Regulators of G Protein Signaling (RGS) Proteins Constitutively Activate Gβγ-gated Potassium Channels*

Moritz Bünemann and M. Marlene Hosey‡

From the Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, Chicago, Illinois 60611

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Here we report novel effects of regulators of G protein signaling (RGS) on G protein-regulated ion channels. RGS3 and RGS4 induced a substantial increase in currents through the Gβγ-regulated inwardly rectifying K⁺ channels, Ik(ACh), in the absence of receptor activation. Concomitantly, the amount of current that could be activated by agonist was reduced. Pretreatment with pertussis toxin or a muscarinic receptor antagonist abolished agonist-induced currents but did not modify RGS effects. Cotransfection of cells with a Gβγ-binding protein significantly reduced the RGS4-induced basal Ik(ACh) currents. The RGS proteins also modified the properties of another Gβγ effector, the N-type Ca²⁺ channels. These observations strongly suggest that RGS proteins increase the availability of Gβγ in addition to their previously described GTPase-activating function.

RGS³ proteins have been shown to serve as GTPase-activating proteins (GAPs) for α-subunits of heterotrimeric Gα, Gβ, Gγ, and G proteins in vitro (1). The affinity of RGS proteins for Gα proteins is highest in the presence of both GDP and AlF₄⁻ suggesting that RGS act by stabilizing the transition state of the GTPase (2). The first crystal structure of the core domain of RGS4 confirmed this hypothesis (3). Because RGS proteins can act as GAPs for heterotrimeric G proteins, they have been considered to be a missing link that might explain unresolved discrepancies in the deactivation kinetics of G protein signaling that have been observed in studies in vivo and in vitro (4–8).

Ion channels that are directly gated by G proteins allow for the resolution of the kinetics of G protein signaling in intact cells. The prototype of such a channel is the cardiac atrial potassium channel that is activated by muscarinic acetylcholine receptors (Ik(ACh), channel) (9). Ik(ACh) channels are formed by heteromultimers of G protein-regulated inwardly rectifying K⁺ channels (GIRKs) 1 and 4 (Kir 3.1 and Kir 3.4) (10–12). Upon activation of Gβγ-coupled receptors, such as the M₂ muscarinic acetylcholine receptors (mAChRs) in membranes of atrial myocytes, Gβγ-subunits have been shown to directly bind to the channels and to increase their open probability (9, 13, 14).

When GIRK channels are studied in heterologous expression systems they exhibit a much slower turn off (deactivation) compared with the rapid deactivation that is observed in native atrial myocytes (6, 7). However, several recent studies have demonstrated that RGS3, RGS4, and RGS8 can induce rapid deactivation of heterologously expressed Ik(ACh) (6, 7). These studies were the first to demonstrate that RGS proteins may function as GAPs in intact cells. However, the RGS proteins also caused an unexpected acceleration of the kinetics of activation of the channels (6, 7). Furthermore, the RGS proteins did not cause a reduction in peak currents, an effect that would be predicted if the sole effect of RGS is to act as a GAP (8). These observations suggested that RGS may have additional roles.

In other studies, RGS proteins inhibited Gα-activated pathways in intact cells (15–19). These results could be explained in two ways: 1) RGS act as GAPs in vivo and decrease the concentration of active Gα (GTP-Gα), or 2) RGS bind to Gα and prevent binding of Gα to its effectors. RGS proteins bind with high affinity to the transition state of GTP-Gα, but they can also bind with lower affinity to the GTP- and GDP-bound form of Gα (1, 20, 21). The analysis of RGS effects on Gβγ signaling should help to elucidate which of these two possibilities occur in vivo, because structural studies indicate that it is unlikely that Gβγ and RGS can bind to the same Gα at the same time (1, 3, 22). Therefore, if RGS inhibit signaling by binding to Gα, RGS will increase the concentration of free Gβγ and activate Gβγ-signaling pathways. On the other hand, if RGS act solely as GAPs, there should be a decrease in free Gβγ by promoting reassociation to heterotrimerics (23). In this study we tested these hypotheses by examining the effect of RGS proteins on Gβγ-mediated signaling by measuring kinetics as well as amplitudes of basal and agonist-activated Ik(ACh).

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 cells either nontransfected, stably expressing the M₂ mAChR (400 fmol of receptor/mg of protein) (24), or stably expressing Ca²⁺ channel subunits α₁d, β₁, and δ (G1A1 cells kindly provided by Dr. R. J. Miller with permission from SIBIA) (25)) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.). Chinese hamster ovary (CHO K1) cells were grown in Ham’s F-12 medium (Life Technologies, Inc.). The media were supplemented with 10% fetal bovine serum and streptomycin/penicillin (100 units each). Cells were grown under 10% CO₂ at 37°C. In all transfections for electrophysiological studies the CD8 reporter gene system was used to visualize transfected cells (26). Dynabeads coated with anti-CD8 antibodies were purchased from Dynal. The calcium phosphate method was used for transient transfection of HEK cells. The following amounts of cDNAs were used (unless otherwise indicated): mH3-CD8 (human), 1 µg; pC1-GIRK (rat), 1 µg; pCDNA1-GIRK4 (rat), 1 µg; pCDNA3-M₁ mAChR (human), 2 µg; pCDNA3.1-RGS4 (rat), 4 µg; perCMV-RGS3 (rat), 4 µg; pCDNA3B-CD8-βARK-c, 4 µg. Typically the total amount of cDNA used for transfection of one 10-cm dish was 9 µg. CHO K1 cells were transfected using transfectACE (27) or Superfect (Qiagen) using the following amounts of cDNAs/6-cm dish: CD8, 0.3 µg; GIRK1, 1 µg;
GIRK4, 1 µg; M2, 1 µg; and RGS4, 2 µg. All assays were performed 48–72 h post-transfection. The CDNAs for the GIRK1 and -4 were gifts from F. Lesage and M. Lazdunski, CD8 was from G. Yellen, CDS-BARKR-ct was from S. Gutkind, RGS3 was from K. Druey and J. Kehrl, and RGS4 was from P. Koif and H. A. Lester. For measurement of N-type Ca2+ channels, OIA1 cells were transiently transfected with RGS4 (4 µg) or empty pcDNA 3.1 (4 µg) using calcium phosphate.

Solutions—For the measurement of K+ currents an extracellular solution of the following composition was used (mM): NaCl, 120; KCl, 20; CaCl2, 2; MgCl2, 1; Hepes-NaOH, 10; pH 7.4. The internal (pipette) solution contained (mM): potassium glutamate, 100; KCl, 40; MgATP, 5; Hepes-KOH, 10; NaCl, 5; EGTA, 2; MgCl2, 1; GTP, 0.01; pH 7.4. For measurement of barium currents through N-type Ca2+ channels, the extracellular solution contained (mM): NaCl, 105; CsCl, 25; BaCl2, 10; MgCl2, 1; Hepes-NaOH, 10; pH 7.4. The intracellular solution contained: CsCl, 130; EGTA, 5; Hepes-CsOH, 5; MgATP, 5; MgCl2, 1; GTP, 0.01; pH 7.3. All standard salts as well as acetylcholine (ACh) were from Sigma.

Measurement of Membrane Currents—Membrane currents were recorded under voltage clamp using conventional whole cell patch-clamp techniques (26). Patch pipettes were fabricated from borosilicate glass capillaries (GF-150-10, Warner Instrument Corp.) using a horizontal puller (P-95 Fleming & Poulson). The DC resistance of the filled pipettes ranged from 3 to 6 MΩ. Membrane currents were recorded using a patch-clamp amplifier (Axopatch 200, Axon Instruments). Signals were analog-filtered using a lowpass Bessel filter (1–3 kHz corner frequency). Data were digitally stored using an IBM compatible PC equipped with hardware/software package (ISO2 by MPF, Frankfurt/Main, Germany) for voltage control, data acquisition, and data evaluation. I_{K(ACh)} was measured as an inward current using a holding potential of −90 mV as described (29). Voltage ramps (from −120 mV to +60 mV in 500 ms, every 10 s) were used to determine current-voltage (I-V) relationships.

Barium currents through N-type calcium channels were measured as described (25). Briefly, Ba2+ currents were measured every 10 s using two 25-ms test pulses of +10 mV that were interrupted by a 70-ms period in which the potential was clamped either to the holding potential (without prepulses) or for a 65-ms long prepulse to +80 mV followed by 5-ms period at the holding potential of −90 mV. All measurements were performed at room temperature. Summarized results are presented as mean values ± S.E. Statistically significant differences were analyzed using Student’s t test.

RESULTS AND DISCUSSION

RGS Has Stimulatory Effects on I_{K(ACh)}—To measure agonist-induced I_{K(ACh)} we routinely applied a saturating concentration of ACh (2 µM for HEK cells and 1 µM for CHO K1 cells) to fully activate I_{K(ACh)}: To measure basal (nonagonist-dependent) currents, we measured the difference in currents before and after application of 0.2 mM Ba2+, which will completely block inwardly rectifying potassium currents. We first observed that RGS4 induced an unexpected increase in basal currents. In HEK cells transfected with GIRK1 and -4, only a small amount of basal current was observed as Ba2+ reduced the current in the absence of ACh by 4.3 ± 0.8 pA/pF, n = 16 (Fig. 1, A and D). In marked contrast, in HEK293 cells transfected with GIRK1 and -4 plus RGS4, Ba2+ decreased the basal currents by 14.3 ± 2.7 pA/pF (n = 14) (Fig. 1, B and D). In nontransfected cells, Ba2+ did not affect background currents (five cells tested for each cell line). That the Ba2+ blockable currents were attributable to expression of GIRK1 and -4 was confirmed by demonstrating that the I-V relationships for both the agonist-induced currents and basal currents were identical (Fig. 1C). Concomitant to the RGS-induced increase of basal currents, the ACh-induced currents were reduced in RGS4-expressing cells (12.3 ± 2.4 pA/pF in RGS-expressing cells versus 24.1 ± 8.8 pA/pF in control cells), whereas the total (agonist + basal) currents were not altered (Fig. 1, B and D). Thus expression of RGS4 induced activation of 53 ± 8% of total I_{K(ACh)} compared with basal activation of 19 ± 4% of I_{K(ACh)} in control cells. Similar results were obtained using RGS3 instead of RGS4 (basal activation, 43 ± 5%, n = 4).

Previously it was reported that RGS4 accelerated the kinetics of heterologously expressed I_{K(ACh)} in Xenopus oocytes, as well as in CHO K1 cells (6); however, stimulation of basal currents was not apparent. To test whether or not the observed increase in basal I_{K(ACh)} in HEK 293 cells was a cell type-specific effect, we tested the effect of RGS4 on I_{K(ACh)} in transiently transfected CHO K1 cells. Upon cotransfection with
RGS Proteins Activate Gβγ-gated K+ Channels

![Graph](image)

**Fig. 2. Effects of RGS4 on I\textsubscript{K(ACh)} in CHO cells.** M\textsubscript{2} mAChR, GIRK1, and GIRK4, with (B) or without (A) RGS4, were transiently expressed in CHO K1 cells using liposome-mediated gene transfer. ACh (1 mM) or Ba\textsuperscript{2+} (0.2 mM) were used to determine the amplitude of agonist-induced and basal I\textsubscript{K(ACh)}\textsubscript{bg} respectively. The dotted lines indicate zero current level.

RGS4, most cells exhibited a large increase of ACh-independent, Ba\textsuperscript{2+}-sensitive inwardly rectifying K+ currents (Fig. 2B, 27 ± 20 pA/PF, n = 5), whereas in the absence of expressed RGS4, the detected Ba\textsuperscript{2+}-sensitive background currents were negligible (Fig. 2A, 2 ± 0.5 pA/PF, n = 5). These results mirrored those from the HEK cells and demonstrated that RGS expression led to agonist-independent activation of I\textsubscript{K(ACh)}\textsubscript{bg}. We cannot explain why others did not detect an increase in basal current (6, 7), however, one obvious explanation is that there are differences in the heterologous expression systems used. In our experience the vast majority of the RGS4-transfected cells exhibited increased basal I\textsubscript{K(ACh)}\textsubscript{bg}.

Expression of RGS proteins also caused a small acceleration of the activation of I\textsubscript{K(ACh)} (Table I); however, this effect was less significant than previously reported in studies of other RGS-sensitive inwardly rectifying K+ currents and on acceleration of deactivation. When the amount of RGS cDNA was reduced as low as 0.1 μg, the RGS effects on basal currents were still obvious, whereas the effects on deactivation were reduced (data not shown). Thus the newly appreciated effects of RGS to increase channel currents did not appear to be an artifact of overexpression.

**Table I**

| Cells          | Activation kinetics (τ\textsubscript{1/2 act}) | Deactivation kinetics (τ\textsubscript{1/2 deact}) |
|----------------|-----------------------------------------------|-----------------------------------------------|
| HEK293: control | 1.26 ± 0.15 s, n = 16                          | 7.4 ± 0.6 s, n = 16                            |
| HEK293: +RGS3  | 0.96 ± 0.14 s, n = 9                           | 3.6 ± 1.0 s, n = 8                            |
| HEK293: +RGS4  | 0.89 ± 0.11 s, n = 8                           | 5.2 ± 2.5 s, n = 7                            |
| CHO K1: control| 1.14 ± 0.18 s, n = 7                           | 14.3 ± 1.6 s, n = 7                           |
| CHO K1: +RGS4  | 1.03 ± 0.17 s, n = 7                           | 6.1 ± 1.5 s, n = 7                            |

Induction of Basal I\textsubscript{K(ACh)} by RGS4 Depends on Gβγ but Not on Receptors—An RGS-induced promotion of the coupling of receptor, G protein, and channel was considered as a possible mechanism for the unexpected effects of RGS on basal currents and on activation kinetics (6). To test this, cells were treated with pertussis toxin. The uncoupling of receptors from G proteins by pertussis toxin pretreatment did not reduce the RGS-induced basal I\textsubscript{K(ACh)}\textsubscript{bg} whereas it completely abolished ACh-induced I\textsubscript{K(ACh)}\textsubscript{bg} (data not shown). Therefore, the RGS-induced basal activation of I\textsubscript{K(ACh)} did not depend on the basal activity of G\textsubscript{bg} or G\textsubscript{a} coupled receptors because it was not inhibited by pertussis toxin, which is known to prevent activation of G\textsubscript{bg} and G\textsubscript{b} proteins by G protein-coupled receptors but not GTP hydrolysis and nucleotide exchange (34).

We determined that expression of Gβγ\textsubscript{1}γ\textsubscript{2} resulted in a 3–4-fold increase of total I\textsubscript{K(ACh)} in HEK cells (data not shown) indicating that endogenous G\textsubscript{bg} proteins are limiting for maximal activation of I\textsubscript{K(ACh)} in these cells. Next we tested whether RGS induced I\textsubscript{K(ACh)} by increasing free Gβγ in the cell or by activating GIRK channels via a Gβγ-independent mechanism. To do this we coexpressed the channels and RGS with CD8-βARK-ct, a fusion protein of the α-subunit of the CD8 receptor, and the C terminus of the β-adrenergic receptor kinase 1 (βARK1), which has been demonstrated to act as a Gβγ “sink” and block Gβγ-mediated signaling (35). The expression of CD8-βARK-ct reduced basal I\textsubscript{K(ACh)} in RGS4-expressing cells by 65% (Fig. 3, compare currents in cells expressing CD8 versus CD8-βARK-ct). The expression of the Gβγ-binding protein also reduced the ACh response to the threshold of detection, resulting in a total (basal + agonist-activated) I\textsubscript{K(ACh)} of about 20% compared with the control (Fig. 3). Therefore, we concluded that RGS4 activated I\textsubscript{K(ACh)} via a Gβγ-dependent mechanism.

Because GIRK1 and GIRK4 have been shown to bind Gβγ directly (36) and therefore may act as sink for Gβγ if overexpressed, it was possible that the observed RGS-induced increase in basal I\textsubscript{K(ACh)} was the result of an RGS-induced increase in GIRK expression. Immunoblotting experiments using antibodies against GIRK1, GIRK4, and hemagglutinin-tagged RGS4 demonstrated no increase of GIRK1 and GIRK4 expression upon cotransfection with hemagglutinin-tagged RGS4 (data not shown). Therefore, an increase of GIRK1 and GIRK4 expression as the mechanism by which RGS4 induces basal I\textsubscript{K(ACh)} can be ruled out. An increase in the expression level of the endogenous Gβγ upon coexpression of RGS proteins could be another potential explanation for the observed increase in basal I\textsubscript{K(ACh)}\textsubscript{bg}. However, we have observed that the total I\textsubscript{K(ACh)} is limited by the expression level of endogenous Gβγ proteins, as currents are greatly increased upon expression of Gβγ (data not shown). Because neither RGS3 nor RGS4 increased total I\textsubscript{K(ACh)} (Fig. 1), an increase of Gβγ expression induced by RGS proteins seems unlikely. The possibility that expression of RGS proteins caused down-regulation of the Gα, proteins was not tested.

RGS4 Increases the Concentration of Free Gβγ—Because the increase of basal I\textsubscript{K(ACh)} by RGS was dependent on Gβγ, a reasonable hypothesis was that the underlying mechanism for the RGS-induced I\textsubscript{K(ACh)}\textsubscript{bg} was an increase in the concentration of free Gβγ in the cell. To obtain further support of this hypothesis, we analyzed another Gβγ-regulated effector system that allowed for quantitative measurement of basal (nonagonist regulated) effects of Gβγ. N-type Ca\textsuperscript{2+} channels are known to be inhibited by Gβγ, and this inhibition can be partially reversed by applying strong positive voltages prior to measuring channel currents with a procedure termed prepulse facilitation (25, 37–40). Although prepulse facilitation is usually more apparent in the presence of an agonist that activates G\textsubscript{bg} coupled receptors, we attempted to reveal effects of RGS proteins on basal currents in the absence of an agonist. The effects of RGS on basal inhibition of N-type Ca\textsuperscript{2+} currents were tested in HEK293 cells stably expressing α\textsubscript{1b}, β\textsubscript{1}, and ε\textsubscript{1,2} calcium channel subunits (25) using Ba\textsuperscript{2+} as the charge carrier. Ba\textsuperscript{2+} currents were measured using a double test pulse proto-
col in which two successive measurements of current test pulses were separated by a 65-ms period during which the cells were or were not exposed to a strong depolarization (prepulse to +80 mV) to test for relief of constitutive Gβγ-mediated inhibition. Prepulse facilitation was determined by the relative current amplitude of the second test pulse without a preceding positive prepulse. In the control condition, if there was no positive prepulse applied prior to the second test pulse, the current amplitude of Ba2+ currents was 76 ± 3%, n = 11 compared with the current measured during the first test pulse (Fig. 4A, compare the first and second test pulses). In cells not transfected with RGS a positive prepulse did not increase \( I_{\text{Ba}} \) (Fig. 4F). The test pulses ± the prepulse were completely superimposable (Fig. 4, A and C, prepulse facilitation, 0.95 ± 0.04, n = 11). However, in cells transfected with RGS4, positive prepulses resulted in a small but reproducible and significant facilitation of \( I_{\text{Ba}} \) (Fig. 4H). The effect of RGS4 to induce constitutive inhibition of the N-type channel was probably larger than it appeared in our measurements, because in independent experiments we determined that the prepulse led only to a ~60% release of the Gβγ-mediated inhibition of the channels. These results suggested that a Gβγ-dependent “basal” inhibition of channels was occurring in RGS4-transfected cells but not in control cells, as reflected by the increase of currents after reversal of the Gβγ-mediated inhibition by positive prepulses. These results further suggested that RGS proteins cause constitutive Gβγ signaling in HEK cells.

Both the N-type Ca2+ channels as well as the \( I_{\text{K(ACh)}} \) channels are well accepted to be direct effectors of Gβγ-subunits of pertussis toxin-sensitive G proteins (38, 41, 42). Because RGS4 in both cases induced a response that is usually mediated by Gβγ-subunits, we concluded that the expression of RGS4 increased the concentration of free Gβγ in the cell and constitutively modulated the activation state of both channels. It should be mentioned that constitutive signaling through Gβγ was not observed in previous studies where the effects of RGS proteins were assessed in systems involving Gβγ signaling. Thus, an inhibition, rather than a constitutive activation, of agonist-induced mitogen-activated protein kinase activation was observed upon coexpression of RGS1 in HS-Sultan cell lines (16) or with RGS4 and SST2 in yeast (16, 43). Although we have no definitive explanation for the differences between our observations and those made with the mitogen-activated protein kinase-linked systems, the different experimental conditions and the fact that the signaling cascades that lead to activation of mitogen-activated protein kinases are complex and involve considerable amplification make it difficult to compare results obtained in this system with ion channels that are directly Gβγ-modulated. It seems possible that the net effect of RGS proteins in different systems will reflect a summation of inhibitory (GAP activity) and stimulatory (increased availability of Gβγ) processes. This appears to be the case with the receptor-mediated activation of Gβγ-gated K+ channels. In all of the studies reported so far, although the kinetics of channel deactivation were increased, there was no depression of the peak currents upon expression of RGS proteins (6, 7) in contrast to what one would expect if the RGS proteins were solely acting as GAPs (see discussion in Ref. 8). Thus, in the mitogen-activated protein kinase systems studied previously (16, 43), it is not possible to know if the inhibitions observed were also influenced by stimulatory actions of RGS proteins.

**A Newly Appreciated Action of RGS**—Several studies of intact cells have shown that RGS proteins inhibit Go, Gsa, or Gq signaling (15–19, 21, 44, 45). These results could be explained by enhanced deactivation of the G proteins due to the GAP activity of RGS or by binding of RGS to Go and consequent block of effector activation. The efficiency of RGS proteins to act as GAPs in intact cells will strongly depend on the relative affinities of RGS to the different conformations of Gα proteins. Our approach to measure basal and receptor-activated Gβγ signaling in intact cells allowed for detection of GAP effects by RGS (i.e. the acceleration of \( I_{\text{K(ACh)}} \)) kinetics) as well as for indirect detection of RGS binding to Gα proteins in the absence of agonists, by measuring basal Gβγ-induced currents. The concentration of free Gβγ will most likely be increased if RGS...
proteins bind to Gα proteins, because Gβγ will not be able to bind to RGS-occupied Gα-subunits (3). Assuming that the underlying mechanism for the turn off of Gβγ-mediated signaling is reassociation with the deactivated Gα-subunit, the GAP function of RGS should speed up deactivation of Gα and consequently also turn off Gβγ-mediated signals. Considering this, we speculate that the observed and previously described acceleration of $I_{K(ACh)}$ deactivation by RGS is because of the GAP activity of these proteins (6, 7). The increase of basal Gβγ signaling upon coexpression of RGS3 or RGS4 may reflect binding of RGS to a substantial portion of the Gα protein pool and a consequent decrease in the sequestering of Gβγ by Gα-subunits. Our studies report a novel effect of RGS on Gβγ signaling and suggest that in addition to causing desensitization because of GAP activity, RGS proteins may also positively impact Gβγ signaling.

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