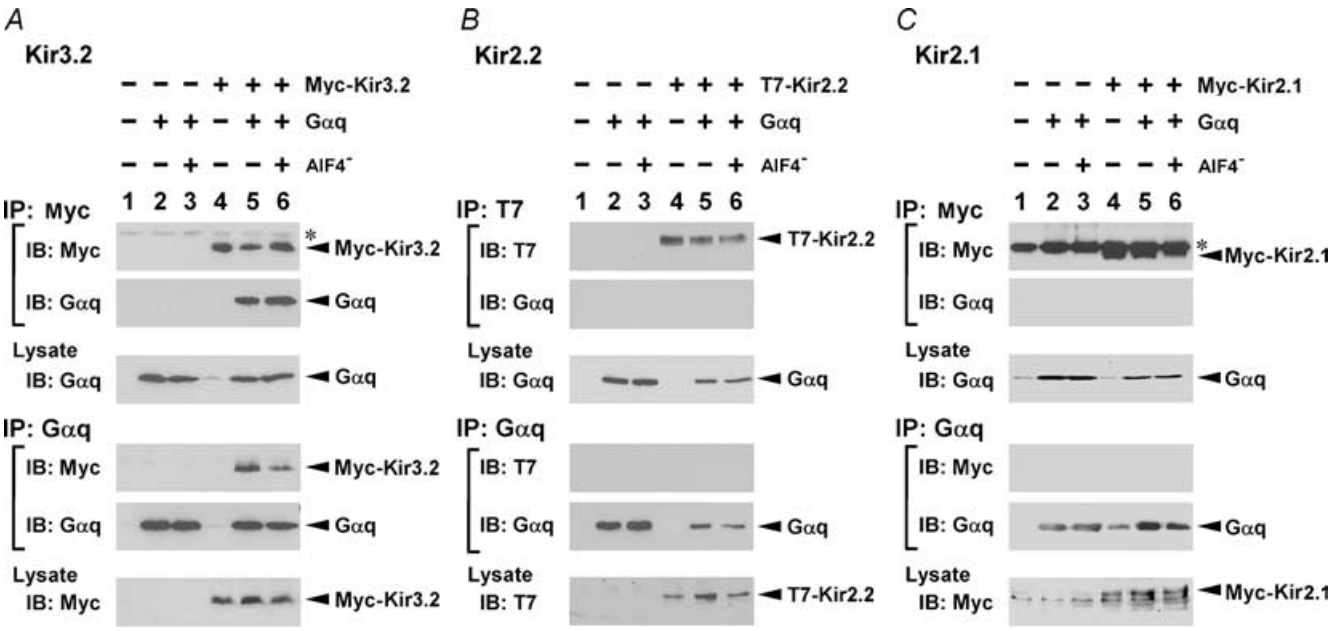

Figure 6. Co-immunoprecipitation between $G\alpha_q$ and Kir3.2, Kir2.2 or Kir2.1
A, $G\alpha_q$ associates with Myc-Kir3.2. (IP: Myc) Anti-Myc antibody co-precipitated $G\alpha_q$ from cells expressing both $G\alpha_q$ and Myc-Kir3.2 (lane 5 and lane 6). (IP: $G\alpha_q$) Anti-$G\alpha_{q/11}$ antibody co-precipitated Myc-Kir3.2 from cells expressing both $G\alpha_q$ and Myc-Kir3.2. Both inactive (without AlF_4^-) and active (with AlF_4^-) forms of $G\alpha_q$ were co-immunoprecipitated with Kir3.2. Experiments were performed using two different protocols: (i) wild type $G\alpha_q$ with AlF_4^- (lane 6) or without AlF_4^- (lane 5), and (ii) constitutively active $G\alpha_q$ (R183C) (without AlF_4^-). The results were the same for the two protocols. A representative result from 4 experiments (2 from each one of the protocols) is shown. *B,* $G\alpha_q$ does not co-precipitate with Kir2.2. (IP: T7) Anti-T7 antibody did not co-precipitate $G\alpha_q$ from cells expressing both $G\alpha_q$ and T7-Kir2.2, while it co-precipitated T7-Kir2.2 from cells expressing T7-Kir2.2 alone (lane 4) or together with $G\alpha_q$ (lane 5 and lane 6, top panel). (IP: $G\alpha_q$) Anti-$G\alpha_{q/11}$ antibody did not co-precipitate T7-Kir2.2 from cells expressing both $G\alpha_q$ and T7-Kir2.2 (lane 5 and lane 6), whereas it co-precipitated $G\alpha_q$ from cells expressing $G\alpha_q$ alone (lane 2 and lane 3) and $G\alpha_q$ with T7-Kir2.2 (lane 5 and lane 6). A representative result from 3 experiments is shown; each includes data with and without GDP-AlF_4^-. In addition two experimental runs were performed using $G\alpha_q$(R183C). These two different protocols produced the same result. *C*, $G\alpha_q$ does not co-precipitate with Kir2.1. A representative result from 5 experiments is shown. * in *A* and *C* indicates IgG heavy chains of anti-Myc antibody.

unchanged. As for experiments on Kir2.1(R218Q) (Fig. 4), the antibody-induced inhibition rate of Kir3.1/Kir3.2 was 2.5 times slower than that of Kir2.1(R218Q) (Fig. 4*B*), whereas the SP-induced inhibition of Kir3.1/Kir3.2 was 7.5 times faster than that of Kir2.1(R218Q). Hence, the SP-induced Kir3 inhibition is ∼19 times faster than expected from PIP_2 binding characteristics. This kind of comparison, assuming a very strong affinity of the antibody to PIP_2 and linear relations over large ranges, would have a considerable quantitative inaccuracy. Nonetheless, it indicates that the rapidity of the SP-induced Kir3 inhibition is by no means trivial. Thus, PIP_2 reduction cannot account for the direct cause for the SP-induced inhibition of Kir3.1/Kir3.2 channels. There must be a mechanism in Kir3 channels which renders them capable of responding so rapidly to the transmitter. It is unlikely that this mechanism involves PKC. The experiments by Lei *et al.* (2001) and ours (Fig. 5) on the PKCδ inhibitor bisindolylmaleimide I indicate that, unlike the results obtained using *Xenopus* oocyts (Sharon *et al.* 1997; Hill & Peralta, 2001; Mao *et al.* 2004), PKC seems to play a relatively small role in the SP-induced inhibition of activated Kir3 channels expressed in mammalian host cells.

Recently, a GFP-tagged PH domain of PLC has been used as an optical probe to observe PIP_2 hydrolysis (Runnels *et al.* 2002; Rohacs *et al.* 2002; Zhang *et al.* 2003; Suh *et al.* 2004). The data show that PIP_2 hydrolysis by receptor activation occurs as fast as the rate of channel inhibition. These data support the notion (Suh & Hille, 2002; Zhang *et al.* 2003; Suh *et al.* 2004) that transmitter-induced inhibition of KCNQ2/3 is primarily caused by depletion of PIP_2. Is our conclusion compatible with these findings? A crucial factor to consider is the large variation in the PIP_2 affinity among different types of channels. The PIP_2 affinity of KCNQ2/3 is much less than that of Kir2.1, with an EC_{50} of ∼90 μM for KCNQ2/3, and ∼5 μM for Kir2.1 (EC_{50} is measured with a water-soluble diC_8-PIP_2; Rohacs *et al.* 2002; Zhang *et al.* 2003). This means that KCNQ2/3 could be inhibited by a small decline of PIP_2, while Kir3 (in the presence of G$\beta\gamma$) or Kir2, with its inherently high PIP_2 affinity (Huang *et al.* 1998), may remain active even with a considerably reduced level of PIP_2. Therefore, the conclusion drawn from KCNQ studies does not necessarily contradict our conclusion from Kir3 studies. Importantly, the optical experiments do not describe the relative amount of PIP_2 remaining inside the membrane after transmitter application.

Previously, Huang *et al.* (1995) and Peleg *et al.* (2002) showed, using the glutathione *S*-transferase (GST) pull down assay, that Gα_i and/or G$\beta\gamma$ interact with the Kir3 N-terminus. Furthermore, Dascal's group (Schreibmayer *et al.* 1996; Peleg *et al.* 2002) has shown that Gα_i inhibits the basal Kir3 activity independent of its ability to sequester G$\beta\gamma$. Thus, Gα_q and Gα_i, despite mediating opposite gross effects on Kir3 channel activity, may actually share certain signalling capabilities. The question is to explain these opposite effects on channel activity despite the fact that both Gα_i and Gα_q seem to be capable of inhibiting channel activity. Furthermore, channel-activating G$\beta\gamma$ is liberated regardless of the G protein system under stimulation. The functional differences may arise simply due to the quantitative strengths of the involved G protein subunits. The capability of Gα_q to inhibit Kir3 is strong enough to nullify the activating effect of G$\beta\gamma$ (Velimirovic *et al.* 1995), whereas channel inhibition by Gα_i is so weak as to be easily overcome by G$\beta\gamma$.

We have presented two novel findings from the present research: (a) a unique mechanism must exist between Gα_q and Kir3.1/Kir3.2 to produce inhibition of Kir3. This mechanism does not exist between Gα_q and Kir2.1; (b) there is a physical interaction between Gα_q and Kir3.2, but not between Gα_q and Kir2.1 or Kir2.2. Furthermore, the literature shows that there is some similarity between Gα_i and Gα_q in their capacity to inhibit Kir3 activity (Peleg *et al.* 2002) and to physically interact with Kir3 (Huang *et al.* 1995; Peleg *et al.* 2002). A simple hypothetical mechanism that explains all these facts might be a signalling pathway that includes a protein–protein interaction between Gα_q and Kir3, resulting in closure of the channel gate.

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