Synergistic Activation of G Protein–gated Inwardly Rectifying Potassium Channels by the βγ Subunits of G Proteins and Na^+ and Mg^{2+} Ions

Jérôme Petit-Jacques, Jin Liang Sui, and Diomedes E. Logothetis

From the Department of Physiology and Biophysics, Mount Sinai School of Medicine of the New York University, New York, New York 10029

abstract Native and recombinant G protein–gated inwardly rectifying potassium (GIRK) channels are directly activated by the βγ subunits of GTP-binding (G) proteins. The presence of phosphatidylinositol-bis-phosphate (PIP_2) is required for G protein activation. Formation (via hydrolysis of ATP) of endogenous PIP_2 or application of exogenous PIP_2 increases the mean open time of GIRK channels and sensitizes them to gating by internal Na^+ ions. In the present study, we show that the activity of ATP- or PIP_2-modified channels could also be stimulated by intracellular Mg^{2+} ions. In addition, Mg^{2+} ions reduced the single-channel conductance of GIRK channels, independently of their gating ability. Both Na^+ and Mg^{2+} ions exert their gating effects independently of each other or of the activation by the $G_{\beta\gamma}$ subunits. At high levels of PIP_2, synergistic interactions among Na^+, Mg^{2+}, and $G_{\beta\gamma}$ subunits resulted in severalfold stimulated levels of channel activity. Changes in ionic concentrations and/or G protein subunits in the local environment of these K^+ channels could provide a rapid amplification mechanism for generation of graded activity, thereby adjusting the level of excitability of the cells.

key words: G protein–gated inwardly rectifying potassium channels • phosphatidylinositol-bis-phosphate • $G_{\beta\gamma}$ gating • Mg^{2+} gating • Na^+ gating

INTRODUCTION

In atrial tissue, acetylcholine released by the vagus nerve binds to muscarinic type 2 receptors, activates K_{ACh} channels via pertussis toxin–sensitive G proteins, and slows the heart rate. Upon activation, the heterotrimeric G protein dissociates, allowing the $G_{\beta\gamma}$ subunits to directly activate the K_{ACh} channel (Logothetis et al., 1987; Krapivinsky et al., 1995b). K_{ACh} has been shown to be composed of two types of G protein–gated inwardly rectifying potassiumb channels (GIRK1 and GIRK4),[1] associated in a heterotetrameric complex (Krapivinsky et al., 1995a; Silverman et al., 1996; Corey et al., 1998). Recombinant (GIRK) channels expressed in oocytes are also directly activated by G protein βγ subunits (Reuveny et al., 1994). In addition, GIRK channels appear to be activated independently of G proteins. In the absence of agonist, ATP hydrolysis leads to an increase in the mean open time and sensitizes channels to gating by Na^+ ions (Sui et al., 1996). Recently, it was shown that the ATP modification of GIRK channels is mediated via phosphatidylinositol phosphates such as phosphatidylinositol-bis-phosphate (PIP_2) (Huang et al., 1998; Sui et al., 1998). PIP_2 has been implicated in the regulation of the sodium–calcium exchanger (Hilgemann and Ball, 1996), the K_{ATP} channel (Hilgemann and Ball, 1996; Fan and Makielski, 1997; Baukrowitz et al., 1998; Shyng and Nichols, 1998), the inwardly rectifying ROMK1 and IRK1 channels (Huang et al., 1998) and other Na^+-gated nonselective cation channels (Zhainazarov and Ache, 1999). Moreover, PIP_2 appears to be essential for GIRK channel activation by the G protein βγ subunits (Sui et al., 1998).

Here, using both native and recombinant GIRK channels, we show that Na^+ as well as Mg^{2+} ions gate the ATP- or PIP_2-modified channels. While the two ions seem to exert their effects at distinct sites on the channel protein, they showed synergistic effects on gating. In the presence of exogenous PIP_2, $G_{\beta\gamma}$ and Na^+ and Mg^{2+} ions showed great synergism in activating the channel. However, in the absence of exogenous PIP_2, preactivation by G protein βγ subunits sensitized the channel to gating by Na^+ but not Mg^{2+} ions. These data suggest that the synergism between Mg^{2+} and $G_{\beta\gamma}$ subunits in gating GIRK channels shows a much greater dependence on PIP_2 levels than the synergism between Na^+ and $G_{\beta\gamma}$. The synergism among ions and $G_{\beta\gamma}$ proteins in the gating of GIRK channels implies that variations of the concentrations of these molecules in the local environment of these channels could play an important role in the "fine tuning" of their activity.

Dr. Sui's present address is Cambridge Neuroscience, Cambridge, MA 02139.

Address correspondence to Diomedes E. Logothetis, Department of Physiology and Biophysics, Mount Sinai School of Medicine of the New York University, New York, NY 10029. Fax: 212-860-3369; E-mail: logothetis@msvax.mssm.edu

[1]*Abbreviations used in this paper:* ACh, acetylcholine; βARK-PH, β-adrenergic receptor kinase plekstrin homology domain; GIRK channel, G protein–gated inwardly rectifying potassium channel; PIP_2, phosphatidylinositol-bis-phosphate.

J. Gen. Physiol. © The Rockefeller University Press • 0022-1295/99/11/673/12 $5.00
Volume 114 November 1999 673–684
Released online 11 October 1999
http://www.jgp.org

MATERIALS AND METHODS

Expression of Recombinant Channels in Xenopus Oocytes

Recombinant channel subunits (GIRK1, GenBank accession No. U39196; GIRK4, GenBank accession No. U39195) were expressed in *Xenopus* oocytes as described previously (Chan et al., 1996). Channel subunit coexpression was accomplished by coinjection of equal amounts of each cRNA (~4 ng). The human muscarinic receptor type 2 was coexpressed with the channel subunits (~1.5 ng injected per oocyte). The β-adrenergic receptor kinase (βARK)–PH construct, altered to incorporate the 15 NH_2-terminal residues of Src for membrane targeting, was generously provided by Dr. E. Reuveny (Weizmann Institute of Science, Rehovot, Israel). cRNA concentrations were estimated from two successive dilutions that were electrophoresed on formaldehyde gels in parallel and compared with known concentrations of a RNA marker (GIBCO BRL). Oocytes were isolated and microinjected as described previously (Logothetis et al., 1992). The oocytes were maintained at 18°C, and electrophysiological recordings were performed 2–6 d after injection at room temperature (20–22°C).

Preparation of Chicken Atrial Myocytes

The procedure used for isolating cardiac myocytes from chicken embryos has been described previously (Sui et al., 1996). In brief, atrial tissue was selected using chicken embryos from eggs incubated 14–18 d. Atrial tissue was incubated for 20–30 min at 37°C in 5 ml of Mg^{2+}- and Ca^{2+}-free PBS supplemented with 1–2% trypsin/EDTA solution (10×, GIBCO BRL). Isolated myocytes were collected by triturating the digested tissue in 5 ml of trypsin-free solution and stored in a high potassium (KB) solution (Isenberg and Klöckner, 1982) at 4°C for up to 36 h. The cells were allowed to settle on poly-lysine–coated coverslips in the recording chamber before experiments.

Reagents

General chemical reagents, including GTP and ATP, were purchased from Sigma Chemical Co. PIP_2 (Boehringer Mannheim) was sonicated on ice for 30 min before application. Purified recombinant G protein subunits dimer $\beta_1\gamma_7$ was kindly provided by Dr. J. Garrison (University of Virginia, Charlottesville, VA). The stock of $\beta_1\gamma_7$ (0.86 μg/μl) was dissolved in 20 mM HEPES, 1 mM EDTA, 200 mM NaCl, 0.6% CHAPS, 50 mM $MgCl_2$, 10 mM NaF, 30 μM $AlCL_3$, 3 mM dithiothreitol (DTT), 3 μM GDP, pH 8.0. The final concentration was 20 nM in a solution containing 0.012% CHAPS, and 20 μM DTT. QEHA peptide (Chen et al., 1995) was kindly provided by Dr. R. Iyengar (Mount Sinai School of Medicine) and was used at a final concentration of 50 μM.

Single-Channel Recording and Analysis

Single-channel activity was recorded in the cell-attached or inside-out patch configurations (Hamill et al., 1981) using an Axopatch 200B amplifier (Axon Instruments). All pipettes used in the experiments were pulled using the WPI-K borosilicate glass (World Precision Instruments) and gave resistances of 2–8 MΩ. All experiments were conducted at room temperature (20–22°C). Single-channel recordings were performed at a membrane potential of −80 mV with acetylcholine (ACh, 5 μM) in the pipette, unless otherwise indicated. Single-channel currents were filtered at 1–2 kHz, sampled at 5–10 MHz, and stored directly into the computer's hard disk through the DIGIDATA 1200 interface (Axon Instruments). PCLAMP (v. 6.03; Axon Instruments) was used for data acquisition.

To remove the vitelline membrane, *Xenopus* oocytes were placed in a hypertonic solution (Stühmer, 1992) for 5 min. Shrunk oocytes were transferred into a V-shaped recording chamber and the vitelline membrane was partially removed, exposing just enough plasma membrane for access with a patch pipette (Sui et al., 1996). This procedure increased the success rate of forming gigaseals.

The pipette solution contained 96 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.35. The bath solution contained 96 mM KCl, 5 mM EGTA, and 10 mM HEPES, pH 7.35. When high concentrations of Mg^{2+} ions (>5 mM) were used in the bath solution, the KCl concentration was reduced accordingly to maintain osmolarity. Gadolinium chloride at 100 μM was routinely added to the pipette solution to suppress native stretch channel activity in the oocyte membrane. For chick atrial cells, the experimental solutions were the same as those used with oocyte recordings, except that the KCl concentration was 140 mM without gadolinium chloride.

Free Mg^{2+} and ATP concentrations were estimated as described previously (Vivaudou et al., 1991). Single-channel records were analyzed using PCLAMP software, complemented with our own analysis routine, as described previously (Sui et al., 1996). Parameters used for single-channel analysis include activity of all channels in the patch (or the total open probability, NP_o), the total frequency of opening (NF_o), and the mean open time (MT_o), and averages over 5-s bins are displayed.

In experiments shown in Fig. 7, where exogenous PIP_2 was applied throughout the experiment (i.e., Fig. 7, A and C), occasional applications of the same ion as a function of time in the experiment were used as control to ascertain that the synergistic effects described were not due to a time-dependent accumulation of PIP_2 in the membrane patch. Similar precautions were taken in the experiments shown in Fig. 4. Experiments used to generate the data shown in these two figures were never longer than 14 min (usually 10–13 min). Na^+ and/or Mg^{2+} ions were applied for 30 s.

RESULTS

MgATP/Na Activation of the K_{ACh} Channel Can Proceed Independently of the Involvement of G Proteins

It has been shown previously that Na^+ ions can stimulate K_{ACh} activity in an ATP-dependent manner in the absence of agonist and internal GTP (Sui et al., 1996). The ATP-dependent modification of the channel is thought to work via the production of membrane phosphoinositide phosphates (e.g., PIP_2), which interact directly with members of the inwardly rectifying K^+ channel family (Huang et al., 1998; Sui et al., 1998). PIP_2 also appears to be essential for G protein regulation of the K_{ACh} channel (Sui et al., 1998).

To further test for a dependence of the MgATP/Na^+ activation on G protein gating of K_{Ach}, we designed experiments where G protein–dependent activation of the channel was impaired. As shown in Fig. 1 A, the K_{ACh} channel in an inside-out atrial myocyte patch was activated persistently by 10 μM GTPγS, a nonhydrolyzable analogue of GTP. Activation of the channel by GTPγS was blocked upon perfusion of the QEHA peptide. QEHA is a 27 amino acid long peptide derived from the COOH terminus of the $G_{\beta\gamma}$-sensitive adenylate cyclase 2 isoform. It has been shown to block $G_{\beta\gamma}$ activation of several different effectors, including the K_{ACh} channel

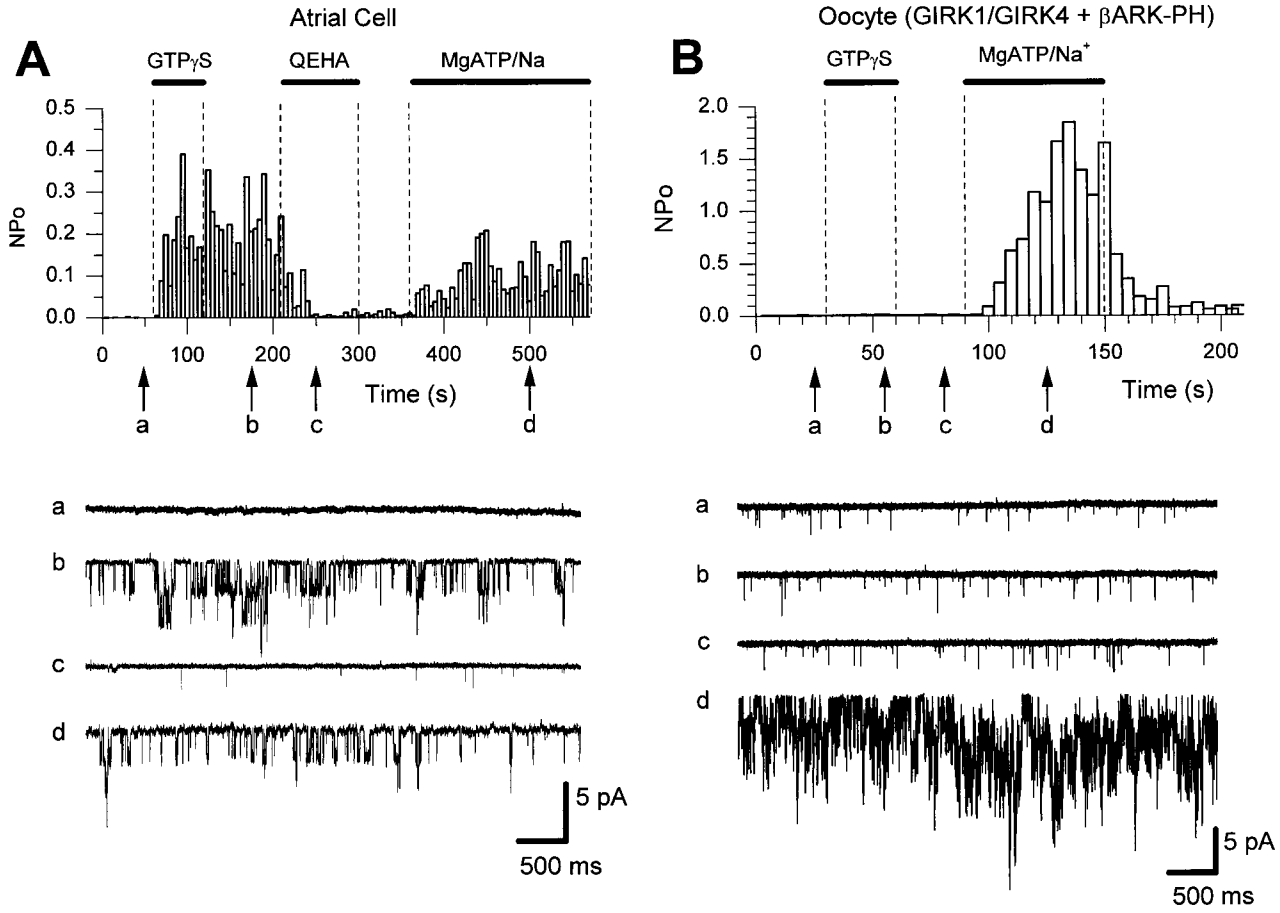

Figure 1. Impairment of G protein signaling does not affect the activation of K_{ACh} by MgATP and Na^+. (A) Single channel activity (top; NP_o, bin = 5 s) plotted as a function of time. The data were obtained from an inside-out patch excised from an atrial cell. The K_{ACh} channel was stimulated by maintaining the membrane at −80 mV and by the presence of 5 μM acetylcholine in the pipette. 10 μM GTPγS, 50 μM QEHA, and 5/20 mM MgATP/Na^+ were applied for the duration indicated by the bars. Sample single-channel currents in each condition at the time marked by the arrows are shown under the plot (bottom). (B) NP_o plot of K_{ACh} channel activity (top, bin = 5 s) in an inside-out patch from an oocyte expressing the human GIRK1/GIRK4 and the construct βARK-PH. The membrane was clamped at −80 mV and 5 μM acetylcholine was present in the pipette. Application of 10 μM GTPγS and 5/20 mM MgATP/Na^+ are illustrated by the bars. Labeled arrows correspond to the sample single-channel currents shown under the plot (bottom).

(Chen et al., 1995). QEHA (50 μM) application abolished the GTPγS activation of K_{ACh} in <2 min (n = 3). After washout, the channel activity remained very low, suggesting the persistence of the QEHA-blocking effect. However, under these conditions, the K_{ACh} channel could be activated by MgATP/Na^+ (5/20 mM). QEHA coapplication with MgATP/Na^+ failed to block channel activation, whereas QEHA did block GTPγS-induced activation in the same oocyte patches (n = 3) (data not shown).

Another way we impaired the G protein regulation of the GIRK channels was by coexpressing them in oocytes with a βγ-binding protein. We used the PH domain of βARK (βARK-PH), which specifically binds the βγ subunits of G proteins, and thus acts as a "βγ sink" (Koch et al., 1993; He et al., 1999). In oocytes coexpressing the recombinant channels GIRK1/GIRK4 and the construct βARK-PH, 10 μM GTPγS did not induce channel activity. This suggests that the βARK-PH protein bound the oocyte endogenous G proteins, such that no βγ subunits were available for channel activation (Fig. 1 B). However, in the same patches, MgATP/Na^+ (5/20 mM) caused a >30-fold increase in channel activity. Summary data revealed that channel activities (NP_o) before, during, and after GTPγS application were similar, 0.0070 ± 0.0039, 0.0077 ± 0.0037, and 0.0097 ± 0.0045, respectively (mean ± SEM, n = 4). During application of MgATP/Na^+, NP_o was 0.313 ± 0.221 (n = 4).

In control experiments using inside-out patches from oocytes of the same batch that coexpressed the recombinant channels GIRK1/GIRK4 alone, GTPγS caused great channel activation (n = 3, data not shown). Similar results were obtained in experiments in which we applied Na^+ ions with PIP_2 rather than MgATP (n = 4, data not shown).

These results suggest that even when G protein regulation is impaired, Na^+ ions are still able to activate the channel. Thus, Na^+ gating of the channel can indeed proceed independently of $G_{\beta\gamma}$ gating.

$G_{\beta\gamma}$ Subunits Sensitize GIRK Channels to Gating by Na^+ Ions

Na^+ ions can gate GIRK channels when membrane PIP_2 levels are maintained (i.e., via hydrolysis of ATP). We next tested under conditions that did not maintain PIP_2 at a constant high level whether Na^+ ions could gate these channels after $G_{\beta\gamma}$ activation.

Fig. 2, A and B, show representative and summary data from experiments where Na^+ ions gated GIRK1/GIRK4 channels after activation by G proteins. Inside-out patches from oocytes expressing these channels showed no channel activity upon application of 20 mM Na^+. This result suggested a low presence of PIP_2 in the membrane. However, this PIP_2 concentration was sufficient to allow persistent channel activation by a brief exposure to 10 μM GTPγS. Reapplication of Na^+ ions produced a more than fourfold increase in the channel activity above the level obtained with GTPγS. It should be noted that the effect of Na^+ ions on the basal channel activity was variable from patch to patch, presumably reflecting different levels of endogenous PIP_2 at the time of Na^+ application.

Na^+ ions also gated GIRK channels after stimulation of activity by purified $G_{\beta\gamma}$ subunits. In Fig. 2, C and D, Na^+ ions (20 mM) applied on an inside-out patch did not affect significantly the basal activity of the channel. After washout of the Na^+ ions, recombinant $G_{\beta1\gamma7}$ was applied on the patch at a concentration of 20 nM, causing a slow channel activation. After washout of $G_{\beta1\gamma7}$, and as activity stabilized, a second application of Na^+ ions produced a more than threefold increase in channel activity, above the level obtained with $\beta_1\gamma_7$. Combined together, these data suggested that the G protein βγ subunits sensitized GIRK channels to gating by Na^+ ions. It has been shown that the mean open time (MT_o) increased in the presence of PIP_2 that is generated by hydrolysis of ATP or exogenous application (Sui et al., 1996, 1998). In the present experiments, no change in the channel MT_o was observed in the different solutions perfusing the patches (data not shown). This suggests that the levels of PIP_2 in the membrane were not altered, and thus could not account for the $G_{\beta\gamma}$-dependent gating effects of Na^+ ions.

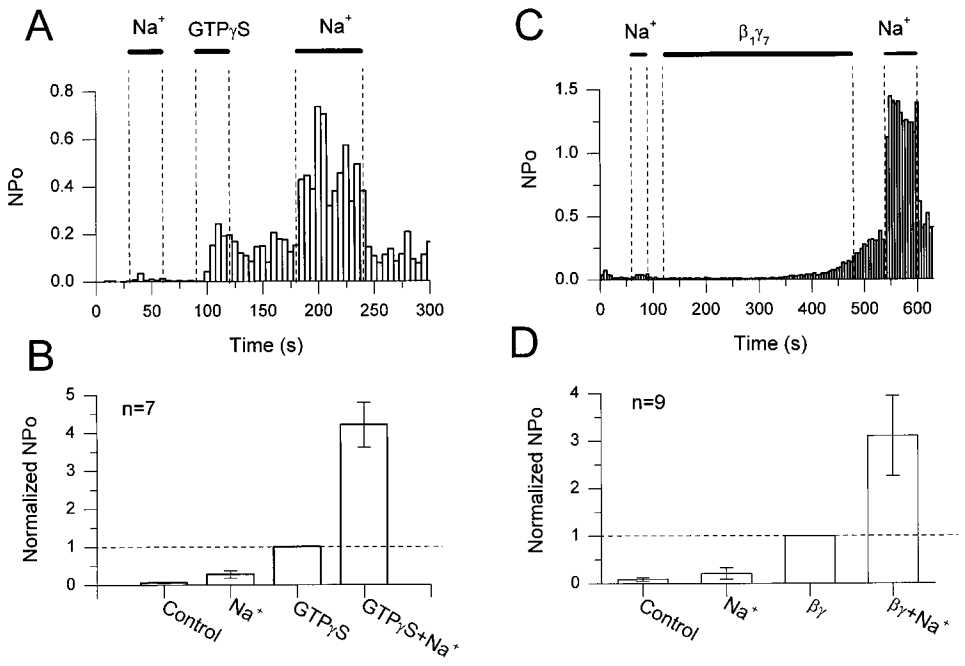

Figure 2. Na^+ ions gate GIRK channels after activation by G protein βγ subunits. (A) Single-channel activity (NP_o, bin = 5 s) plotted as a function of time. The data were obtained from an inside-out patch excised from an oocyte expressing the recombinant channel GIRK1/GIRK4. 20 mM Na^+ and 10 μM GTPγS were applied as indicated by the bars. The membrane was clamped at −80 mV and 5 μM acetylcholine was in the pipette solution. (B) The mean NP_o for seven patches is plotted for different conditions. Steady state channel activity after activation by GTPγS was taken as reference (GTPγS) and NP_o were normalized to it. Na^+ concentration was 20 mM and GTPγS was 10 μM. GTPγS+Na^+ corresponds to the application of 20 mM Na^+ after the washout of the GTP analogue. SEM are indicated by the vertical bars. The normalized mean NP_o was 0.057 ± 0.018 (mean ± SEM) in control solution, 0.277 ± 0.095 in the presence of 20 mM Na^+ ions, 1 after the application of 10 μM GTPγS, and 4.21 ± 0.59 in the presence of 20 mM Na^+ ions after channel activation by GTPγS. (C) NP_o vs. time plot for the channel activity recorded in an inside-out patch from an oocyte expressing GIRK1/GIRK4. 20 mM Na^+ and 20 nM $\beta_1\gamma_7$ purified subunits were applied via the bath as indicated by the bars. V_m = −80 mV. 5 μM acetylcholine was present in the pipette. (D) The mean NP_o for nine patches are plotted for different conditions. Steady state channel activity after $\beta_1\gamma_7$ activation (after $\beta_1\gamma_7$ washout) was taken as reference and NP_o was normalized to it. Na^+ concentration was 20 mM and $\beta_1\gamma_7$ was 20 nM. βγ+Na^+ refers to the application of 20 mM Na^+ after the washout of βγ. The vertical bars represent SEM. The normalized mean NP_o was 0.084 ± 0.039 (mean ± SEM) in control solution, 0.206 ± 0.12 in the presence of 20 mM Na^+ ions, 1 after the application of 20 nM $\beta_1\gamma_7$, and 3.1 ± 0.84 in the presence of 20 mM Na^+ ions after activation of the channel by the G protein subunits.

It has been shown that Li^+ ions stimulate GIRK channels modified by ATP to ~10% the activity level achieved by comparable Na^+ ion concentrations (Sui et al., 1996). However, Li^+ ions were unable to increase the activity of the channel after activation by GTPγS. In three patches, the mean NP_o of the GIRK channel was 0.028 ± 0.013 in control conditions, 0.127 ± 0.035 after application of 10 μM of GTPγS, 0.578 ± 0.15 in the presence of 20 mM Na^+ ions, and 0.099 ± 0.045 in the presence of 20 mM Li^+ ions (data not shown). When applied together, Li^+ ions were also unable to affect the gating of the GIRK channel by Na^+ ions. This suggests that the gating effect of Na^+ ions on the GIRK channel activated by G protein βγ subunits is specific to Na^+ ions.

Mg^{2+} Ions Gate GIRK Channels After Channel Modification by ATP or PIP_2

In certain experiments, 5 mM MgATP increased the activity of the GIRK channels in the absence of Na^+ ions (e.g., Sui et al., 1996). 5 mM MgATP in the solution corresponds to a free Mg^{2+} ion concentration of ~2.1 mM (Vivaudou et al., 1991). This observation prompted us to test whether Mg^{2+} ions alone were able to gate the channel that had been modified by ATP. In Fig. 3, in an inside-out patch from an oocyte coexpressing the channel subunits GIRK1/GIRK4, Mg^{2+} ions (10 mM) had no significant effects on channel activity in the absence of ATP. After washout of Mg^{2+}, the channel was activated by the combination of MgATP (2.5 mM; corresponding to ~1.1 mM free Mg^{2+}) and Na^+ ions (20 mM). MgATP application was maintained and, upon withdrawal of Na^+ ions, channel activity became comparable to basal levels. Application of Mg^{2+} ions (10 mM), in the continuous presence of MgATP (2.5 mM), increased channel activity to levels similar to those obtained with Na^+ ions (as confirmed by sequential application of 10 mM of each of the ions at the end of the experiment). Withdrawal of Mg^{2+} ions caused channel activity to return to basal levels ($n = 3$). The MT_o of the channel activity was increased from ~1 to ~2 ms by the application of MgATP, but was not further modified during the gating by Mg^{2+} or Na^+ ions.

Using PIP_2, we could test the ability of different Mg^{2+} concentrations to activate the GIRK channels. Fig. 4 represents normalized activity of GIRK channels for different concentrations of Mg^{2+} ions. The NP_o for each concentration was calculated in reference to the NP_o measured at 1 mM Mg^{2+}. Mg^{2+} ions could activate the GIRK channels at concentrations as low as 100–300 μM. Maximal activity could be obtained at a concentration of ~7 mM Mg^{2+}. At higher concentrations (e.g., 20 mM), Mg^{2+} ions resulted in a decrease of channel activity relative to lower concentrations (e.g., 7 mM). It has been shown that, at high concentrations, divalent cations can trigger aggregation of PIP_2 molecules (Flanagan et al., 1997), a result that could account for the effects of high Mg^{2+} concentrations on channel activity.

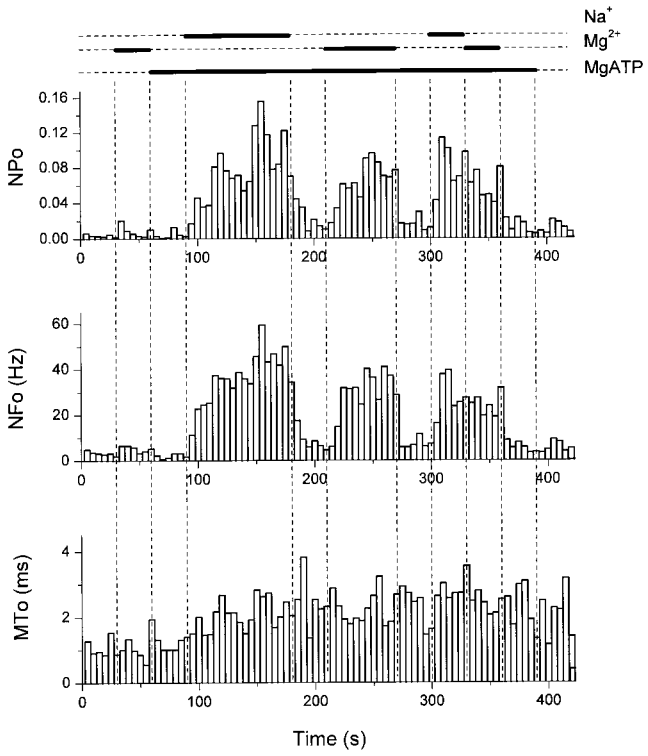

Figure 3. Mg^{2+} ions gate the ATP-modified GIRK channels. NP_o, NF_o, and MT_o plots (bin = 5 s) of GIRK channel activity in an inside-out patch excised from an oocyte expressing GIRK1/GIRK4. 10 mM Mg^{2+}, 2.5 mM MgATP, and 20 mM Na^+ were applied via the bath as indicated by the bars. The membrane was clamped at −80 mV and 5 μM acetylcholine was present in the pipette. NP_o, NF_o, and MT_o in 10 mM Mg^{2+} were calculated such that the amplitude levels were set to match the reduced amplitude of the channel openings in 10 mM Mg^{2+}.

In another set of experiments, we showed that Mg^{2+}, like Na^+ gating, can occur independently of G proteins. Patches excised from oocytes coexpressing the βARK-PH domain and GIRK channels were exposed to PIP_2 (2.5 μM) and subsequently to Mg^{2+} ions. In these patches, GTPγS (10 μM) was unable to activate the GIRK channels, giving a NP_o of 0.08 ± 0.03, identical to the NP_o measured in PIP_2 (0.078 ± 0.02). Mg^{2+} ions (1 mM) could increase the channel activity approximately sixfold ($n = 4$, data not shown) above the activity measured in PIP_2, showing that Mg^{2+} gating could proceed independently of $G_{\beta\gamma}$ gating.

These results suggest that when modified by ATP or PIP_2, GIRK channels become sensitive to either Na^+ or Mg^{2+} ions.

Mg^{2+} Ion Gating Occurs at a Site Distinct from that of Na^+ Action

Recent work has identified an aspartate amino acid residue as the site of action of Na^+ ions on GIRK channels,

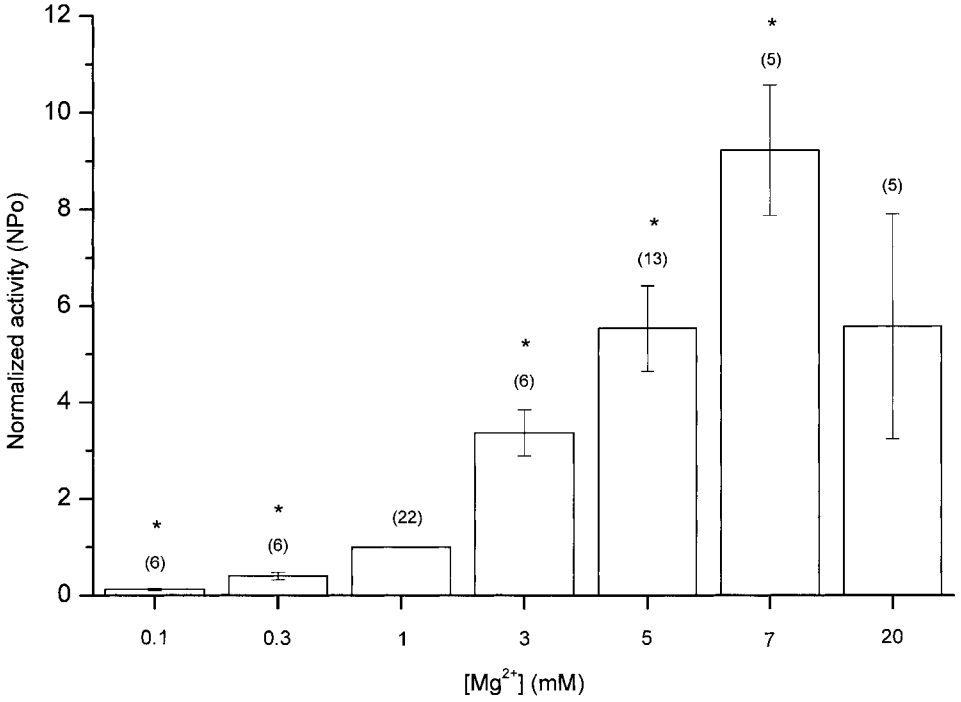

Figure 4. Concentration dependence of GIRK channel activation on internal Mg^{2+} ions. Normalized activity (NP_o) of GIRK1/GIRK4 channels is plotted for different Mg^{2+} concentrations. Inside-out patches were exposed to 2.5 μM PIP_2 before and during application of Mg^{2+}. Responses were expressed in fold increase above the activity in the presence of PIP_2 and were normalized to those recorded in the presence of 1 mM Mg^{2+}. Vertical bars represent SEM. The responses obtained at the low concentrations of Mg^{2+} tested (<1 mM) were significantly higher than those with PIP_2 alone ($P < 0.05$, paired t test). The holding potential was at −80 mV. 5 μM ACh was present in the pipette. *Significant differences from 1 mM Mg^{2+} ($P < 0.01$; paired t test).

GIRK2 (D228) and GIRK4 (D223) (Ho and Murrell-Lagnado, 1999; Zhang et al., 1999). Moreover, it was shown that Na^+ sensitivity lies entirely with the heteromeric partners of GIRK1, as this channel possesses an asparagine instead of an aspartate residue at the equivalent position. We used the point mutant GIRK4(S143T) (referred to as GIRK4*) that allows for high levels of activity of homotetrameric GIRK4 channels (Vivaudou et al., 1991) to test for Na^+ and Mg^{2+} sensitivity. GIRK4* channel activity shows high sensitivity to both Na^+ and Mg^{2+}. Fig. 5 shows that indeed GIRK4*(D223N) loses its sensitivity to Na^+ ions (20 mM). However Mg^{2+} ion (1 mM) sensitivity was intact (Fig. 5 A). Summary data are shown in Fig. 5 B. These data indicate that Na^+ and Mg^{2+} ions act at distinct sites to activate GIRK channels.

Mg^{2+} Ions Reduce the Conductance of the GIRK Channels

We observed, particularly at high concentrations (>5 mM), that internal Mg^{2+} ions reduced the amplitude of single GIRK channel currents. In Fig. 6 A, the activity of the coexpressed channel subunits GIRK1/GIRK4 from an inside-out patch was recorded at −120 mV. After activation by 10 μM GTPγS, channel activity was recorded in a solution containing 1 mM Mg^{2+} ions, showing an approximate amplitude of −3.2 pA. When the solution applied to the patch was switched to one containing 20 mM Mg^{2+} ions, the amplitude of the single openings was rapidly reduced to a lower value, approximately −2.5 pA ($n = 5$). In Fig. 6 B, the activity of native K_{ACh} channels in an inside-out patch from an atrial cell was recorded at −90 mV. After exposure to 5 μM PIP_2, the patch was perfused with a solution containing 20 mM Mg^{2+} ions, giving an amplitude of approximately −2.2 pA. When the solution applied to the patch was switched to one containing 20 mM Na^+ and 1 mM Mg^{2+} ions, the channel amplitude immediately increased to a value of approximately −3.5 pA. This amplitude was also obtained in control conditions, where 1 mM Mg^{2+} ions were present ($n = 5$). The reduction in the single-channel amplitude was observed at various voltages. Since it was present at negative potentials (i.e., −80, −90, and −120 mV) where no rectification occurs, it is likely to proceed by a mechanism distinct from that of the rectification phenomenon. Mg^{2+} ions at high concentrations also decreased the amplitude of GIRK single channels when applied together with Na^+ ions (data not shown). Thus, regardless of their ability to gate GIRK channels (see Figs. 3 and 7), Mg^{2+} ions at high concentrations (>5 mM) show a clear inhibition on single-channel current amplitudes. These data suggest that the inhibitory effect of Mg^{2+} ions on the single-channel amplitude was not dependent on their ability to gate the channel.

Synergistic Interactions among Ions and G Protein Subunits in Gating GIRK Channels

ATP modification of GIRK channels (native or recombinant) is likely to proceed through changes in the level of membrane PIP_2 in the local environment of the channel (Huang et al., 1998; Sui et al., 1998). In Fig. 7

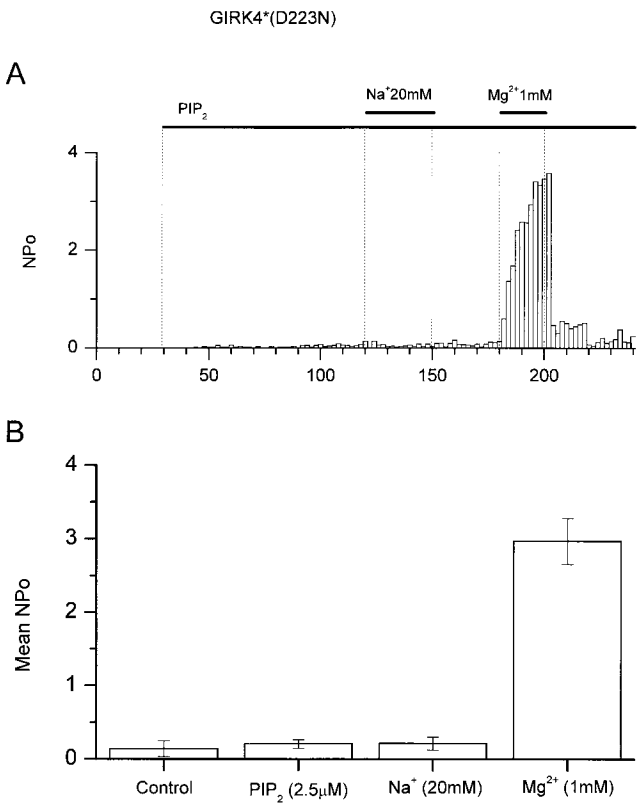

Figure 5. Mg^{2+} ions act at a site distinct of that used by Na^+ ions. (A) Single channel activity (NP_o, bin = 5 s) plotted as a function of time. An inside-out patch from an oocyte expressing GIRK4*(D223N) (see text) and human muscarinic receptor type 2 receptor was exposed to 2.5 μM PIP_2 and 20 mM Na^+ or 1 mM Mg^{2+} ions, and channel activity was recorded. The membrane potential was kept at −80 mV. The pipette solution contained 5 μM ACh. (B) Summary data plotting mean NP_o of four experiments such as that shown in A. The mean NP_o values were 0.14 ± 0.1 (mean ± SEM) in control conditions, 0.21 ± 0.06 in the presence of PIP_2, 0.21 ± 0.08 in the presence of PIP_2 and Na^+ ions, and 2.97 ± 0.31 in the presence of PIP_2 and Mg^{2+} ions.

A, the activity of recombinant GIRK1/GIRK4 channels was not increased by the application of 2.5 μM PIP_2 alone. Mg^{2+} ions (10 mM), applied with PIP_2, stimulated activity >40-fold. As shown previously (Sui et al., 1998), Na^+ ions (10 mM) were able to gate the channel in the presence of PIP_2, resulting in activity equivalent to that obtained with Mg^{2+} ions. When applied together, in the presence of PIP_2, Mg^{2+} and Na^+ ions (10 mM each) showed a synergistic effect stimulating channel activity >200-fold.

We next tested whether Mg^{2+} ions, like Na^+ ions (Fig. 2), can further gate GIRK channels after channel activation by GTPγS, under conditions that do not maintain PIP_2 at constant high levels. Fig. 7 B shows that the basal activity of GIRK1/GIRK4 recombinant channels was not affected by Mg^{2+} ions. 10 μM GTPγS increased the activity of the channel approximately fivefold above basal levels. After GTPγS washout, the channel activity was stable and, when applied to the patches, Mg^{2+} ions were unable to increase channel activity further. In contrast, Na^+ ions (10 mM) increased activity by another twofold above the GTPγS effect. When Mg^{2+} ions were applied together with Na^+ ions, no further increase in channel activity above the levels obtained with Na^+ ions was seen. Thus, G protein activation sensitized the GIRK channels to gating by Na^+ ions, but not Mg^{2+} ions.

In Fig. 7 C, we show the effects of Mg^{2+} and Na^+ ions after stimulation of the channel by GTPγS under conditions that kept PIP_2 at a constant high level. As shown earlier, in the absence of Mg^{2+} and Na^+ ions, PIP_2 was not able to increase the basal activity of the GIRK channels. When Mg^{2+} ions (10 mM) were applied to the patches in the presence of PIP_2, a greater than eightfold increase over control or PIP_2 activity levels occurred. Mg^{2+} and Na^+ ions (each 10 mM) in combination could raise channel activity by 50-fold over control levels. We then applied GTPγS and studied the effects of ions on G protein–stimulated channel activity in the continuous presence of PIP_2. GTPγS was able to activate the channel >14-fold above control basal levels. After washout of GTPγS, channel activity was stable. When Mg^{2+} ions (10 mM) were applied to the patches after the GTPγS treatment in the continuous presence of PIP_2, they could enhance channel activity to levels >100-fold higher than those obtained under control conditions. Thus, in the continuous presence of PIP_2, this high level of activity was greater than that obtained with Mg^{2+} or GTPγS alone or their sum, suggesting synergistic interactions among the three molecules. Finally, when Mg^{2+} and Na^+ ions were applied together, the channel total activity was increased 400-fold compared with control.

Similar data were obtained when the G protein $\beta_1\gamma_7$ subunits rather than GTPγS were used. In three cells, the total channel activity measured as the mean NP_o was 0.027 ± 0.023 in control conditions, 0.022 ± 0.02 in the presence of 2.5 μM PIP_2, 0.12 ± 0.09 in the presence of PIP_2 and 10 mM Mg^{2+} ions, and 1 ± 0.55 in the presence of PIP_2 and Mg^{2+} and Na^+ ions. When 20 nM $\beta_1\gamma_7$ was applied in the presence of PIP_2, it gave a steady state activity of the channel corresponding to a mean NP_o of 0.25 ± 0.12. In the continuous presence of PIP_2 and after stimulation of the channel by βγ subunits, the mean NP_o was 1.23 ± 0.23 in the presence of Mg^{2+} ions and 2.61 ± 0.24 in the presence of Mg^{2+} and Na^+ ions. It should be noted that the differences in channel activity (mean NP_o) for the same condition applied to the patches (for example PIP_2 + Mg^{2+} in Fig. 7, A and C) may be related to differences in the level of channel expression between different batches of oocytes. Taken together, these data make four points. (a) Mg^{2+} ions can gate the channel after modification by

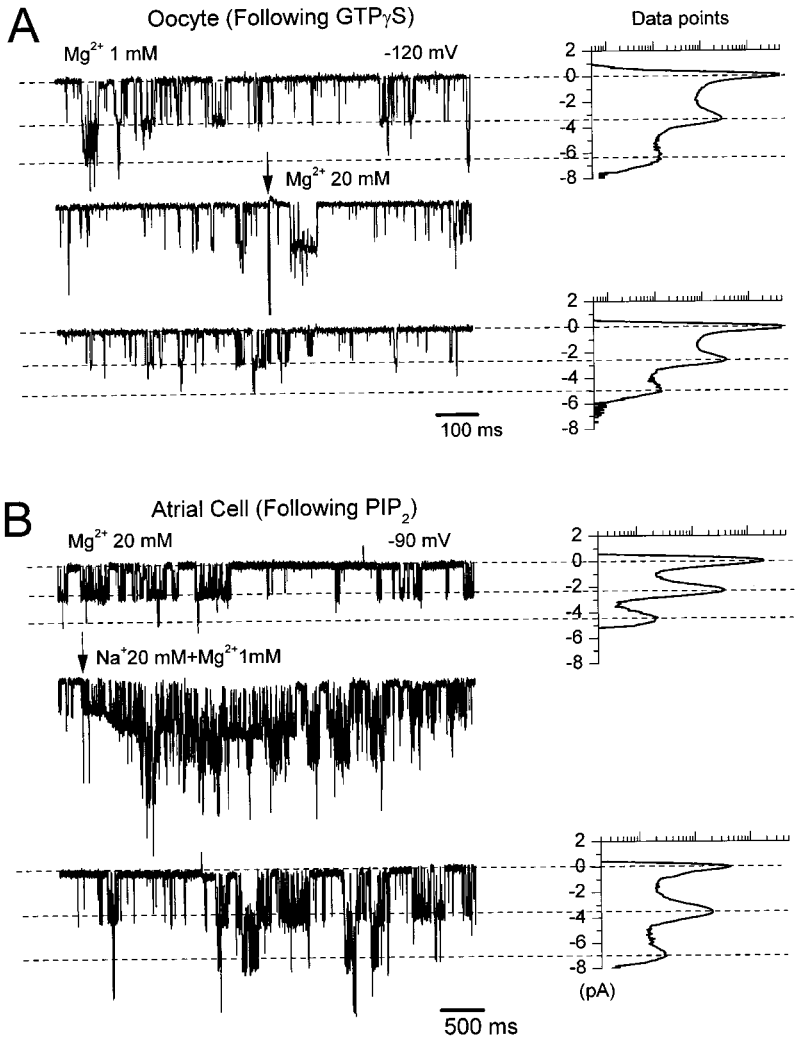

Figure 6. High concentrations of Mg^{2+} ions reduce the GIRK single-channel currents. (A) Single-channel records of GIRK channels in an inside-out patch excised from an oocyte expressing GIRK1/GIRK4. The membrane was clamped at −120 mV and 5 μM acetylcholine was present in the pipette. The channel was preactivated by 10 μM GTPγS and the current traces shown were recorded after washout of the GTP analogue. The switch between bath solutions containing 1 and 20 mM Mg^{2+} is visualized by the arrow (and by the corresponding electrical artifact on the record) on top of the second current trace. Associated all-point histogram plots indicate the amplitudes resulting from the various activity levels ranging from closed to multiple open levels and are shown for the first (1 mM Mg^{2+}) and third (20 mM Mg^{2+}) current traces. Data points are shown on a logarithmic scale ranging from 5 to 50,000. (B) K_{ACh} channel activity in an inside-out patch from a cardiac cell. The membrane was held at −90 mV and the pipette contained 5 μM acetylcholine. The patch was preincubated with 5 μM PIP_2 and the current traces shown were recorded after the washout of PIP_2. The arrow on top of the second current trace visualizes the switch between bath solutions containing 20 mM Mg^{2+} and 20 mM Na^+ + 1 mM Mg^{2+}. Associated all-point histogram plots are shown for the first (20 mM Mg^{2+}) and third (20 mM Na^+ + 1 mM Mg^{2+}) current traces. Data points are shown on a logarithmic scale ranging from 20 to 50,000.

PIP_2. At a concentration of 10 mM, their gating potency is comparable with that of 10 mM Na^+ ions. (b) When applied together, Mg^{2+} and Na^+ ions show synergistic effects, resulting in levels of activity higher than those induced by each of the ions separately or their summed responses. (c) After activation by GTPγS (in the absence of exogenous PIP_2 or MgATP), the GIRK channels are not further gated by Mg^{2+} ions, suggesting an important difference between Mg^{2+} and Na^+ ions in gating these channels. (d) After channel modification by the combination of PIP_2 and GTPγS, Mg^{2+} ions do stimulate the GIRK channel activity to higher levels than those obtained with PIP_2 alone, suggesting that PIP_2 renders the βγ-activated channels sensitive to gating by Mg^{2+} ions.

DISCUSSION

In the present study, we have shown that Mg^{2+} ions at physiological concentrations are additional activators of G protein–gated potassium channels. These K^+ channels can be activated independently either by the βγ subunits of GTP-binding proteins (Logothetis et al., 1987) or by intracellular ions, such as Na^+ (Sui et al., 1996) or Mg^{2+} ions. Activation by either G protein subunits or ions shows an absolute dependence on the presence of PIP_2 (Sui et al., 1998). Specific combinations of these molecules show synergism and suggest differential dependence on the level of PIP_2 for channel activation. This complex dependence of K^+ channel activity on G proteins, Mg^{2+}, Na^+ ions, and PIP_2 could serve to "fine tune" channel activity during physiological and pathophysiological conditions, where changes in the relative concentrations of these molecules might occur.

GIRK Channel Activation by Na^+ Ions Can Be Independent of G Protein Subunit Involvement

Previous results from our laboratory showed that intracellular solution containing MgATP/Na^+ was able to stimulate K^+ channel activity in the absence of acetylcholine in the pipette, suggesting a G protein–independent mechanism of activation (Sui et al., 1996). Subse-

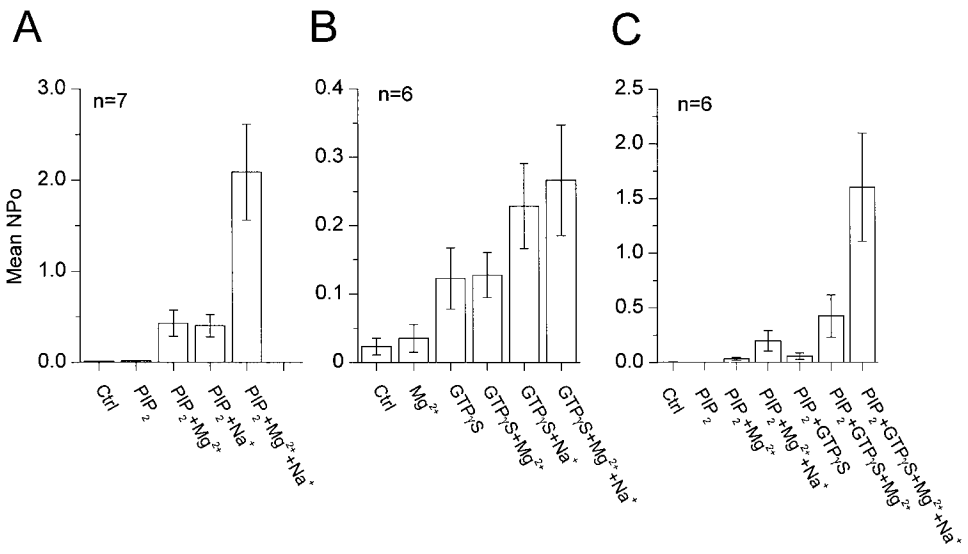

Figure 7. Synergistic effects of $G_{\beta\gamma}$, Na^+, and Mg^{2+} ions in activating GIRK channels. (A) The mean NP_o for seven patches are plotted for different conditions. The data were obtained from inside-out patches excised from oocytes expressing the recombinant channel GIRK1/GIRK4. The membrane was held at −80 mV and 5 μM acetylcholine was present in the pipette. PIP_2 was 2.5 μM, Mg^{2+} was 10 mM, and Na^+ was 10 mM. SEM are indicated by the vertical bars. The mean NP_o for the channel activity was 0.011 ± 0.003 in control conditions, and 0.01 ± 0.006 during the application of 2.5 μM PIP_2. When 10 mM Mg^{2+} ions were applied with PIP_2, the mean NP_o was 0.43 ± 0.14. 10 mM Na^+ ions gave a mean NP_o of 0.40 ± 0.12. When applied together, in the presence of PIP_2, Mg^{2+} and Na^+ ions (10 mM each) yielded a mean NP_o of 2.08 ± 0.52. (B) Mean NP_o plots for six inside-out patches from oocytes expressing GIRK1/GIRK4. V_m was −80 mV. 5 μM acetylcholine was in the pipette. Mg^{2+} was 10 mM, Na^+ was 10 mM, and GTPγS was 10 μM. The columns GTPγS and GTPγS+x depict the channel activity measured after the GTP analogue was washed out and substance(s) x were added. SEM are indicated by vertical bars. The mean NP_o of the channel was 0.023 ± 0.012 in control conditions and 0.035 ± 0.02 in the presence of 10 mM Mg^{2+} ions. 10 μM GTPγS gave a mean NP_o of 0.12 ± 0.045. After GTPγS washout, Mg^{2+} ions gave a mean NP_o of 0.12 ± 0.03. 10 mM Na^+ ions gave a mean NP_o of 0.23 ± 0.06. Coapplication of Mg^{2+} and Na^+ ions resulted in a mean NP_o of 0.26 ± 0.08. (C) Mean NP_o plots for six patches. The inside-out patches were excised from oocytes expressing GIRK1/GIRK4. V_m was −80 mV. 5 μM ACh present in the pipette. PIP_2 was 2.5 μM, Mg^{2+} was 10 mM, Na^+ was 10 mM, and GTPγS was 10 μM. PIP_2+GTPγS refers to the channel activity (at steady state) during the application of the GTP analogue. PIP_2+GTPγS+x columns depict the channel activity measured after the washout of the GTP analogue and addition of substance(s) x. In absence of 10 mM Mg^{2+}, all solutions contained 50 μM Mg^{2+}. This low concentration of Mg^{2+} was necessary to render GTPγS effective. Vertical bars represent SEM. The mean NP_o for the channel activity was 0.004 ± 0.002 in control conditions and 0.0007 ± 0.0002 in the presence of 2.5 μM PIP_2. When 10 mM Mg^{2+} ions were applied to the patches in the presence of PIP_2, a mean NP_o of 0.034 ± 0.013 was obtained. Although this activity appeared small, it was significantly higher than that in PIP_2 alone ($P < 0.005$, paired *t* test, log scale). 10 mM each of Mg^{2+} and Na^+ ions in combination yielded a mean NP_o of 0.2 ± 0.09. GTPγS gave a mean NP_o of 0.058 ± 0.03. Again, although this activity appeared relatively small, it was significantly higher than that in PIP_2 alone ($P < 0.005$, paired *t* test, log scale). When 10 mM Mg^{2+} ions were applied to the patches after the GTPγS treatment a mean NP_o of 0.42 ± 0.19 was obtained. Mg^{2+} and Na^+ ions applied together resulted in NP_o of 1.6 ± 0.49.

quently, it was further demonstrated that the ATP dependence of G protein–sensitive K^+ channels, as well as of other inwardly rectifying channels, involved phosphoinositide formation, particularly PIP_2 (Huang et al., 1998; Sui et al., 1998). In addition, it was reported that G protein activation of the K^+ channel showed an absolute dependence on PIP_2 (Sui et al., 1998). In the present study, we show that impairment of G protein subunit activation of the channel (by binding and competing away $G_{\beta\gamma}$ from the channel with either QEHA perfusion or βARK-PH coexpression) did not prevent the MgATP/Na^+ stimulation of activity (Fig. 1). Thus, we have provided further evidence that Na^+ ion gating of the channel modified by ATP (or PIP_2) can be independent of G protein subunit activation.

Mg^{2+} Gating of the G Protein-gated K^+ Channel

Mg^{2+} ions have been shown to play an essential role in the rectification properties of inwardly rectifying K^+ channels. Unitary current–voltage relations for G protein–sensitive K^+ channels become ohmic if the internal face of the patch is exposed to Mg^{2+}-free solutions. Inward rectification is restored when Mg^{2+} is reintroduced in the bathing solutions (Matsuda, 1991; Kurachi et al., 1992; Nichols and Lopatin, 1997).

Mg^{2+} ions are involved in many other reactions as essential cofactors. Kurachi et al. (1986) showed that G protein activation of the native G protein–sensitive K^+ channel was absolutely dependent on Mg^{2+}, possibly due to the requirement of Mg^{2+} for the binding of GTP to the G_α subunit (also see Logothetis et al., 1987). More recently, it has been appreciated that Mg^{2+}-dependent processes of ATP hydrolysis (likely to be involved in phosphorylation–dephosphorylation of phosphoinositides) regulate channel activity (Sui et al., 1996, 1998; Huang et al., 1998).

Our present data show that Mg^{2+} ions, in addition to their involvement in the processes mentioned above, are able to activate the ATP- or PIP_2-modified G protein–sensitive channel (Figs. 3 and 7). In the presence of PIP_2, similar concentrations of Mg^{2+} and Na^+ ions

yielded comparable levels of channel activity, suggesting equivalent gating abilities for both ions. Since PIP_2 mimics the MgATP effects on the channel, we have been able to study directly Mg^{2+} gating effects.

Mutation of the amino acid responsible for Na^+-ion activation of GIRK channels did not interfere with Mg^{2+}-ion activation. This result strongly suggests that Mg^{2+} and Na^+ ions act on distinct sites to gate the channel.

Our data also show that Mg^{2+} ions reduced the conductance of the G protein–gated channels in a manner independent of their stimulatory effect on gating. Since this inhibitory effect of Mg^{2+} ions on conductance was present at negative potentials (-120, -90, and -80 mV), where no rectification is occurring (Kurachi et al., 1992), it is unlikely that the two processes proceed through a single mechanism. This effect of partial block on channel conductance suggests that Mg^{2+} ions act at a site located very near the pore. Chuang et al. (1997) described a chronic inhibition of the IRK3 inward rectifier channel by internal Mg^{2+} ions, which is independent of the rectification process and is voltage independent. However, the on and off rates of this inhibition were slow (in the minute range) and no reduction of the single-channel conductance was reported. Under our conditions, the blocking effect of Mg^{2+} ions occurred much more rapidly, in the range of seconds (Fig. 6).

Synergism Among G Proteins and Ions in the Gating of GIRK Channels

At higher PIP_2 concentrations, the combination of Na^+ and Mg^{2+} ions resulted in a stimulation of channel activity that was greater than the sum of their individual effects, suggesting synergistic interactions of these ions on channel gating (Fig. 7 A).

Na^+ ions gate the K^+ channel in the presence of hydrolyzable ATP or PIP_2 (Sui et al., 1996, 1998; Figs. 3 and 7 A). In the present study, we show that (shortly after patch excision in solutions that do not replenish or supply PIP_2) application of $G_{\beta\gamma}$ subunits, but not of Na^+ or Mg^{2+} ions, results in stimulation of channel activity (Figs. 2 and 7 B). Our previous study (Sui et al., 1998) showed that in the absence of PIP_2 in the membrane (e.g., by its complete hydrolysis by exogenous $PLC\beta_2$) no gating molecule (e.g., $G_{\beta\gamma}$ or Na^+) could activate the channel. In the present experiment under conditions that we do not expect to have depleted PIP_2, $G_{\beta\gamma}$ subunits caused a much greater stimulation of activity than Na^+ or Mg^{2+} ions. This result suggests that the dependence on PIP_2 for channel gating is greater for Mg^{2+} and Na^+ than for $G_{\beta\gamma}$. Under these conditions, we find that Na^+ ions do stimulate channel activity after preactivation by GTPγS or by purified $G_{\beta\gamma}$ subunits (Figs. 2 and 7 B). This result suggests that $G_{\beta\gamma}$ activation sensitizes the K^+ channel gating to Na^+ ions. Moreover, in such experiments, $G_{\beta\gamma}$ subunits and Na^+ ions act synergistically in gating the channel. Interestingly, Mg^{2+} ions were unable to gate the channel after channel preactivation by GTPγS. These data underscore an interesting difference in the gating of this channel by ions, namely at low PIP_2 levels $G_{\beta\gamma}$ subunits synergize with Na^+ but not Mg^{2+} ions to gate the channel.

This difference of the two ions on channel gating is lost at higher PIP_2 concentrations (Fig. 7 C). In such experiments, not only were the synergistic effects of the ions shown in Fig. 7 A reproduced, but also Mg^{2+} as well as Na^+ ions cooperated with $G_{\beta\gamma}$. When applied in combination, all three gating particles showed synergistic effects (Fig. 7 C).

We have previously shown that block of the Na^+/K^+ pump activates K_{ACh} in atrial myocytes with kinetics similar to those seen for Na^+ accumulation resulting from the block of the pump (Sui et al., 1996). Thus, it is likely that the effects of cardiac glycosides on cardiac rhythm involve the Na^+-sensitive K_{ACh} channels. Under physiological conditions, local variations of $[Na^+]_i$ (e.g., during an action potential) and possibly $[Mg^{2+}]_i$ could provide a sensitive and fast control of the GIRK channel gating and activity. The synergism among ions and $G_{\beta\gamma}$ subunits implies that variations in the local levels of these molecules could have a profound impact on the dynamic range of GIRK channel activity under normal or pathophysiologic states.

A Gating Model for GIRK Channels

Channel binding sites for PIP_2, $G_{\beta\gamma}$, and Na^{2+} ions have been identified (Huang et al., 1995; 1997; Kunkel and Peralta, 1995; He et al., 1999). We postulate that additional distinct sites exist to completely account for the effects of gating molecules on channel activity. Fig. 8 shows the closed channel state C_0 in the absence of PIP_2. GIRK channels interact weakly with PIP_2, and as a result PIP_2 does not directly activate these channels (closed state C_1). In the absence of PIP_2, gating molecules such as $G_{\beta\gamma}$, Na^+, or Mg^{2+} are unable to activate the channel (closed state C_2). However, in the presence of PIP_2, any of the gating molecules can cause channel activation.

We envision two possible mechanisms for the synergistic action of gating molecules to activate the channel. Ions and $G_{\beta\gamma}$ subunits maybe exerting their combined effects by synergistic interactions of channel sites with PIP_2. Published reports have already suggested a stronger interaction of channel with PIP_2 in the presence of either $G_{\beta\gamma}$ subunits or Na^+ ions (Huang et al., 1998; Zhang et al., 1999) Alternatively, the gating molecules could be inducing conformational changes, affecting gating directly, independently of PIP_2 interactions. Although PIP_2 is absolutely required for gating

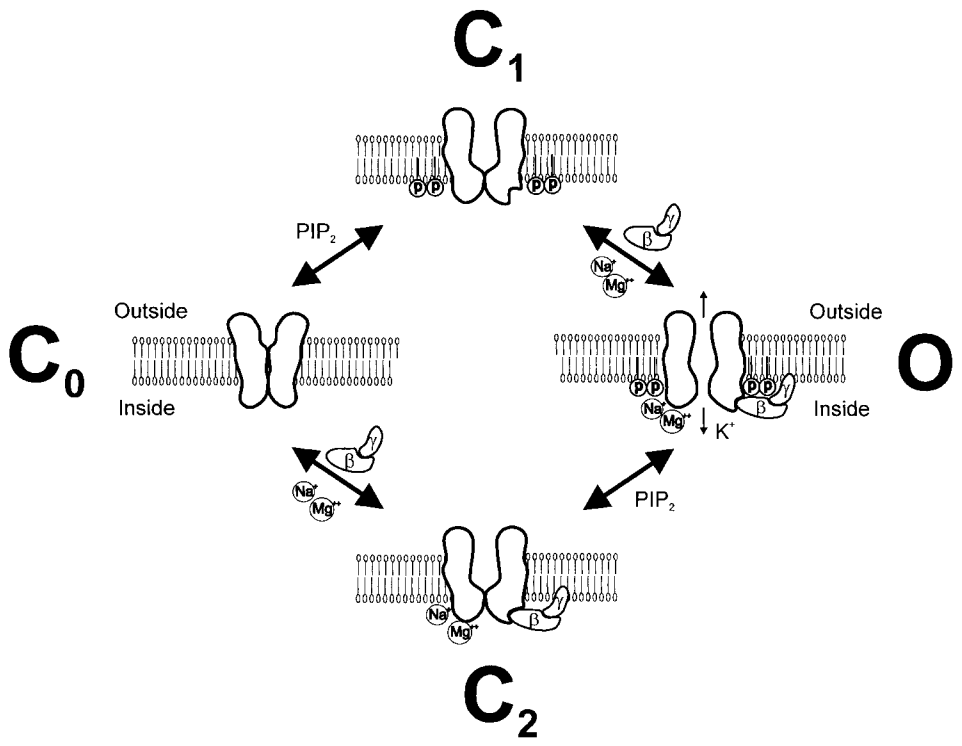

Figure 8. A model depicting gating of GIRK channels by the combination of PIP_2 with gating molecules $G_{\beta\gamma}$ and/or Na^+ and Mg^{2+} ions. (C_o) Channel closed state, in the absence of PIP_2 in the plasma membrane and gating molecules. (C_1) Channel closed state, in the presence of PIP_2 in the membrane GIRK channels experience weak interactions that in the absence of gating molecules are not of sufficient strength to gate the channel. (C_2) Channel closed state, gating molecules can interact with the channel at distinct sites but in the absence of PIP_2 they fail to gate the channel. (O) Channel open state, gating molecules in the presence of membrane PIP_2 can activate the channel and show synergism.

molecules to be effective, we have seen that at low PIP_2 concentrations $G_{\beta\gamma}$, unlike Na^+ or Mg^{2+} ions, can still gate the channels. This result suggests a stronger influence of $G_{\beta\gamma}$ than of Na^+ or Mg^{2+} ions on channel gating, possibly proceeding in a PIP_2-independent manner. Further work will be required to distinguish between these possibilities.

We are grateful to Lidiya Lontsman and Xixin Yan for technical support and preparation of the oocytes. We thank Drs. James Garrison, Eitan Reuveny, and Ravi Iyengar for their kind gifts of $G_{\beta1\gamma7}$ subunits, the βARK-PH construct and QEHA peptide, respectively. We also thank Drs. Vladimir Brezina, Sherman Kupfer, Tooraj Mirshahi, Tibor Rohacs, and Hailin Zhang for critical comments on the manuscript.

This work was supported by grants from the Aaron Diamond Foundation (J.L. Sui), the National Institutes of Health (HL54185), and the American Heart Association, National Center (96011620) (D.E. Logothetis).

Submitted: 16 July 1999 Revised: 25 August 1999 Accepted: 26 August 1999 Released online: 11 October 1999

REFERENCES

Baukrowitz, T., U. Schulte, D. Oliver, S. Herlitze, T. Krauter, S.J. Tucker, J.P. Ruppersberg, and B. Fakler. 1998. PIP_2 and PIP as determinants for ATP inhibition of K_{ATP} channels. *Science.* 282: 1141–1144.

Chan, K.W., M.N. Langan, J. Sui, J.A. Kozak, A. Pabon, J.A.A. Ladias, and D.E. Logothetis. 1996. A recombinant inwardly rectifying potassium channel coupled to GTP-binding proteins. *J. Gen. Physiol.* 107:381–397.

Chen, J., M. DeVivo, J. Dingus, A. Harry, J. Li, J.L. Sui, D. Carty, J.L. Blank, J. Exton, R.H. Stoffel, et al. 1995. A region of adenylyl cyclase 2 critical for regulation by G protein βγ subunits. *Science.* 268:1166–1169.

Chuang, H., Y.N. Jan, and L.Y. Jan. 1997. Regulation of IRK3 inward rectifier K^+ channel by m1 acetylcholine receptor and intracellular magnesium. *Cell.* 89:1121–1132.

Corey, S., G. Krapivinsky, L. Krapivinsky, and D.E. Clapham. 1998. Number and stoichiometry of subunits in the native atrial G protein–gated K^+ channel, I_{KACh}. *J. Biol. Chem.* 273:5271–5278.

Fan, Z., and J.C. Makielski. 1997. Anionic phospholipids activate ATP-sensitive potassium channels. *J. Biol. Chem.* 272:5388–5395.

Flanagan, L.A., C.C. Cunningham, J. Chen, G.D. Prestwich, K.S. Kosik, and P.A. Janmey. 1997. The structure of divalent cation–induced aggregates of PIP_2 and their alteration by gelsolin and tau. *Biophys. J.* 73:1440–1447.

Hamill, O.P., A. Marty, E. Neher, B. Sakmann, and F.J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* 391:85–100.

He, C., H. Zhang, T. Mirshahi, and D.E. Logothetis. 1999. Identification of a potassium channel site that interacts with G protein βγ subunits to mediate agonist-induced signaling. *J. Biol. Chem.* 274:12517–12524.

Hilgemann, D.W., and R. Ball. 1996. Regulation of cardiac Na^+, Ca^{2+} exchange and K_{ATP} potassium channels by PIP_2. *Science.* 273:956–959.

Ho, I.H.M. and R.D. Murrell-Lagnado. 1999. Molecular determinants for sodium-dependent activation of G protein–gated K^+ channels. *J. Biol. Chem.* 274:8639–8648.

Huang, C., S. Feng, and D.W. Hilgemann. 1998. Direct activation of inward rectifier potassium channels by PIP_2 and its stabilization by Gβγ. *Nature.* 391:803–806.

Huang, C.L., Y.N. Jan, and L.Y. Jan. 1997. Binding of the G protein βγ subunit to multiple regions of G protein–gated inward-rectifying K^+ channels. *FEBS. Lett.* 405:291–298.

Huang, C.L., P.A. Slesinger, P.J. Casey, Y.N. Jan, and L.Y. Jan. 1995. Evidence that direct binding of $G_{\beta\gamma}$ to the GIRK1 G protein–gated inwardly rectifying K^+ channel channel is important for channel activation. *Neuron*. 15:1133–1143.

Isenberg, G., and U. Klöckner. 1982. Calcium tolerant ventricular myocytes prepared by preincubation in a "KB medium." *Pflügers Arch.* 395:6–18.

Koch, W.J., J. Inglese, W.C. Stone, and R.J. Lefkowitz. 1993. The binding site for the βγ subunits of heterotrimeric G proteins on the β-adrenergic receptor kinase. *J. Biol. Chem*. 268:8256–8260.

Krapivinsky, G., E.A. Gordon, K. Wickman, B. Velimirovic, L. Krapivinsky, and D.E. Clapham. 1995a. The G protein–gated atrial K^+ channel I_{KACh} is a heteromultimer of two inwardly rectifying K^+-channel proteins. *Nature.* 374:135–141.

Krapivinsky, G., L. Krapivinsky, K. Wickman, and D.E. Clapham. 1995b. $G_{\beta\gamma}$ binds directly to the G protein–gated K^+ channel, I_{KACh}. *J. Biol. Chem.* 270:29059–29062.

Kunkel, M.T., and E.G. Peralta. 1995. Identification of domains conferring G protein regulation on inward rectifier potassium channels. *Cell.* 83:443–449.

Kurachi, Y., T. Nakajima, and T. Sugimoto. 1986. Role of intracellular Mg^{2+} in the activation of muscarinic K^+ channel in cardiac atrial cell membrane. *Pflügers Arch*. 407:572–574.

Kurachi, Y., R.T. Tung, H. Ito, and T. Nakajima. 1992. G protein activation of cardiac muscarinic K^+ channels. *Prog. Neurobiol.* 39: 229–246.

Logothetis, D.E., Y. Kurachi, J. Galper, E.J. Neer, and D.E. Clapham. 1987. The βγ subunits of GTP-binding proteins activate the muscarinic K channel in heart. *Nature.* 325:321–326.

Logothetis, D.E., S. Movahedi, C. Satler, K. Lindpaintner, and B. Nadal-Ginard. 1992. Incremental reductions of positive charge within the S4 region of a voltage-gated K^+ channel result in corresponding decreases in gating charge. *Neuron*. 8:531–540.

Matsuda, H. 1991. Magnesium gating of the inwardly rectifying K^+ channel. *Annu. Rev. Physiol.* 53:289–298.

Nichols, C.G., and A.N. Lopatin. 1997. Inward rectifier potassium channels. *Annu. Rev. Physiol.* 59:171–191.

Reuveny, E., P.A. Slesinger, J. Inglese, J.M. Morales, J.A. Iniguez-Lluhi, R.J. Lefkowitz, H.R. Bourne, Y.N. Jan, and L. Y. Jan. 1994. Activation of the cloned muscarinic potassium channel by G protein βγ subunits. *Nature.* 370:143–146.

Shyng, S.L., and C.G. Nichols. 1998. Membrane phospholipid control of nucleotide sensitivity of K_{ATP} channels. *Science.* 282:1138–1141.

Silverman, S.K., H.A. Lester, and D.A. Dougherty. 1996. Subunit stoichiometry of a heteromultimeric G protein–coupled inward-rectifier K^+ channel. *J. Biol. Chem.* 271:30524–30528.

Stühmer, W. 1992. Electrophysiological recording from *Xenopus* oocytes. *Methods Enzymol*. 207:319–339.

Sui, J.L., K.W. Chan, and D.E. Logothetis. 1996. Na^+ activation of the muscarinic K^+ channel by a G protein–independent mechanism. *J. Gen. Physiol.* 108:381–391.

Sui, J.L., J. Petit-Jacques, and D.E. Logothetis. 1998. Activation of the atrial K_{ACh} channel by the βγ subunits of G proteins or intracellular Na^+ ions depends on the presence of phosphatidylinositol phosphates. *Proc. Natl. Acad. Sci. USA*. 95:1307–1312.

Vivaudou, M.B., C. Arnoult, and M. Villaz. 1991. Skeletal muscle ATP-sensitive K^+ channels recorded from sarcolemmal blebs of split fibers: ATP inhibition is reduced by magnesium and ADP. *J. Membr. Biol*. 122:165–175.

Zhainazarov, A.B., and B.W. Ache. 1999. Effects of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate on a Na^+-gated nonselective cation channel. *J. Neurosci.* 19:2929–2937.

Zhang, H., C. He, X. Yan, T. Mirshahi, and D.E. Logothetis. 1999. Specific PIP_2 interactions with inwardly rectifying K^+ channels determine distinct activation mechanisms. *Nat. Cell Biol.* 1:183–188.