Ca\textsuperscript{2+} Elevation Evoked by Membrane Depolarization Regulates G Protein Cycle via RGS Proteins in the Heart

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Abstract—Regulators of G protein signaling (RGS), which act as GTPase activators, are a family of cytosolic proteins emerging rapidly as an important means of controlling G protein-mediated cell signals. The importance of RGS action has been verified in vitro for various kinds of cell function. Their in situ modes of action in intact cells are, however, poorly understood. Here we show that an increase in intracellular Ca\textsuperscript{2+} evoked by membrane depolarization controls the RGS action on G protein activation of muscarinic K\textsuperscript{+} (K\textsubscript{G}) channel in the heart. Acetylcholine-induced K\textsubscript{G} current exhibits a slow time-dependent increase during hyperpolarizing voltage steps, referred to as “relaxation.” This reflects the relief from the decrease in available K\textsubscript{G} channel number induced by cell depolarization. This phenomenon is abolished when an increase in intracellular Ca\textsuperscript{2+} is prevented. It is also abolished when a calmodulin inhibitor or a mutant RGS4 is applied that can bind to calmodulin but that does not accelerate GTPase activity. Therefore, an increase in intracellular Ca\textsuperscript{2+} and the resultant formation of Ca\textsuperscript{2+}/calmodulin facilitator GTPase activity of RGS and thus decrease the available channel number on depolarization. These results indicate a novel and probably general pathway that Ca\textsuperscript{2+}-dependent signaling regulates the G protein cycle via RGS proteins. (Circ Res. 2001;89:1045-1050.)

Key Words: G protein–activated K\textsuperscript{+} channel ■ regulators of G protein signaling ■ Ca\textsuperscript{2+} ■ calmodulin ■ cell excitation

G protein–gated inward rectifier K\textsuperscript{+} (K\textsubscript{G}) channels, which are directly activated by the βγ subunits released from pertussis toxin–sensitive G proteins (designated G\textsubscript{βγ}), contribute to acetylcholine (ACH)–induced deceleration of heartbeat and neurotransmitter-evoked slow inhibitory postsynaptic potentials in different neurons.\textsuperscript{1–3} The cardiac K\textsubscript{G} channel is a heterotetramer composed of two kinds of inward rectifier K\textsuperscript{+} channel (Kir) subunits, GIRK1/Kir3.1 and GIRK4/Kir3.4,\textsuperscript{4} which can be reconstituted by expressing the Kir subunits and m\textsubscript{2}-muscarinic receptors in Xenopus oocytes. The reconstituted current, however, lacks several of the characteristic features of native K\textsubscript{G} currents. One of these features is an agonist concentration-dependent slow increase during hyperpolarized potentials, which is referred to as “relaxation.”\textsuperscript{5,6} Since its first description in sinoatrial node cells,\textsuperscript{5} the molecular mechanism underlying this characteristic feature of the K\textsubscript{G} current has remained an enigma.

Recently a family of cytosolic proteins that act as regulators of G protein signaling (RGS) has been identified.\textsuperscript{6,7} These proteins accelerate GTP hydrolysis on α subunits of G\textsubscript{α} and/or G\textsubscript{βγ} and are supposed to play essential roles in the negative regulation of various G protein–mediated cell-signaling systems. In reconstituted systems, RGS proteins have been reported to accelerate the time course of activation and deactivation of K\textsubscript{G} currents induced by agonists.\textsuperscript{8–10} We have shown that one RGS protein, RGS4, restores the feature of relaxation to the reconstituted K\textsubscript{G} current\textsuperscript{11} and that this effect was mediated exclusively by the interaction of RGS domain with pertussis toxin–sensitive G\textsubscript{α} subunit.\textsuperscript{12} The question of how the cytosolic RGS protein confers this membrane potential–dependent feature to K\textsubscript{G} channels, however, remained a mystery.

In this report, using native cardiac atrial myocytes, we show for the first time that this characteristic can be imputable to the voltage-dependent behavior of RGS proteins probably caused by depolarization-induced formation of the Ca\textsuperscript{2+}/calmodulin (CaM) complex. This result not only reveals the molecular mechanism of the relaxation of native K\textsubscript{G} current, but also provides us with a novel concept that intracellular Ca\textsuperscript{2+}-dependent pathways dynamically modulate G protein signaling via RGS proteins.

Materials and Methods
Preparation of Isolated Atrial Myocytes
Single atrial myocytes were enzymatically isolated from hearts removed from adult male Wistar-Kyoto rats as described elsewhere.\textsuperscript{13} Briefly, rats were deeply anesthetized by intraperitoneal injection of pentobarbital. A cannula was inserted into the aorta, and the heart was perfused in a retrograde manner through the coronary arteries. The heart was digested by collagenase (Boehringer Mannheim) in nominally Ca\textsuperscript{2+}-free solution at 37°C for 10 minutes.

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Dissociated myocytes were seeded on glass coverslips (15 mm in diameter) that had been coated with poly-D-lysine (Sigma), kept in a humidified environment of 0.5% CO₂ at 37°C, and fed with medium M199 (PAA Laboratories) containing gentamycin and kanamycin (25 mg/L each).

**Electrophysiological Recordings**

Whole-cell currents were measured at room temperature by a patch-clamp amplifier (Axon 200A, Axon Instruments) and recorded on videocassette tape with a PCM converter system (VR-10B, Instrutech). For analysis, data were reproduced, low-pass-filtered at 1 kHz (−3 dB) by an eight-pole Bessel filter, sampled at 5 kHz, and analyzed offline on a computer with commercially available software (Patch Analyst Pro, MT Corp). The control bathing solution contained the following (in mmol/L): 115 NaCl, 20 KCl, 5 MgCl₂, 5.5 glucose, and 5.5 HEPES-NaOH (pH 7.4). The pipette (internal) solution contained the following (in mmol/L): 150 NaCl, 1 DTT, 10 HEPES-NaOH (pH 7.3). In some experiments, BAPTA reagent with CaM-agarose beads (20 mg/L each). Binding buffer to adjust its free Ca²⁺ concentration calculated to be ~10 μmol/L or <0.5 nmol/L. CaM kinase II (290 to 309) CaM antagonist (Biomol Research Laboratories) was added to make a final concentration of 1 μmol/L. The beads were then extensively washed and incubated with SDS-PAGE loading buffer for 5 minutes at 95°C. The bound protein was separated by SDS-PAGE and visualized by Coomassie brilliant blue R-250 staining.

**Results**

**Slow Increase of KG Current at Hyperpolarized Potentials (Relaxation) Depends on Voltage-Dependent Ca²⁺ Influx Across the Membrane**

Figure 1A illustrates a current induced by 10⁻⁷ mol/L of ACh in a rat atrial myocyte. When the membrane potential is clamped to +40 mV for 1 second, little outward current flows through KG channels. On hyperpolarization to ~100 mV the inward KG current instantaneously jumps to one level (Iₘᵢ₈) and then slowly increases to a steady level (Iₘᵢ₉). The immediate increase in current reflects the rapid relief from the blockade of outward KG channel gating that is associated with depolarization. Figure 1B shows that with 10⁻⁷ mol/L ACh, modulation of the prepulse potential influenced the amplitude of Iₘᵢ₈ without affecting Iₘᵢ₉, an effect that was essentially abolished with 10⁻⁸ mol/L ACh. The ratio Iₘᵢ₈/Iₘᵢ₉ shows the amount of KG current available during each prepulse relative to that at ~100 mV (Figure 1C). Thus in 10⁻⁷ mol/L ACh, KG current channel availability was reduced with depolarization such that at +40 mV this was ~30% of that at ~100 mV (Figure 1C, open circles), whereas with 10⁻⁸ mol/L of ACh KG channel availability at +40 mV had been increased to ~80% (Figure 1C, closed circles). These results indicate that at low concentrations of ACh the KG channels are inhibited on cell depolarization and that the slow increase in current (referred to as relaxation⁳) on hyperpolarization reflects relief from this

[Figure 1](#)
The membrane potential was held at either 0 or -80 mV, and 10^{-7} mol/L ACh was applied using a rapid perfusion system. In our assay system, T_{1/2} of activation (on) time courses was estimated as ~400 to 800 ms, which was almost compatible with the values reported in previous measurements.16-18 Figure 2A shows that, under control conditions, whereas the on time course of the current induced by ACh was nearly the same at 0 and at -80 mV, the off time course was significantly faster at 0 than at -80 mV (Figures 2C and 2D). When BAPTA was included in the pipette solution, the off time course was significantly prolonged at both voltages (Figure 2B), although more so at 0 than at -80 mV, such that the difference between them was reduced (Figures 2B and 2D). These results may suggest that the action of intrinsic RGS proteins on deactivation of K_o channel seems to be more augmented at 0 than at -80 mV and that the increase in intracellular Ca^{2+} may be involved in this voltage-dependent phenomenon. On the other hand, difference was not evident during the on time courses in this system (Figure 2C). The effect of extracellular application of EGTA on activation and deactivation time course of K_o was also examined, and these time courses were shown to be much slower (T_{1/2} on=40±10 seconds, T_{1/2} off=35±7 seconds; not shown in Figure). These extremely slow time courses could be explained by the low binding affinity between receptor (m2R) and agonist (ACh) in the absence of extracellular Ca^{2+}.19

Effect of Intracellular BAPTA, a Rapid Ca^{2+}-Chelating Agent, on Rapid Deactivation of Native K_o Current
In the context of the interaction between K_o currents and RGS, it is now widely accepted that the deactivation of ACh-induced K_o current on washout of the agonist is accelerated by RGS proteins.8-10 Therefore we examined the effect of membrane potential and intracellular BAPTA on the deactivation (off) time course of the K_o current (Figure 2).
antagonist. Recent structural analyses of RGS4 and Gαi2 indicate that residue N128 of RGS4 is located facing the switch II region of Gαi, and plays a crucial role in facilitating GTP hydrolysis.22 and the substitution of N128 into histidine completely abolishes its function.23 Thus, the mutant RGS4 may compete with intrinsic RGS proteins in binding to the Ca2+/CaM complex but cannot accelerate the GTPase activity of α subunit of Gαi protein. Therefore, it is expected that the acceleration by intrinsic RGS proteins of GTP hydrolysis on Gαi is directly disturbed by the injected mutant RGS4 in a competitive manner.

Discussion
In this study, we showed that depolarization-induced decrease in available Kᵦ channel number, which caused relaxation, depended on extracellular Ca²⁺ (blocked by extracellular EGTA), an increase in intracellular Ca²⁺ (blocked by intracellular BAPTA), formation of Ca²⁺/CaM complex (blocked by a CaM antagonist), and RGS action on accelerating GTP hydrolysis of Gαᵦ subunit (blocked by a mutant RGS4). It is widely accepted that the deactivation time course of Kᵦ current on washout of agonist reflects the GTPase activity of Gαᵦ and is accelerated by RGS proteins.8-10 We showed that the deactivation was faster at 0 than at −80 mV, and that the rapid deactivation was also disturbed by intracellular BAPTA, the CaM antagonist, and the mutant RGS4. These results indicate that depolarization-induced decrease in available Kᵦ channel number is mainly due to GTPase-accelerating action of intrinsic RGS proteins at depolarized potentials, and intracellular Ca²⁺ elevation and formation of Ca²⁺/CaM are required for this RGS action. In the preliminary experiments, we observed that neither the internal solution containing BAPTA (5 mmol/L) nor that containing Ca²⁺/CaM (100 nmol/L) affected GTP, GTPγS, or Gβγ activation of Kᵦ channel in the inside-out patches of atrial cell membrane. These data show that lowering Ca²⁺ or Ca²⁺/CaM does not directly modulate G protein activation of the Kᵦ channel. We also observed that inhibitors of CaM-dependent protein kinase and calcineurin did not affect the relaxation of the Kᵦ current in the whole-cell recording, which may suggest that phosphorylation or dephosphorylation of the Kᵦ channel is not involved in the phenomenon. There is, however, still one of two possibilities for the action of Ca²⁺/CaM, as follows: (1) Ca²⁺/CaM somehow activates RGS, which causes the acceleration of the Gᵦ protein cycle, or (2) Ca²⁺/CaM modulates the Gᵦ protein cycle to be more receptive to interaction with RGS. Because Ca²⁺/CaM can bind to RGS4 proteins in vitro14 (Figure 3C), we think the former is more likely, although we cannot completely exclude the latter possibility. On the basis of these possibilities, we propose the following signaling cascade in formation of relaxation gating of cardiac Kᵦ channel (Figure 4).

When membrane potential is depolarized, intracellular Ca²⁺ beneath the plasma membrane of cardiac myocytes increases. Ca²⁺ binds to CaM and Ca²⁺/CaM complex leads to the facilitation of RGS action to accelerate hydrolysis of Gαᵦ-GTP to Gαᵦ-GDP. Gαᵦ-GDP binds free Gβγ to form trimeric G proteins, which therefore decreases the number of free Gβγ and thus the number of available Kᵦ channels at depolarized potentials. The time-dependent increase in Kᵦ current on hyperpolarization can then be interpreted as the reflection of the reverse reactions of these events, ie, less Ca²⁺ influx, less Ca²⁺/CaM, lesser activity of RGS, and more available Gβγ.

There still remain, however, two major questions in this reaction scheme. The first one is how Ca²⁺/CaM facilitates RGS action. It was reported that Ca²⁺/CaM binds RGS but does not change its GTPase accelerating activity in vitro.14 Therefore, some mechanisms other than direct facilitating action of Ca²⁺/CaM on the GTPase-accelerating function of RGS should be involved. One possibility is that Ca²⁺/CaM might cause rapid translocation of RGS proteins to the plasma membranes.24 It might also be the case that some unidentified factor(s) may be needed for Ca²⁺/CaM to facilitate the GTPase-accelerating action of RGS proteins. Further studies are needed to examine these possibilities. This will lead us to reach the final conclusion on the role of Ca²⁺/CaM in the formation of apparent voltage-dependent behavior of RGS action.
The second remaining question is how membrane depolarization causes Ca\textsuperscript{2+} elevation beneath the cell membrane. Because removal of Ca\textsuperscript{2+} from the extracellular solution abolished the relaxation, one of the major mechanisms may be depolarization-induced Ca\textsuperscript{2+} influx across the cell membrane. The depolarization-induced Ca\textsuperscript{2+} influx in cardiac cell membrane is carried mainly through the voltage-gated (L-type) Ca\textsuperscript{2+} channel and/or the reverse mode of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. The voltage-gated Ca\textsuperscript{2+} channel, however, cannot be mainly responsible for the Ca\textsuperscript{2+} influx, because (1) the KG channel availability decreased monotonically as the membrane was depolarized and did not show such a bell-shaped voltage dependence that is observed in the current flowing through L-type Ca\textsuperscript{2+} channel, and (2) nifedipine (≈100 nmol/L to 1 μmol/L) did not significantly affect the relaxation property, although it inhibited channel current amplitude directly (not shown). The Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger may also not be mainly responsible, because (1) the relaxation was detected in nominally Na\textsuperscript{+}-free pipette solution and a decrease in extracellular Na\textsuperscript{+} did not affect the relaxation property, and (2) KB-R7943 \textsuperscript{25} (a blocker of the reverse mode of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger) did not significantly affect the relaxation property (not shown). We may therefore need to consider the possibility that some other mechanisms mediate the depolarization-induced Ca\textsuperscript{2+} elevation beneath the cell membrane for control of RGS action. The candidates might include TRP-family Ca\textsuperscript{2+}-permeable channels showing voltage-dependent gating property.\textsuperscript{26}

In addition to depolarization-induced Ca\textsuperscript{2+} elevation, it should be noted that the basal level of Ca\textsuperscript{2+} seems to play a significant role in controlling RGS action. As depicted in Figure 2, even at −80 mV the deactivation (off) time course of KG current became slower with the BAPTA-containing pipette solution than with the EGTA-containing (control) one, although the effect was not prominent compared with that at 0 mV. This suggests that even a very small amount of basal Ca\textsuperscript{2+} at the restricted domain probably just beneath the sarcolemma may contribute to the control of RGS action. Because neither voltage-dependent Ca\textsuperscript{2+} channels nor Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is supposed to carry significant Ca\textsuperscript{2+} influx across the cell membrane at −80 mV, a leak Ca\textsuperscript{2+} influx through, eg, nonselective cation channels\textsuperscript{27,28} and/or a basal Ca\textsuperscript{2+} release from internal pool might be involved in maintaining the basal Ca\textsuperscript{2+} level at the restricted domain. The mechanisms for Ca\textsuperscript{2+} increase on depolarization and those for control of basal Ca\textsuperscript{2+} level are not necessarily the same. Further studies are needed to clarify both mechanisms.

Nevertheless, this study in native cardiac myocytes provides the answer for the long-sought-after molecular mechanism underlying the characteristic voltage-dependent relaxation gating of KG channels. Also we show, in situ, the mode of action of negative regulators of the G protein cycle, which unexpectedly provides us with a novel principle, ie, that the cell signaling through Ca\textsuperscript{2+}-dependent pathways can dynamically regulate G protein signaling via cytosolic RGS proteins. In this context, the KG channel can be regarded as an example of a G protein effector molecule through which we can detect the G protein cycle in real time with high temporal resolution. Therefore, the principle proposed here should not be limited only to the KG channel system but must also be applicable to other G protein signaling machinery controlling such target proteins as neuronal (N- and P/Q-type) Ca\textsuperscript{2+} channels, phospholipase C, and adenylyl cyclases. Adenylyl cyclases are often subject to dual regulation by stimulatory Gs subunits of GTP-binding proteins activate the muscarinic K\textsubscript{G} channel in heart. Nature, 1987;325:321–326.

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References


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