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Co-expression of Gβ5 Enhances the Function of Two Gγ Subunit-like Domain-containing Regulators of G Protein Signaling Proteins*

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Regulators of G protein signaling (RGS) stimulate the GTPase activity of G protein Gα subunits and probably play additional roles. Some RGS proteins contain a Gγ subunit-like (GGL) domain, which mediates a specific interaction with Gβ5. The role of such interactions in RGS function is unclear. RGS proteins can accelerate the kinetics of coupling of G protein-coupled receptors to G-protein-gated inwardly rectifying K^+ (GIRK) channels. Therefore, we coupled m2-muscarinic acetylcholine receptors to GIRK channels in *Xenopus* oocytes to evaluate the effect of Gβ5 on RGS function. Co-expression of either RGS7 or RGS9 modestly accelerated GIRK channel kinetics. When Gβ5 was co-expressed with either RGS7 or RGS9, the acceleration of GIRK channel kinetics was strongly increased over that produced by RGS7 or RGS9 alone. RGS function was not enhanced by co-expression of Gβ1, and co-expression of Gβ5 alone had no effect on GIRK channel kinetics. Gβ5 did not modulate the function either of RGS4, an RGS protein that lacks a GGL domain, or of a functional RGS7 construct in which the GGL domain was omitted. Enhancement of RGS7 function by Gβ5 was not a consequence of an increase in the amount of plasma membrane or cytosolic RGS7 protein.

G protein-coupled receptors (GPCRs)[1] form the largest family of cell surface receptors and mediate cellular responses to diverse signals that include neurotransmitters, hormones, and sensory stimuli (1, 2). These receptors are proteins with seven membrane-spanning regions and can interact with intracellular, heterotrimeric G proteins. When an agonist-bound or activated GPCR encounters a trimeric G protein, it catalyzes the exchange of GTP for GDP at the α subunit of the G protein (Gα). This leads to activation of the Gα and dissociation of the activated GTP-bound Gα subunit from the Gβγ dimer. Both the activated GTP-bound Gα subunit and the freed Gβγ dimers regulate the activity of many intracellular effector molecules. The intrinsic GTPase activity of Gα mediates signal termination. The Gα subunit hydrolyzes the bound GTP to GDP to produce the inactive GDP-Gα subunit, which then reassociates with high affinity to the Gβγ dimer.

The intrinsic GTPase activity of purified Gα measured *in vitro* is much slower than the rate of termination of many cellular G protein responses such as phototransduction (3) and ion channel modulation (4). This suggested that cells contained factors that accelerated the GTPase activity of Gα. Subsequently, a new gene family called "regulators of G protein signaling" (RGS) was identified (3, 5, 6). RGS proteins may have several functions (7); one function of RGS proteins is to serve as "GTPase-activating proteins" (GAPs) for Gα, hence to accelerate signal termination. Swift transduction kinetics allow cells to read or detect rapidly changing concentrations of a signaling molecule, while slow transduction kinetics cause them to integrate or filter such a signal. Therefore, RGS proteins are important components of the programs that determine how the temporal information specified in the release and removal of an extracellular signaling molecule is translated into an appropriate cellular response.

RGS proteins vary in size and sequence but all have a common, conserved core "RGS domain" of ~120 amino acids, which is necessary and sufficient for their GAP activity. The RGS family can be subclassified into small (160–220-residue) and large (380–1400-residue) proteins. Small RGS proteins consist largely of the RGS domain flanked by short N termini and even shorter C termini. The larger RGS proteins contain additional sequence-definable domains and motifs that predict interactions with proteins other than Gα subunits (7). These additional domains underscore the current view that some RGS proteins are not simply GAPs but have separate functions that link them to other signaling pathways. For example, the RGS protein p115RhoGEF acts as a GAP for $G\alpha_{13}$ and as a GDP-GTP exchange factor for the small GTPase RhoA (8, 9).

Sequence analysis has revealed that a subset of the large RGS proteins, RGS6, -7, -9, and -11, contain a G protein γ subunit-like (GGL) domain between the N terminus and the RGS domain (10, 11). This GGL domain allows these RGS proteins to complex specifically with the atypical G protein β subunit, Gβ5 (10–12). Gβ5 is significantly different from the other Gβ proteins in structure and cellular localization. Gβ5 has only about 50% identity with Gβ subunits 1–4, which themselves are about 90% identical (13, 14). Gβ5 is found predominantly in neuronal tissues. Gβ5-long, the long form of Gβ5, is found in the outer segment membranes of retinal photoreceptors (14). Gβ5-short, a shorter form of Gβ5 is found in brain and in the inner retina (13). Although the interaction of Gβ5 with the GGL domains of RGS proteins has been well established, the effect of such interaction on RGS function is presently uncertain. Levay *et al.* (12) reported that the formation of the Gβ5-RGS7 complex blocked the binding of RGS7 to the $G\alpha_o$ subunit in purified preparations and concluded that Gβ5 is a specific RGS inhibitor (12). On the other hand, Snow

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; RGS, regulators of G protein signaling; GAP, GTPase-activating protein; ACh, acetylcholine; m2-mAChR, m2-muscarinic acetylcholine receptor; GGL, G protein γ subunit-like domain; GIRK, G protein-gated inwardly rectifying K^+ channel.

et al. (10) showed that the complex between Gβ5 and a partial RGS11 construct (lacking a 200-amino acid stretch at the N terminus) had GAP activity. In addition, recent work performed with rod photoreceptor preparations indicates that the RGS9-Gβ5 complex is a GAP for transducin in the presence of the effector, the γ subunit of rod phosphodiesterase (15). However, the latter two studies did not compare the GAP activity of the RGS-Gβ5 dimer with that of the RGS protein alone (10, 15). Thus, the effect of Gβ5 on RGS function remains to be clarified.

A useful way to study the kinetics of the G protein cycle is to follow the kinetics of activation and deactivation of G-protein-gated inwardly rectifying K^+ (GIRK) channels. GIRK channels are heterotetrameric channels that are widely expressed in the brain and in the heart and are activated by G protein-coupled receptors, such as m2-muscarinic acetylcholine receptor (m2-mAChR) and D2-dopamine receptor, that couple to pertussis-sensitive G proteins (16). Free Gβγ subunits that are released from activated Gα G proteins activate or gate the GIRK channels (16). The key step in G protein signal termination occurs when the GTPase activity of the Gα subunit hydrolyzes the bound GTP to GDP. This produces the inactive GDP-Gα-G protein that then reassociates with high affinity to the Gβγ dimer. Thus, the deactivation kinetics of the GIRK channels are governed either by the rate of GTP hydrolysis by the Gα G protein or by the rate at which Gβγ subunits dissociate from the GIRK channels. In atrial myocytes and in neurons, GIRK currents activate and deactivate with time constants <1 s after application, and removal of receptor agonist occurs in less than 1 s (4, 17). When the channels were expressed in *Xenopus* oocytes or other systems that have low endogenous levels of RGS proteins, the currents recovered much more slowly, over many seconds (18, 19). The rate was comparable with the rate of GTP hydrolysis by purified $G\alpha_i$/$G\alpha_o$ measured *in vitro*, suggesting that that GTP hydrolysis determines the rate of channel deactivation. Two studies showed that the co-expression of RGS proteins with receptors and GIRK channels in *Xenopus* oocytes reconstituted the native kinetics of GIRK channels (18, 19). Point mutations in RGS2 designed to kill its GTPase accelerating activity abolished RGS2 acceleration of GIRK inactivation (20). These results suggest that RGS proteins accelerate GIRK inactivation kinetics by enhancing the rate of GTP hydrolysis by Gα. Whatever the mechanism, it is clear that the time course of GIRK channel deactivation following the unbinding of agonist from receptor may be used as a measure of RGS function (21).

In this study, we expressed m2-mAChR and GIRK channels with either RGS7 or RGS9 alone or in combination with Gβ5 in *Xenopus* oocytes. We observed an enhancement of RGS function in the presence of both forms of Gβ5 and provide data to suggest that the GGL domain is required for the enhancement of RGS function by Gβ5. We also showed that co-expressing Gβ5 did not alter the total concentration of RGS, indicating that the enhancement of RGS function by Gβ5 was not mediated by an increase in the level of RGS protein.

EXPERIMENTAL PROCEDURES

Complementary cDNA Clones and cRNA Synthesis—cRNA was synthesized *in vitro* from plasmids containing the cDNA and appropriate promoters for cRNA transcription. Plasmids were linearized prior to cRNA synthesis, and mMESSAGE MACHINE kits (Ambion) were used to generate capped cRNA. The m2-mAChR receptor was provided by E. Peralta (Harvard University). The cDNA for the GIRK subunits, rat Kir3.1 (22) and mouse Kir3.2 (23), rat RGS4 (18), mouse RGS7, mouse RGS9-2 (long striatally enriched form (24)), and mouse Gβ5 short (13) and long form (14) were generated in our laboratory.

Oocyte Culture and Injection—*Xenopus* oocyte preparation was described previously (25). cRNA was injected into oocytes at a volume of 50 nl/oocyte using a Drummond microinjector. Oocytes were maintained in a saline buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM HEPES, pH 7.5) solution supplemented with sodium pyruvate (2.5 mM) and Gentamycin (50 μg/ml).

Electrophysiology—All oocytes were injected with 0.01 ng of cRNA for the m2-mAChR and 0.02 ng of cRNA each for GIRK1 and GIRK2. 200 nM acetylcholine (ACh) was used as the m2-mAChR agonist. Receptor activation was reversed by simultaneous ACh wash-off and perfusion with the m2-mAChR antagonist, atropine (1 μM). A valve system controlled by the data acquisition software, pCLAMP 6 (Axon Instruments), was used to control solution changes and to minimize wash-in and wash-out times. Two-electrode voltage clamp of the oocytes were performed 36–72 h after cRNA injection. Membrane potential was clamped at −80 mV using a Geneclamp 500 amplifier (Axon Instruments) and pCLAMP 6 software. Electrodes were filled with 3 M KCl and had resistances of 0.5–1.5 megaohms. To reveal inward currents through the inwardly rectifying GIRK channels, recordings were performed in oocyte saline buffer with elevated (16 mM) KCl concentration (other components: 82 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM HEPES, pH 7.5).

Xenopus Oocyte Plasma Membrane and Cytosol Preparation—Plasma membranes were separated mechanically as described (26). Defolliculated oocytes were incubated for 10 min in ice-cold hypotonic solution (5 mM NaCl, 5 mM HEPES, pH 7.5, 1 mM phenylmethanesulfonyl fluoride, 1 μM aprotinin, 1 μM leupeptin, and 1 μM pepstatin). The plasma membranes together with the vitelline membranes (extracellular collagen-like matrix) were removed manually with fine tweezers and appeared as transparent sheets. The remainder of the oocyte (cytosol), consisting of cytoplasm and intracellular organelles, was left as an intact sphere. The two fractions were pelleted separately by centrifugation (10 min at 10,000 × *g* and 4 °C) in a microcentrifuge. Previously, it was shown by electron microscopy that the clear plasma and vitelline membrane sheets were devoid of other cellular components (26).

Metabolic Labeling with [^{35}S]Methionine and Immunoprecipitation—After cRNA injection, 2 mCi/ml [^{35}S]methionine was added to the oocyte incubation solution, and the oocytes were incubated in this solution for 36–48 h. Eight whole oocytes, 30 plasma membranes, or eight cytosolic fractions were homogenized in 100 μl of SDS solubilization buffer (4% SDS, 10 mM EDTA, 50 mM Tris, pH 7.5, 1 mM phenylmethanesulfonyl fluoride, 1 μM aprotinin, 1 μM leupeptin, and 1 μM pepstatin) and boiled for 2 min. 900 μl of detergent containing immunoprecipitation buffer (190 mM NaCl, 6 mM EDTA, pH 7.5, 2.5% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 1 μM aprotinin, 1 μM leupeptin, and 1 μM pepstatin) was then added, and the homogenates were centrifuged for 10 min at 10,000 × *g* at 4 °C to remove insoluble components. The supernatant was precleaned by incubation with protein A-Sepharose slurry for 3 h at 4 °C. The protein A-Sepharose slurry was pelleted by centrifugation and discarded. 10 μg of an antibody to RGS7 was added to each sample group and incubated at 4 °C for 16 h. The RGS7 antibody, R4613, was raised in rabbit against recombinant bovine RGS7. The antigen-antibody complex was captured by incubation with protein A-Sepharose for 4 h at 4 °C followed by centrifugation (30 s at 10,000 × *g*). The antigen-antibody-protein A-Sepharose pellets were washed four times by resuspension and centrifugation in buffer (150 mM NaCl, 6 mM EDTA, 50 mM Tris, pH 7.5, 0.1% Triton X-100, and 0.02% SDS). The supernatant was discarded each time. The pellets were then boiled in SDS-gel loading buffer and subjected to SDS-polyacrylamide gel electrophoresis. The protein bands were visualized by autoradiography, and the relative intensities of the labeled bands were measured using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) and ImageQuant (Molecular Dynamics) software.

Curve Fitting and Statistical Analysis—Kinetic analysis and curve fitting were performed using pCLAMP 6 software. Exponentials were fit to the activation and deactivation phases of the GIRK currents. Cursors were positioned at points on the activation and deactivation curves that corresponded to 20 and 80% of the maximum equilibrium responses, and the exponentials were fitted to the portions of the current trace between these two points. We used Student's *t* test for comparison of the independent means. Two-tailed p value < 0.01 is defined as significantly different.

RESULTS AND DISCUSSION

We used ACh-evoked GIRK currents recorded from *Xenopus* oocytes co-expressing the functional Kir3.1/Kir3.2 channel heteromultimer along with the m2-mAChR to define the role of the G protein Gβ5 subunit in RGS7 function. GIRK channels respond to G protein activation and are useful for following G protein kinetics and in turn as an assay for RGS function (21). This analysis may also be physiologically relevant because the

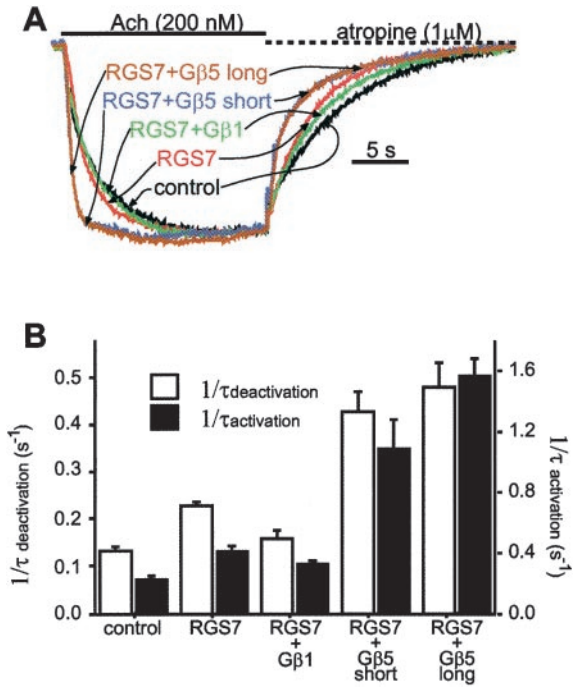

FIG. 1. **Effect of Gβ5 and Gβ1 on RGS7 function.** Control oocytes were injected with 0.01 ng of cRNA for the m2-mAChR and 0.02 ng of cRNA each for GIRK1 and GIRK2. The other oocyte groups tested were injected with the following cRNA combinations in addition to those injected into the control oocytes: RGS7 (10 ng) alone, RGS7 (10 ng) and Gβ1 (10 ng), RGS7 (10 ng) and Gβ5 short form (10 ng), and RGS7 (10 ng) and Gβ5 long form (10 ng). *A*, normalized representative traces of ACh (m2-mAChR agonist, 200 nM)-evoked and atropine (m2-mAChR antagonist, 1 μM)-reversed GIRK currents recorded from the different oocyte groups. The *solid horizontal line above* the *traces* represents ACh application, while the *dashed line* shows atropine application. The traces were normalized to the amplitude of the response just prior to the application of atropine. *B*, comparison of the activation ($1/\tau_{activation}$, *solid black bars*) and deactivation ($1/\tau_{deactivation}$, *open bars*) rate constants derived from the exponential fits of the activation and deactivation phases of the GIRK currents in the different oocyte groups. All *bars* are means ± S.E. from 4–7 oocytes from the same oocyte donor. However, the results were repeated in oocytes from two other donors. The mean steady-state current amplitudes in nA ± S.E. for the different oocyte groups were as follows: control, 271 ± 51; RGS7 alone, 394 ± 28; RGS7 + Gβ1, 245 ± 38; RGS7 + Gβ5 short, 351 ± 88; RGS7 + Gβ5 long, 434 ± 40.

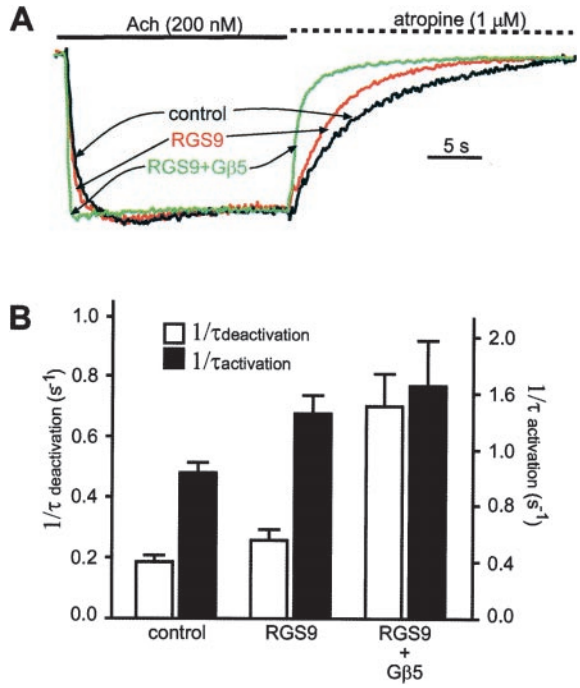

FIG. 2. **Effect of Gβ5 on RGS9 function.** Control oocytes were injected with 0.01 ng of cRNA for the m2-mAChR and with 0.02 ng of cRNA each for GIRK1 and GIRK2. The other oocyte groups tested were injected with the following cRNA combinations in addition to those injected into the control oocytes: RGS9 (alternatively spliced long or striatally expressed form) (10 ng) alone or RGS9 (10 ng) plus and Gβ5 short form (10 ng). *A*, normalized representative traces of ACh (m2-mAChR agonist, 200 nM)-evoked and atropine (m2-mAChR antagonist, 1 μM)-reversed GIRK currents recorded from the different oocyte groups. The *solid horizontal line above* the *traces* represents ACh application, while the *dashed line* shows atropine application. The traces were normalized to the amplitude of the response just prior to the application of atropine. *B*, comparison of the activation ($1/\tau_{activation}$, *solid black bars*) and deactivation ($1/\tau_{deactivation}$, *open bars*) rate constants derived from the exponential fits of the activation and deactivation phases of the GIRK currents in the different oocyte groups. All *bars* are means ± S.E. from 4–7 oocytes from the same oocyte donor. The mean steady-state current amplitudes in nA ± S.E. for the different oocyte groups were as follows: control, 414 ± 26; RGS9 alone, 560 ± 73; RGS9 + Gβ5 short, 489 ± 32.

GIRK channel subunits used in this study, Kir3.1 and Kir3.2, are widely distributed in the brain (27), and their expression overlaps with the expression of RGS7 (28) and Gβ5.

The expression of RGS7 modestly accelerated the deactivation kinetics of the GIRK response when compared with control (Fig. 1, $p < 0.01$), reflecting a stimulation of the GTPase activity of the oocyte Gα-G proteins by RGS7. This acceleration has been independently observed recently (28). In addition, we found that co-expression of both the short and the long forms of the G protein subunit Gβ5 with RGS7 significantly accelerated the speed of GIRK deactivation when compared with RGS7 alone (Fig. 1, $p < 0.01$).

We also found that Gβ5 enhanced the function of another GGL-containing RGS protein, the striatally enriched RGS9-2 (24). The expression of RGS9 with GIRK channels and m2-mAChR accelerated GIRK channel kinetics (Fig. 2, $p < 0.01$). Again, when we co-expressed Gβ5, the acceleration of GIRK channel kinetics was strongly increased over that produced by RGS9 alone (Fig. 2, $p < 0.01$). Expression of Gβ5 in the absence of RGS7 or -9 had no effect on GIRK kinetics (Fig. 4, data not shown), indicating that the Gβ5 isoforms function to enhance the action of these RGS proteins on Gα-G proteins. The functional activity of RGS7 and RGS9 when expressed alone does not appear to be due to any endogenous Gβ5 expressed by *Xenopus* oocytes. Oocyte lysates from uninjected oocytes showed no immunoreactivity to an anti-Gβ5 antibody that recognized Gβ5 from *Xenopus* retina or Gβ5 exogenously expressed in the oocytes by cRNA injection (data not shown).

These results appear to be at odds with those obtained by Levay *et al.* (12), who showed that formation of the RGS7-Gβ5 complex abolished the binding of RGS7 to purified $G\alpha_o$. Their suggestion that Gβ5 serves to inhibit RGS function differed from the findings of Snow *et al.* (10), who showed that the RGS11-Gβ5 complex has GAP activity and hence must interact with Gα. The discrepancy may have arisen because Snow *et al.* (10) used a truncated RGS11 construct, while Levay *et al.* (12) used full-length RGS7. Another explanation for the differing results may be that Levay *et al.* (12) separately synthesized the RGS protein and the Gβ5 subunits in rabbit reticulocyte lysates before allowing complex formation by mixing the two reactions. The assumed RGS7-Gβ5 complex prepared in this manner may differ from the RGS7-Gβ5 complex that is produced in the same cells, so that the former RGS7 conformation may not correspond to that formed within the cell.

Biochemical studies have demonstrated that GGL domains bind only Gβ5 subunits (10–12) and not other Gβ subunits. Similarly, we found that the enhancement of RGS7 action was specific to Gβ5; co-expression of Gβ1 did not increase the rate

constant of GIRK activation or deactivation (Fig. 1). We tested the integrity of the Gβ1 cRNA that was used by coinjecting a subset of the Gβ1 cRNA receiving oocytes with Gγ2. The activation of GIRK channels by G protein-coupled receptors is mediated by Gβγ dimers. As previously reported, the coinjection of cRNA for Gβ1 and Gγ2, but not Gγ2 alone, resulted in a dramatic increase in the basal activation of the GIRK channels and an occlusion of further GIRK activation by coexpressed m2-mAChR (data not shown) (29–31). This result verified that functional Gβ1 protein could be translated from the injected cRNA transcript. In the example shown (Fig. 1*B*), it appears that coinjection of Gβ1 cRNA produces a small but significant slowing of the action of RGS7 on GIRK deactivation kinetics. This slowing may have resulted from a small decrease in RGS7 protein due to competition between RGS7 and Gβ1 cRNA for the oocyte protein translational machinery.

We then tested the effect of Gβ5 on the function of RGS4, which does not contain a GGL domain. Oocytes were injected with two concentrations of RGS4 cRNA. Oocytes that were injected with 30 ng of RGS4 cRNA displayed the fastest activation and deactivation of ACh-elicited GIRK currents. The kinetics of the GIRK current response were significantly slower in oocytes injected with 3 ng of RGS4 cRNA and slowest in the control group. Coinjection of Gβ5 cRNA into oocyte groups injected with 3 ng of RGS4 cRNA did not significantly accelerate the kinetics (Fig. 3). That Gβ5 did not noticeably affect RGS4 function was not due to an inability to measure faster GIRK kinetics because the kinetics of GIRK activation and deactivation were significantly accelerated in oocytes injected with a higher amount of RGS4 cRNA (30 ng). Associated with the extreme acceleration of the GIRK activation phase produced in the oocyte group injected with 30 ng of RGS4 cRNA is a significant decay or sag of the current trace. This has been observed previously and may reflect a desensitization process that is normally obscured by slower rise times (18, 32).

We also tested the effect of Gβ5 on the function of a mutant RGS7 construct, RGS7ΔN, lacking the N terminus and the GGL domain. Oocytes were injected with three concentrations of RGS7ΔN cRNA. Oocytes that were injected with 3 ng of RGS7ΔN cRNA displayed markedly accelerated activation and deactivation of ACh-elicited GIRK currents. These rates exceeded 2 s^{-1} and may have been limited by the exchange time of our chamber; the rates were thus at least as great as those observed with full-length RGS7 coexpressed with Gβ5 or with RGS4. Thus, removal of the N terminus and GGL domain may allow RGS7 to function more efficiently. However, the major point for the present experiments is that GIRK kinetics were slower and well within the resolution of our chamber in oocytes injected with 0.5 ng of RGS7ΔN cRNA, slower still in the group injected with 0.1 ng of cRNA, and slowest in the control group (Fig. 4, $p < 0.01$). Importantly, coinjection of Gβ5 cRNA into control oocytes or groups injected with either 0.1 or 0.5 ng of RGS7ΔN cRNA did not significantly accelerate the kinetics (Fig. 4). These data indicate that Gβ5 cannot accelerate GIRK kinetics when expressed alone and does not enhance the function of RGS proteins that lack GGL domains. Furthermore, these data argue against the possibility that it is the RGS7-Gβ5 complex that specifically interacts with the channel to accelerate the kinetics.

In previous experiments on *Xenopus* oocytes and Chinese hamster ovary cells, the co-expression of RGS proteins increased the rate constants for both GIRK activation and deactivation (18, 19, 28) and also did not change the steady-state activation levels. We obtained similar results in our experiments with RGS7, RGS9, and Gβ5. The expression of RGS7 and RGS9 modestly enhanced the observed activation rate

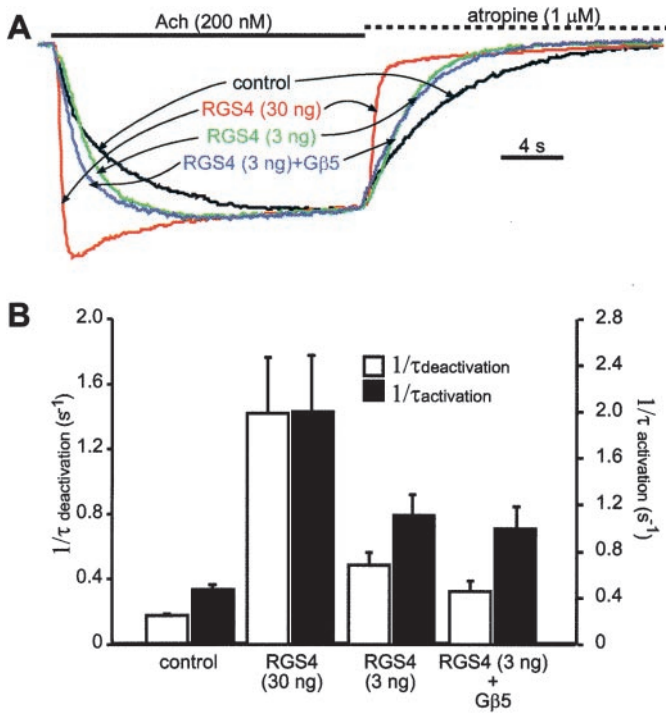

FIG. 3. **Effect of Gβ5 on RGS4 function.** Control oocytes were injected with cRNA for the m2-mAChR (0.01 ng) and 0.02 ng of cRNA each for GIRK1 and GIRK2. The other oocyte groups tested were injected with the following cRNA combinations in addition to those injected into the control oocytes: RGS4 (30 ng), RGS4 (3 ng), Gβ5 (10 ng), and RGS4 (3 ng). *A*, normalized representative traces of ACh (200 nM)-evoked and atropine (1 μM)-reversed GIRK currents. The *solid horizontal line above* the *traces* represents ACh application, and the *dashed line* shows atropine application. The traces were normalized to the amplitude of the response just prior to the application of atropine. *B*, comparison of the activation ($1/\tau_{activation}$, *solid bars*) and deactivation ($1/\tau_{deactivation}$, *open bars*) rate constants derived from the exponential fits of the activation and deactivation phases of the GIRK currents in the different oocyte groups. All *bars* are means ± S.E. from 4–6 oocytes, all from the same oocyte donor. The mean steady-state current amplitudes in nA ± S.E. for the different oocyte groups were as follows: control, 399 ± 38; RGS4 (30 ng), 817 ± 365; RGS4 (3 ng), 563 ± 251; RGS4 (3 ng) + Gβ5 short, 900 ± 97.

constants when compared with control, and these observed activation rate constants were further enhanced by co-expression of Gβ5 (Figs. 1 and 2; $p < 0.01$). Accelerated activation kinetics (an increase in $1/\tau_{activation}$) are in fact an unsurprising and necessary consequence of the accelerated deactivation kinetics (an increase in $1/\tau_{off} = 1/\tau_{deactivation}$), because in most kinetic formulations,

$$1/\tau_{activation} = 1/\tau_{on} + 1/\tau_{off} \quad \text{(Eq. 1)}$$

Thus, point mutations introduced in RGS2 and RGS4 that abolished their GTPase enhancing activity also resulted in the loss of the property of these proteins to enhance both the activation and deactivation kinetics of GIRK channels (20). The surprising aspect of this and previous experiments (18, 19, 21) is that the increased $1/\tau_{off}$ does not lead to a decrease in steady-state activation, since the predicted fraction of open channels f is given by the following.

$$f = 1/\tau_{on}/(1/\tau_{on} + 1/\tau_{off}) \quad \text{(Eq. 2)}$$

In the most likely explanation for this increase in steady-state currents, RGS proteins may be enhancing the rate of GDP-GTP exchange as well as the rate of GTP hydrolysis (33).

We also explored mechanisms by which Gβ5 might enhance RGS7 function. In one possible mechanism, Gβ5 would stabilize the cellular conformation of RGS7, thereby increasing its

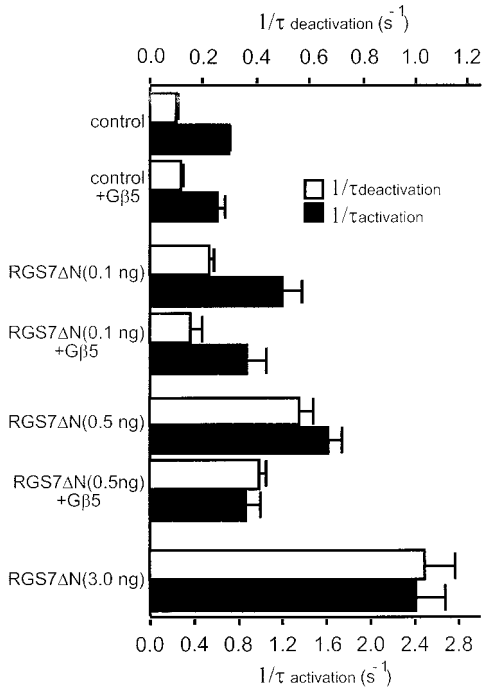

FIG. 4. **Effect of Gβ5 on function of an RGS7 construct lacking the GGL domain.** RGS7ΔN (corresponding to amino acids 322–470 of the wild type clone) is an RGS7 construct created by deleting the N-terminal region containing the GGL domain from the full-length wild type mouse RGS7. Control oocytes were injected with cRNA for the m2-mAChR (0.01 ng) and 0.02 ng of cRNA each for GIRK1 and GIRK2. The other oocyte groups tested were injected with the following cRNA combinations in addition to those injected into the control oocytes: RGS7ΔN (3 ng), RGS7ΔN (0.5 ng), Gβ5 short form (10 ng) and RGS7ΔN (0.5 ng), RGS7ΔN (0.1 ng), Gβ5 short form (10 ng) and RGS7ΔN (0.1 ng), Gβ5 short form alone (10 ng). Comparison of the activation ($1/\tau_{activation}$, *solid bars*) and deactivation ($1/\tau_{deactivation}$, *open bars*) rate constants derived from the exponential fits of the activation and deactivation phases of the GIRK currents in the different oocyte groups is shown. All *bars* are means ± S.E. from 4–6 oocytes, all from the same oocyte donor. The mean steady-state current amplitudes in nA ± S.E. for the different oocyte groups were as follows: control, 1094 ± 103; control + Gβ5, 573 ± 32; RGS7ΔN (0.1 ng), 573 ± 27; RGS7ΔN (0.1 ng) + Gβ5 short, 820 ± 169; RGS7ΔN (0.5 ng), 956 ± 82; RGS7ΔN (0.5 ng) + Gβ5 short, 651 ± 48; RGS7ΔN (3 ng), 736 ± 81.

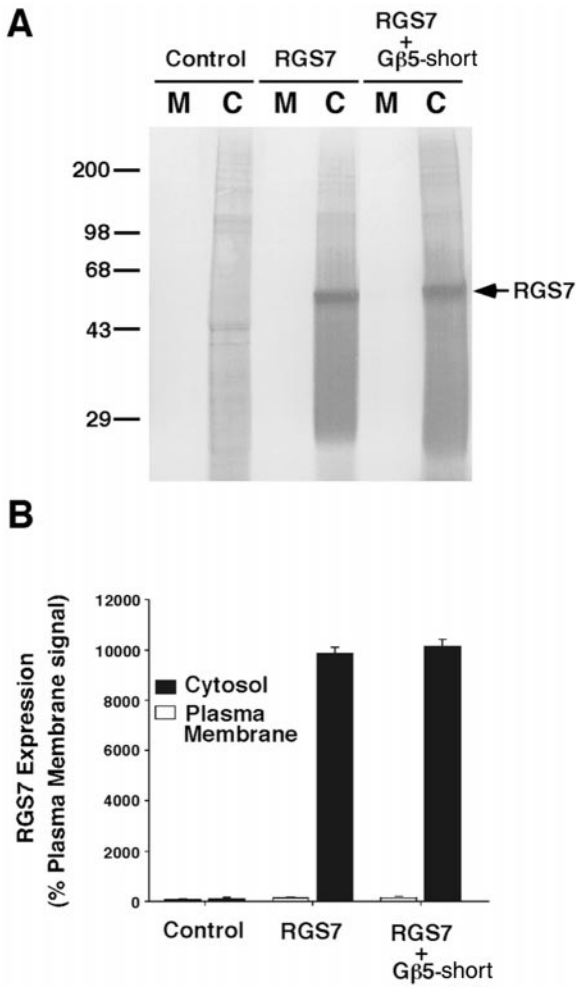

FIG. 5. **Effect of Gβ5 on expression of RGS7 protein.** Control oocytes were injected with 0.01 ng of cRNA for the m2-mAChR and with 0.02 ng of cRNA each for GIRK1 and GIRK2. Other oocyte groups were injected with the following cRNA combinations in addition to the cRNAs injected into the control oocytes: RGS7 (10 ng) alone, RGS7 (10 ng), and Gβ5 short form (10 ng). *A*, autoradiograph showing [^{35}S]methionine-labeled RGS7 immunoprecipitated from cytosolic fraction (*C*) and plasma membrane fraction (*M*). For experimental conditions, see "Experimental Procedures." *B*, *bar graph* showing relative densities of the RGS7 protein bands shown in *A* (see "Experimental Procedures"). Band densities are expressed as a percentage of the density in the portion of the control plasma membrane lane that corresponds to the RGS7 bands in the RGS7-expressing samples. All *bars* are means ± S.E. from three separate experiments.

cellular concentration. Precedent for such a mechanism is provided by the observation that Gβ and Gγ subunits are unstable when unpaired and that co-expression of Gβ and Gγ subunits results in higher cellular protein levels for both subunits (34–36); this is also true for Gβ5 (13). To determine whether Gβ5 increases cellular levels of RGS7 protein, we used an anti-RGS7 antibody to immunoprecipitate ^{35}S-labeled RGS7 from oocyte groups that had been tested electrophysiologically. The immunoprecipitated RGS7 proteins were detected and quantitated after SDS-polyacrylamide gel electrophoresis. Contrary to our expectations, we found that neither the level of total nor cytosolic RGS7 protein was increased by the co-expression of Gβ5 (Fig. 5). The failure to detect an increase in RGS7 levels after co-expression of Gβ5 was not due to saturation of either the anti-RGS7 antibody or the detection technique, because the amount of RGS7 detected was proportionally increased with more starting material (data not shown). Similar results were also obtained when the oocytes were lysed and the immunoprecipitations were performed in buffers containing no detergents, suggesting that Gβ5 did not enhance RGS7 function by altering its solubility (data not shown).

Alternatively, Gβ5 might enhance RGS7 function by targeting RGS7 to the plasma membrane, thus increasing the effective concentrations of RGS7 near the G protein. However, both the long and short forms of Gβ5 contain no predicted membrane targeting sequences (13, 14). Instead, Gβ subunits are thought to associate with membrane by complexing to prenylated Gγ subunits (37, 38). In accordance with the above arguments, we detected no RGS7 signal in the oocyte plasma membranes from any of the oocyte groups that were tested (Fig. 5).

RGS expression may be increased by Gβ5 in other systems; Snow et al. (11) reported that the protein levels of RGS6, which also contains a GGL domain, were elevated in COS cells expressing Gβ5. Consequently, it is notable that our data indicate that Gβ5 enhances the function of the GGL containing RGS7 in the absence of changes in RGS7 protein expression. Instead, we suggest that Gβ5 may alter the conformation of the GGL containing RGS proteins, thereby leading directly to enhanced function. In support of this idea, it has previously been shown that the conformations of monomeric Gβ and Gγ are altered during dimerization (35, 36).

The principal findings of this study are severalfold. First, we

have demonstrated that GIRK kinetics can be accelerated by the co-expression of the GGL domain containing RGS7 and -9. We have addressed uncertainties about the role of Gβ5 in the function of RGS proteins by demonstrating that Gβ5 enhances this action of both RGS7 and RGS9. We provide data to suggest that the GGL domain is required for the enhancement of RGS function by Gβ5. We show that the enhancement of RGS7 function by Gβ5 is not a result of an increase in the total, cytosolic, or plasma membrane RGS7 protein concentration. These results should assist our understanding of the role of GGL-RGS proteins in modulating G protein signaling.

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