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Expression levels of RGS7 and RGS4 proteins determine the mode of regulation of the G protein-activated K^+ channel and control regulation of RGS7 by Gβ5

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Abstract Regulators of G protein signaling RGS4 and RGS7 accelerate the kinetics of K^+ channels (GIRKs) in the *Xenopus* oocyte system. Here, via quantitative analysis of RGS expression, we reveal biphasic effects of RGSs on GIRK regulation. At low concentrations, RGS4 inhibited basal GIRK activity, but stimulated it at high concentrations. RGS7, which is associated with the G protein subunit Gβ5, is regulated by Gβ5 by two distinct mechanisms. First, Gβ5 augments RGS7 activity, and second, it increases its expression. These dual effects resolve previous controversies regarding RGS4 and RGS7 function and indicate that they modulate signaling by mechanisms supplementary to their GTPase-activating protein activity. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: G protein; Regulator of G protein signaling; G protein-activated K^+ channel; Potassium channel; Gβ5; *Xenopus* oocyte

1. Introduction

Regulator of G protein signaling (RGS) proteins accelerate hydrolysis of GTP bound to the Gα subunits of heteromeric G proteins thereby promoting the formation of the inactive $G\alpha_{GDP}\beta\gamma$ heterotrimer, and hence act as negative regulators of G protein-mediated signaling pathways [1–4]. The RGS protein family includes more than 30 members, all of them characterized by a homologous ~120 amino acid region, known as the RGS domain, that is responsible for binding Gα and GTPase-activating protein (GAP) activity [5,6]. A subfamily of RGS proteins that includes RGS6, 7, 9, and 11, has two additional unique domains: the DEP domain (disheveied/egl-10/pleckstrin) of an unknown function, and the GGL (G protein γ-like) domain that has been shown to direct a specific interaction with the G protein subunit Gβ5 [7–9]. RGS6, RGS7 and RGS9 were purified or co-immunoprecipitated from brain and retina only in complex with Gβ5 suggesting that these RGSs and Gβ5 only exist as heterodimers in vivo [10,11]. Complexes of Gβ5 with RGS7 and RGS9 serve as GAPs toward certain Gα subunits [12].

Much of our knowledge about the coupling of RGS proteins to $G_{i/o}$ proteins under physiologic conditions has been derived from the studies of G protein-activated K^+ channels (GIRKs). These channels are activated by direct binding of the βγ subunits of G proteins, $G_{\beta\gamma}$, normally released upon activation of $G_{i/o}$ [13–17]. Deactivation kinetics of neurotransmitter-evoked GIRK currents depend on sequestration of free $G_{\beta\gamma}$ by $G\alpha_{GDP}$ and, therefore, reflect the rate of GTP hydrolysis by Gα [18]. Coexpression of RGS proteins with $G_{i/o}$-coupled neurotransmitter receptors and GIRK channels in *Xenopus* oocytes accelerates the deactivation of GIRK channels [19–21]. Activation is accelerated as well, possibly also as a consequence of the increased GTPase activity, though other factors might be involved [21]. Thus, GIRK current is a sensitive indicator of the RGS activity, and these channels have been extensively utilized to study the roles and physiological effects of RGS proteins. Surprisingly, coexpression of RGS proteins does not decrease (and sometimes increases) the amplitude of agonist-evoked GIRK currents, in contrast to what is predicted from their GAP action [19,20,22,23]. The exact mechanism underlying this phenomenon remains unknown [4]. Recently, Kovoor et al. [21] used the GIRK assay to explore the effect of Gβ5 on the RGS7 function and demonstrated that Gβ5 enhances the RGS7-induced acceleration of deactivation of GIRK. Coexpression of Gβ5 did not change the amount of RGS7 in the oocytes, and it was concluded that the sole effect of RGS–Gβ5 interaction is the enhancement of RGS GAP activity. Another line of evidence supporting the role for Gβ5 as a stimulator of RGS7 GAP activity is the finding that Gβ5 enhanced the inhibitory effect of RGS7 on G_q-mediated Ca^{2+} signaling in transfected CHO cells [10]. However, in contrast to findings of Kovoor et al., Witherow et al. [10] observed 5–10-fold increase in RGS7 expression in the presence of Gβ5. In line with the latter study, Gβ5L disappears from photoreceptors of RGS9 knockout mice [24], supporting the view that the presence of RGS9 improves biosynthesis or slows down degradation of Gβ5.

In this report we attempted to resolve the apparent conflict regarding the effect of Gβ5 on RGS7 expression through a series of experiments titrating the amount of RGS7 expressed in *Xenopus* oocytes. We demonstrate that Gβ5 has a dual

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Abbreviations: ACh, acetylcholine; GAP, GTPase-activating protein; GGL, G protein γ-like (domain); GIRK, G protein-activated K^+ channels; I_{ACh}, agonist-evoked GIRK current; I_{hK}, basal GIRK current; RGS, regulator of G protein signaling

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effect: (i) increase of RGS7 level, which was only observed at submaximal expression of RGS7, and (ii) stimulation of RGS7 effect on GIRK kinetics observed at all levels of RGS7 expression. Titration of the expression of another RGS protein, RGS4, which we initially used as a control for RGS7, helped us to resolve another controversy: previous results of Doupnik et al. in *Xenopus* oocytes [19], showing a decrease in basal GIRK current upon addition of RGS4, were at odds with the increase observed by Bunemann and Hosey in mammalian cells [25]. Here we show that RGS4 can be both inhibitory or stimulatory, depending on its concentration. Thorough analysis of the protein levels not only helps to avoid discrepancies arising from the use of different heterologous expression systems and experimental protocols, but also provides additional evidence that the RGS proteins have functions in addition to their GAP activity.

2. Materials and methods

2.1. cDNA constructs and mRNA

The cDNAs were prepared as described in the previous publications: muscarinic 2 receptor (m2R) [26]; GIRK1 and GIRK2 subunits of the GIRK channel [27]. The coding sequences of the cDNAs of the RGS4 and RGS7 were amplified by polymerase chain reaction with primers containing the desired restriction sites and inserted into high-expression oocyte vectors containing 5′ and 3′ untranslated sequences of *Xenopus* β-globin. The use of these vectors usually improves protein expression in the oocytes [28,29]. RGS4 was subcloned into *Xba*I/*Hin*dIII sites of the pGEMHE vector [28], and bovine RGS7 was inserted into the *Not*I/*Hin*dIII sites of the pBS-MXT vector [29]. The cDNA of the mouse Gβ5 was inserted into the *Not*I/*Hin*dIII sites of the pBS vector (Stratagene). mRNAs were synthesized according to a standard procedure which gives high-quality RNA with preferable capping at the 5′ end [30]. For electrophysiological and biochemical experiments, the following amounts of RNA were injected (per oocyte): GIRK1, GIRK2, m2R, 100 pg; Gβ5, 20 ng.

2.2. Oocytes and electrophysiology

Xenopus oocytes were prepared and injected as described [30], in accordance with the instructions of Tel Aviv University Institutional Animal Care and Use Committee. After injection, the oocytes were incubated for 3 days at 20–22°C in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM Na-pyruvate and 50 μg/ml gentamicin. Two-electrode voltage clamp experiments were performed as described [26]. For measuring the GIRK1/2 currents, the oocytes were placed in a ~70 μl chamber constantly perfused with ND96. The holding potential was set at −80 mV. Basal (I_{hK}) and acetylcholine (ACh)-evoked (I_{ACh}) currents were measured in the hK solution (96 mM KCl, 2 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4–7.5). The perfusion allowed exchange of solution in the bath within ~0.5 s.

2.3. Immunochemistry

The oocytes were homogenized on ice in homogenization buffer (20 mM Tris, pH 7.4, 5 mM EGTA, 5 mM EDTA, 100 mM NaCl) containing the Roche Molecular Biochemicals protease inhibitor mixture. Debris was removed by centrifugation at $8000 \times g$ for 15 min at 4°C [31]. Protein samples (five oocytes per lane) were separated on 10% polyacrylamide–sodium dodecyl sulfate gels. RGS7 and Gβ5 expressed in the oocytes were monitored by Western blotting as described [32]. Antibodies against RGS7 and Gβ5 were described previously [8,33] and used here at 1:3000 dilution. Visualization of protein bands was performed using ECL reagents obtained from Pierce, Inc. The intensity of labeling was quantified using the TINA software (Raytest, Straubenhardt, Germany).

2.4. Statistical analysis

Data are presented as mean ± S.E.M. Comparison between two groups of treatment was done using the two-tailed Student test or, when appropriate, the paired *t*-test. Comparison between several groups of data has been performed using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test.

3. Results

3.1. Regulation of basal activity of GIRK by RGS proteins depends on their expression level

Xenopus laevis oocytes were injected with the RNAs of GIRK1 and GIRK2 subunits (GIRK1/2) of the GIRK channel, the m2R, and different combinations of RGS7 and Gβ5. The ion currents were measured using the two-electrode voltage clamp technique. The basic protocol is shown in Fig. 1A. The oocyte was placed in a low-K^+ solution (ND96), in which the GIRK currents are negligible. Shifting to a high-K^+ solution (hK) caused the appearance of a large inward current (I_{hK}) that reflects the basal activity of the GIRK channels [34]. After the basal currents reached a plateau, 10 μM ACh was applied, to monitor the ACh-evoked current, I_{ACh}. After ~1 min in ACh, the agonist was washed out with hK solution containing 10 μM atropine (a muscarinic antagonist). The speed of deactivation of ACh-evoked response was used as the assay for RGS7 activity. For comparison, we also expressed RGS4, whose effects on GIRK are well characterized [19,21,25].

To characterize and compare the effects of RGS4 and RGS7 at different expression levels, oocytes were injected with standard amounts of RNAs of GIRK1, GIRK2 and m2R (0.1 ng/oocyte) and with increasing amounts of RGS7 or RGS4 RNAs. Fig. 1A presents an example of the effect of RGS4 and RGS7 on GIRK currents in oocytes obtained from one donor ('batch'). In agreement with previous findings [19–22], coexpression of either RGS accelerated both activation and deactivation of I_{ACh}. In addition, RGS4 affected the amplitudes of basal and agonist-evoked currents, but we noticed that the effects varied as a function of the level of RGS4 expression. Thus, at all doses of injected RGS4 RNA, we usually observed a decrease in the amplitudes of I_{hK} and I_{ACh}, whereas after longer periods, especially at the highest doses used (5 or 10 ng RNA/oocyte), both I_{hK} and I_{ACh} were often increased. Fig. 1B compares the changes in amplitudes of I_{hK} (left panel) and I_{ACh} (right panel) at the lowest RNA doses (0.05 and 0.2 ng/oocyte) and the highest doses used (5 and 10 ng). We compared current amplitudes 3 days after RNA injection (black bars) vs. the time when the enhancing effect of 5–10 ng RNA was maximal (4 days in two oocyte batches, 7 days in one batch). This comparison reveals a striking change in the pattern of RGS4 effect: the initial decrease in current amplitudes observed after 3 days of protein expression disappears at later times, and (at higher doses) is replaced by an increase. It is natural to assume that this trend is a result of an increase in the level of RGS4 protein expression. This assumption is further strengthened by the observation that low RGS4 doses still inhibited I_{hK} and I_{ACh} on the same days when the high doses already produced a maximal enhancement of the currents. This can be deduced from Fig. 1B but is best seen in Fig. 1C which reports the dose-dependency of RGS4 effects in the full range of RNA doses used. Currents were measured, in each oocyte batch, at the same day when 5 or 10 ng RNA produced a maximal enhancement (day 4 or day 7 after RNA injection). A statistically significant (25–40%) reduction in I_{hK} was observed at two of the three lowest doses of RGS4 RNA used, 0.05 and

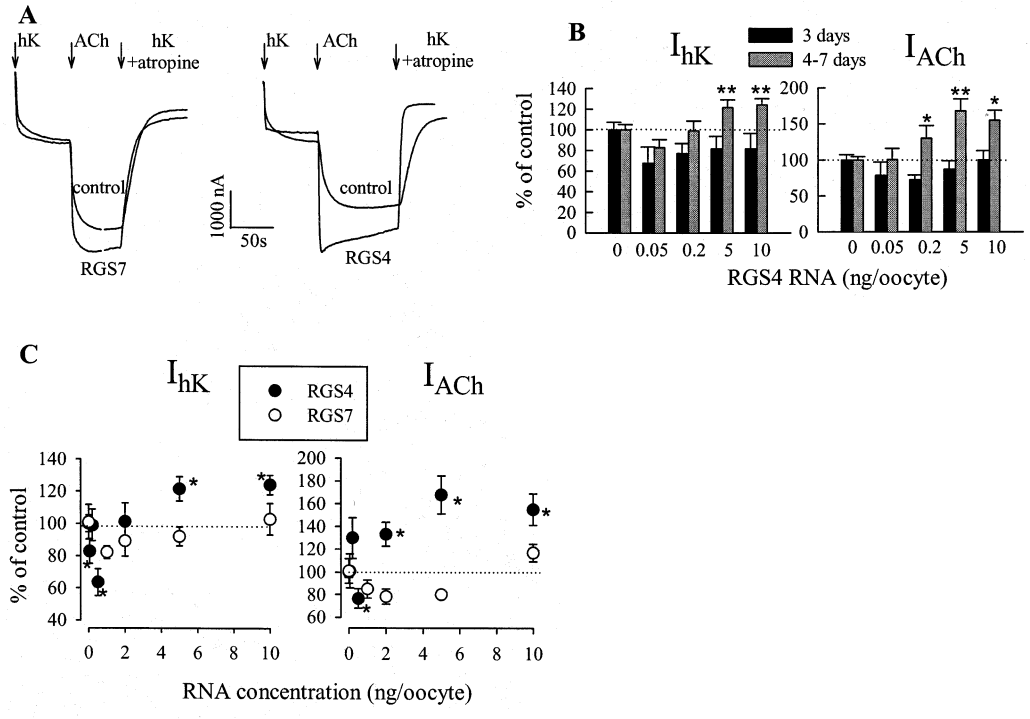

Fig. 1. The effects of RGS4 and RGS7 on the amplitude of GIRK1/2 currents depend on level of expression of RGS proteins. A: GIRK1/2 currents from oocytes coexpressing GIRK1/2 and m2R in the presence or absence of RGS4 (2 ng RNA/oocyte) or RGS7 (10 ng RNA/oocyte). In the left panel, about 15–20 s in each record was omitted, to merge the beginnings of agonist washout. B: Effects of various doses of RGS4 on I_{hK} and I_{ACh} 3 days after RNA injection (black bars; summaries from 5–17 cells of 1–3 batches) or 4–7 days after RNA injection (gray bars; 10–28 cells from 2–3 batches). Pairwise comparisons of black and gray bars' values were done using two-tailed t-test. $^{*}P<0.05$; $^{**}P<0.01$. C: Dose-dependency of the effects of RGS4 (●) and RGS7 (○) on the GIRK1/2 currents amplitude, after 4–7 days of expression (RGS4) or 4 days (RGS7). Each experimental point represents results from 10–28 oocytes from two or three batches (RGS4) or 6–7 oocytes of one batch (RGS7). In each oocyte, the amplitude of the current was normalized to the average amplitude in control group of the same batch. The resulting normalized values were averaged from all oocytes. Asterisks indicate significant difference from control (no RGS) at the $P<0.05$ level.

0.5 ng/oocyte (Fig. 1C, left panel). This decrease was replaced by a clear increase which reached 24 ± 6% at 10 ng RNA ($n=20$; $P<0.05$; one-way ANOVA). The agonist-evoked current, I_{ACh}, clearly showed the same tendency though the increase was more pronounced. The decrease was seen only at 0.5 ng RNA/oocyte, and a significant increase was observed at 2–10 ng RNA, reaching $>50\%$ at 5 and 10 ng (Fig. 1B, right panel). These data resolve the present controversy concerning the effect of RGS4 on basal GIRK activity [19,25] and imply that coexpression of RGS4 affects GIRK in two different ways.

The effect of RGS7 on I_{hK} and I_{ACh} resembled that of RGS4, but the changes were smaller. A trend to decrease was seen at low levels of expression; it tended to disappear at higher doses of RGS7 (Fig. 1B). On average, under the standard conditions used in the following experiments (0.05 and 10 ng RGS7 RNA, 4 days of expression), neither I_{hK} nor I_{ACh} was significantly changed at the fourth day in more than 10 oocyte batches examined (data not shown).

3.2. Dose-dependent acceleration of activation and deactivation of I_{ACh} by RGS4 and RGS7

Activation and inactivation kinetics of the ACh-evoked GIRK current could be approximately described by single exponential functions [19] with time constants $\tau_{activation}$ and $\tau_{deactivation}$, respectively. Fig. 2 describes the dependence of the changes in $\tau_{activation}$ (Fig. 2A) and $\tau_{deactivation}$ (Fig. 2B) on the amounts of coinjected RNAs of RGS4 and RGS7. Activation and deactivation of I_{ACh} were accelerated by both RGS proteins, but RGS4 appeared to be more potent. The effects were quantitated by fitting the curves of τ vs. RNA dose to single exponential functions and calculating the effective doses of RNAs at which the RGSs caused 50% effect (ED_{50}) and the minimal ('saturation') values of τ. Half maximal effects of RGS4 on activation and deactivation occurred at 0.25–0.33 ng RNA/oocyte, whereas those of RGS7 occurred at 5–9-fold higher RNA concentrations. The maximal acceleration was stronger in the presence of RGS4: 1.8 and 2.1 s for $\tau_{activation}$ and $\tau_{deactivation}$, respectively, as compared with 2.4 and 5.3 s for RGS7. The difference in maximal effects suggests that, under the conditions of our experiments, RGS4 is a more potent GAP for *Xenopus* $G_{i/o}$ proteins, which are known to mediate the m2R-directed activation of GIRK in this preparation [16].

3.3. Coexpression of Gβ5 stimulates both RGS7 protein levels and its physiological effect

The lowest concentration of RGS7 RNA causing a statistically significant change in $\tau_{deactivation}$ was 0.5 ng/oocyte, while at 10 ng/oocyte the effect was almost maximal (Figs. 2 and 3). Therefore, we chose these two concentrations to examine the effect of Gβ5 on RGS7. Since 0.5 ng RGS7 RNA/oocyte did not result in a significant change in the rate of activation, we monitored only the changes in deactivation. To describe the

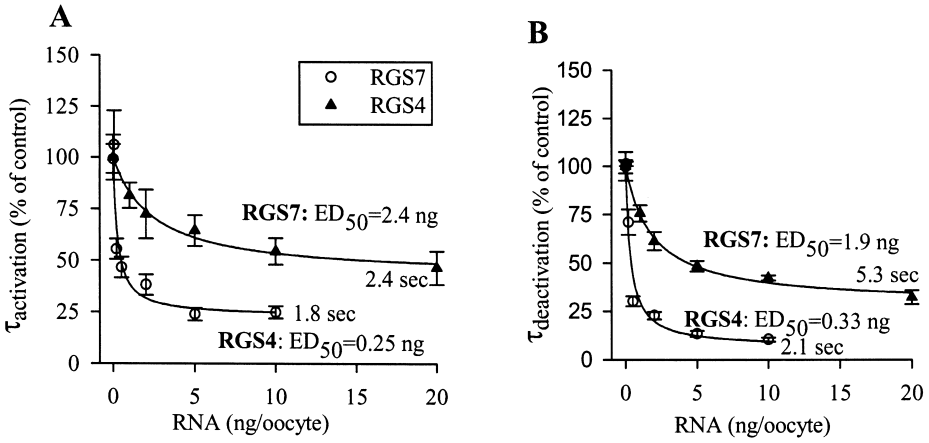

Fig. 2. The effect of RGS4 and RGS7 on the kinetics of GIRK1/2 currents. The calculated (fitted) values of ED_{50} and of the minimal τ are shown near each curve. The data were obtained in oocytes of one batch (5–6 oocytes at each RNA concentration). A: Dose–response of RGS7 and RGS4 effects on $\tau_{activation}$ of I_{ACh}. B: Dose–response of RGS7 and RGS4 effects on $\tau_{deactivation}$ of I_{ACh}.

speed of deactivation, we used the time by which the deactivation of I_{ACh} was 50% complete, $t_{1/2\ deactivation}$, to simplify the analysis.

As shown previously [21], expression of Gβ5 alone (10 or 20 ng RNA/oocyte) did not exert a statistically significant effect on I_{ACh}, but enhanced the accelerating effect of RGS7. As expected, Gβ5 did not modify the effect of RGS4 on GIRK kinetics (data not shown), confirming that its effect on RGS7 was specific. We have first attempted to reproduce the data of Kovoor et al. [21] who utilized high doses of RGS7 RNA. Fig. 3A summarizes the results obtained in one such experiment. Expression of 10 ng RGS7 RNA alone reduced $t_{1/2\ deactivation}$ by 45 ± 6% (Fig. 3A; changes in $t_{1/2\ deactivation}$ are shown as positive values). In agreement with the report of Kovoor et al. [21], expression of Gβ5 further enhanced this effect 1.3-fold. It should be noted here that, since much faster deactivation rate was observed with RGS4, the measured speed of deactivation at high doses of RGS7 with Gβ5 was not limited by our perfusion system (see Section 2). Importantly, when the amount of RGS7 expressed in the oocytes was low (0.5 ng RNA/oocyte), Gβ5 enhanced the RGS7 effect in a more dramatic manner, from 14.5 ± 3.7% to 29 ± 2.4% (i.e. about 2-fold; Fig. 3B). This result implied that Gβ5 could enhance the action of RGS7 by more than one mechanism.

To examine whether expression of Gβ5 influences the amount of RGS7 in the oocyte, either 0.5 or 10 ng/oocyte of RGS7 RNA was injected in the presence or absence of 20 ng RNA of Gβ5. Western blot analysis was used to monitor protein levels. Uninjected oocytes did not show detectable endogenous RGS7 or Gβ5 (Figs. 4 and 5). Fig. 4 shows the results of measurements of RGS7 protein. Injection of 10 ng RNA gave approximately 3-fold higher RGS7 protein level than 0.5 ng (Fig. 4A, upper panel). Gβ5 did not alter the amount of RGS7 in oocytes that expressed the high amount of RGS7 (summary of three experiments is shown in the lower panel of Fig. 4A). This is in agreement with a previous report

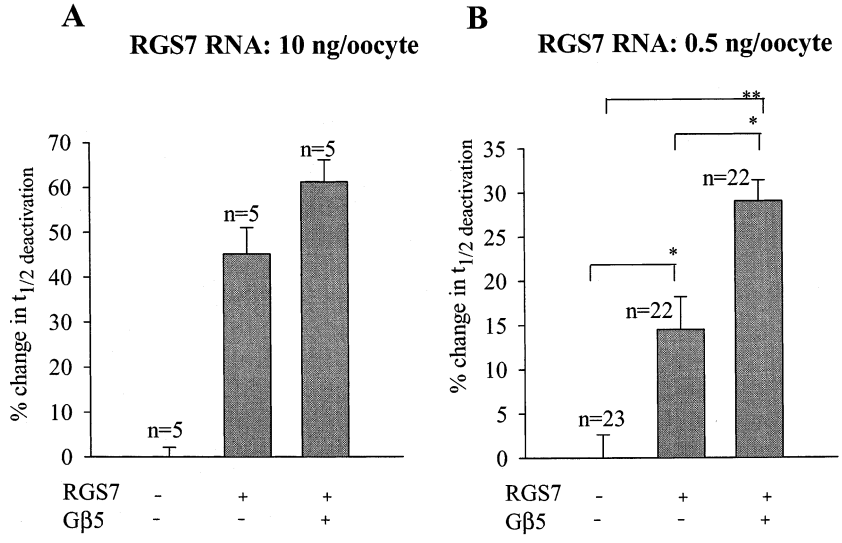

Fig. 3. Gβ5 enhances the effect of RGS7 on deactivation of I_{ACh}. Changes in $t_{1/2\ deactivation}$ (% of control) are shown at the Y axes. The oocytes coexpressed GIRK1/2 with m2R in the presence or absence of Gβ5 and different doses of RGS7. The bars show mean ± S.E.M., n = no. of oocytes. In A, the oocytes were injected with 10 ng/oocyte RGS7 RNA. Results from one oocyte batch are shown. Similar results were obtained in another batch of oocytes. In B, the oocytes were injected with 0.5 ng/oocyte RGS7 RNA. Data from three batches are summarized. Asterisks above bars indicate statistically significant differences (*$P < 0.05$; **$P < 0.01$).

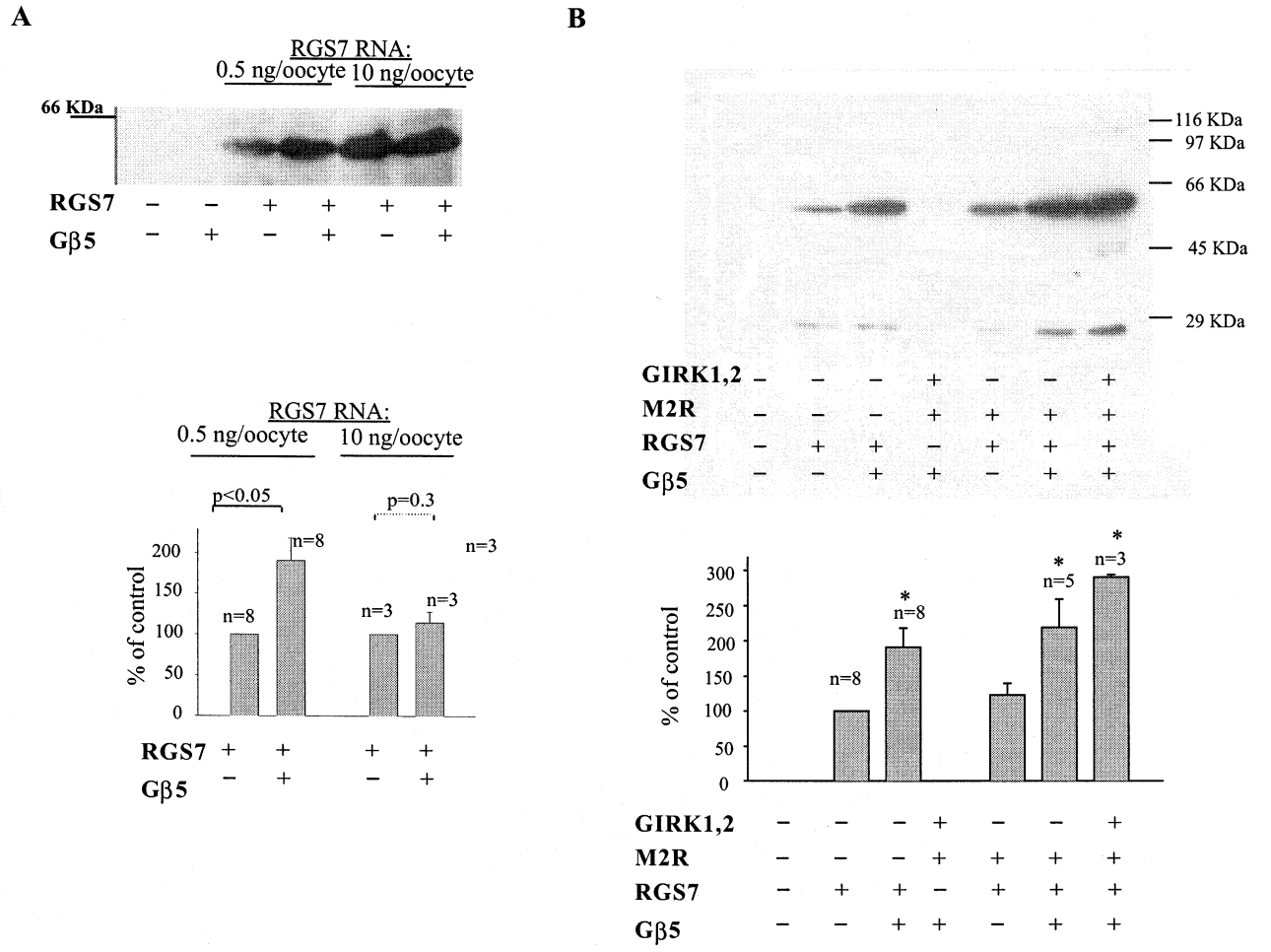

Fig. 4. Expression of RGS7 protein in oocytes and the effect of coexpression of Gβ5, m2R and GIRK. Upper panels show the Western blots from representative experiments, and the bars in the lower panels below summarize the amount of RGS7 protein in oocytes, calculated from densitometric analyses of the images. The bars show mean ± S.E.M., n = no. of experiments. A: Comparison of the effect of coexpression of Gβ5 on the protein level of RGS7 at low (0.5 ng RNA/oocyte) and high (10 ng RNA/oocyte) levels of RGS7. B: Western blot analysis of 0.5 ng/oocyte RGS7 RNA coexpressed with different combinations of GIRK1/2, m2R and Gβ5. Asterisks above bars indicate statistically significant differences, at $P < 0.05$, from control (0.5 ng/oocyte RGS7 RNA alone) by one-way ANOVA test followed by Student–Newman–Keuls test.

by Kovoor et al. [21], who also injected 10 ng RGS7 RNA per oocyte. However, when the dose of RGS7 RNA was 0.5 ng/oocyte, Gβ5 significantly increased the amount of RGS7 (191 ± 27% of control, $n = 8$ experiments; Fig. 4A). An increase in the amount of RGS7 by coexpression of Gβ5 was also observed at 2 ng RGS7 RNA/oocyte (data not shown).

Western blot analysis was further used to examine whether other participants of GIRK1/2 activation pathway can alter the level of the expressed RGS7. RGS7 (at 0.5 ng RNA/oocyte) was coexpressed with Gβ5, GIRK1/2 and m2R in different combinations (Fig. 4B). We found that the main factor that increased the level of RGS7 expression was Gβ5 (1.9-fold). Coexpression of m2R alone did not significantly alter the level of RGS7 which was 123 ± 17% of control ($n = 3$; Fig. 4B). Coexpression of GIRK1/2 together with m2R also did not lead to changes in the level of RGS7 (130 ± 9%, $n = 4$, $P > 0.05$). On the average, coexpression of m2R also did not substantially alter the effect caused by Gβ5 alone (Fig. 4B). On the other hand, when GIRK1/2 and m2R were coexpressed along with Gβ5 and RGS7, the amount of RGS7 protein further rose to 291 ± 4% of control ($n = 3$). This difference was statistically significant compared with the effect of Gβ5 alone ($P < 0.05$ using the ANOVA test). However, further statistical analysis by paired t-test in the three experiments where both groups were present (RGS7+Gβ5 vs. RGS7+Gβ5+GIRK+m2R) did not show a significant difference in RGS7 levels ($P = 0.256$). Therefore, we conclude that Gβ5 is the main factor that causes the observed increase in RGS7 levels, although the presence of all the components of the signaling pathway, including the receptor and the effector, may potentiate the effect of Gβ5.

We have also sought for a possible reciprocal effect of RGS7 on the expression of Gβ5. The control group contained oocytes in which Gβ5 was expressed at the same level as in the electrophysiological experiments (20 ng RNA/oocyte). The amount of expressed Gβ5 protein was not altered by coexpression of either RGS7 (0.5 ng; 110 ± 8% of control, $n = 6$ experiments), GIRK1/2 (103 and 130% of control, $n = 2$), m2R (102 ± 3%, $n = 4$), m2R+GIRK1/2 (93 and 121% of control, $n = 2$), or RGS7+GIRK1/2 (85 and 122% of control, $n = 2$). However, joint expression of RGS7 with m2R, with or without the channel, produced a significant increase in the amount

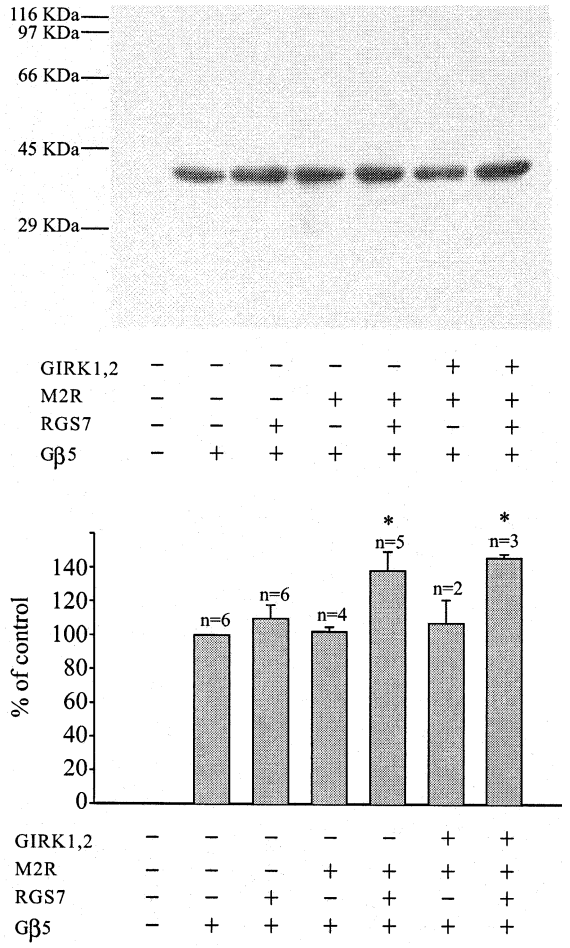

Fig. 5. Expression of Gβ5 protein in oocytes and the effect of coexpression of RGS7, m2R and GIRK. Gβ5 was coexpressed along with different combinations of GIRK1/2 (0.1 ng RNA/oocyte), m2R (0.1 ng RNA/oocyte) and RGS7 (0.5 ng RNA/oocyte). The bars of the lower panel summarize the amount of Gβ5 protein in oocytes, calculated from densitometric analyses of the images. The bars show mean ± S.E.M., n = no. of experiments. Asterisks above bars indicate statistically significant differences from control (the amount of protein measured from oocytes that expressed Gβ5 alone) at $P < 0.05$ level (one-way ANOVA followed by Student–Newman–Keuls test).

of Gβ5 ($P < 0.05$ by one-way ANOVA; Fig. 5). The presence of all the components of the pathway had the most consistent impact on the protein level of Gβ5 (a 46 ± 2% increase above control). Both ANOVA and paired t-test confirmed that the concomitant presence of m2R and GIRK1/2 significantly improved the effect of RGS7 ($P = 0.021$, three experiments). An improvement of RGS7 effect by m2R alone was less obvious. Although the ANOVA test performed on the total summary of all experiments showed $P < 0.05$, paired t-test comparison of the two groups of interest (Gβ5+RGS7 vs. Gβ5+RGS7+m2R) in three experiments where both were present did not show a significant difference ($P = 0.19$, $n = 5$).

4. Discussion

GIRK1/2 channels mediate the inhibitory effects of many neurotransmitters [16,17]. They are widely distributed in the brain, and their expression overlaps with that of RGS7 [22]. Since RGS7 regulates GIRK currents [21,22], and in native tissues it is associated with Gβ5 [11,33,35], the analysis of RGS–Gβ5 interaction is important for understanding regulation of GIRK currents in vivo. The use of heterologous expression systems yielded conflicting results, hindering further progress. The present study resolves the controversy and establishes a dual effect of Gβ5 on RGS7: both protein levels and the GTPase activity of RGS7 are increased by Gβ5.

4.1. Dual effects of RGS proteins of GIRKs

This study was initiated to characterize the influence of Gβ5 on RGS7 expression and its GAP activity in *Xenopus* oocytes, and in an attempt to understand the reasons for the controversy over the question whether Gβ5 affects the expression of RGS7 [10,21]. We were able to demonstrate a dual effect of Gβ5 on RGS7. As assayed by monitoring the rate of deactivation of agonist-induced GIRK current, Gβ5 augments the effect of RGS7, perhaps by stimulating its GAP action. In addition, when the protein level of RGS7 is submaximal, Gβ5 increases RGS7 expression. In the course of the study, as a control for RGS7, we used the much better studied and structurally less complicated RGS protein, RGS4. From these experiments we realized that RGS4 had a dual effect on the basal activity of the GIRK channel which depended on the dose of the expressed RGS4. Furthermore, RGS7 showed a similar trend. The very notion of the possibility that RGS4 would have a dual effect on the amplitude of GIRK currents in the same cell, depending on the expression levels of participating proteins, is not trivial. The same is true regarding the effect of Gβ5 on RGS7. By resolving the existing controversies, our findings present a step forward in the understanding of mechanisms of action and of the physiological roles of RGS proteins and of Gβ5.

Basal activity of GIRK depends on the presence of free $G_{\beta\gamma}$ arising due to basal activity of G proteins [16]. Being a GAP, an RGS is expected to increase the fraction of Gα proteins in their GDP-bound form which sequesters $G_{\beta\gamma}$, leading to a decrease in I_{hK} (the basal current). Such a decrease by RGS1, 2, 3 and 4 has indeed been reported by Doupnik et al. [19] in *Xenopus* oocytes. However, an opposite effect was observed in human embryonic kidney cells expressing GIRK and RGS4 [25]. Bunemann and Hosey [25] proposed that RGS has an additional action: occlusion of $G_{\beta\gamma}$ binding to $G\alpha_{GDP}$, which results in a net increase in free $G_{\beta\gamma}$ concentration and thus a 'constitutive' activation of GIRK. Our data show that both the inhibition and stimulation can be observed at different expression levels of RGS in a single expression system, resolving the controversy between the results obtained by the two groups [19,25]. The resolution of this question also has important mechanistic implications regarding the ways in which RGS may regulate GIRK channels. The dose-dependency of the two opposing effects of RGS (Fig. 1B) supports the Bunemann–Hosey hypothesis. The decrease in I_{hK}, associated with the GAP activity, requires lower levels of RGS4 or RGS7. This corresponds to the high affinity of binding of RGS to the transition, short-lived state of Gα (GTP-bound, in the process of hydrolysis) [1,36]. On the other hand, RGS binds to the $G\alpha_{GDP}$ form of Gα with low affinity; thus, higher doses of RGS are needed to effectively compete with $G_{\beta\gamma}$. This is in line with the increase in I_{hK} observed at high expression levels of RGS4. While we have not observed a consistent increase in I_{hK} at higher doses of RGS7, this could be due

to a weaker coupling of this RGS to the relevant Gα, or to a limited saturating expression level (see below).

4.2. GAP activity of RGS proteins vs. their effects on the amplitude of agonist-evoked currents

As expected, RGS4 and RGS7 accelerated activation and deactivation of agonist-evoked GIRK current, I_{ACh}. For each RGS, the dose-dependencies of changes in activation and deactivation of I_{ACh} were almost identical. In case of RGS4, both kinetic effects were observed already at a low dose of this protein, coinciding with the GAP-related decrease in the basal GIRK currents. This supports the idea that the acceleration of the activation is the consequence of the same mechanism as the acceleration of the deactivation, i.e. increased GTPase rate of Gα [19–21].

On the other hand, RGS7 did not reduce the amplitude of the agonist-evoked current, I_{ACh}. Low levels of RGS4 decreased I_{ACh}, but caused an increase at higher expression levels, instead of the decrease predicted from their GAP action. An increase, or at least the absence of a decrease of the agonist-evoked GIRK currents by coexpressed RGS proteins, has been observed in all previous studies and remains unexplained (see Section 1). The GAP action of RGS also cannot explain the absence of a negative shift in the dose-dependency of the response to agonist [19,20]. Therefore, additional mechanisms of RGS action must be sought for. Unlike the change in I_{hK}, the increase in the amplitude of I_{ACh} cannot be explained by the Bunemann–Hosey hypothesis. The argument is as follows: after application of the agonist, the rate of GDP/GTP exchange at Gα is greatly increased [37,38], and a substantial fraction of Gα would be in the GTP-bound form. Binding of RGS4 to $G\alpha_{GDP}$ is expected to have a much smaller impact on the amplitude of I_{ACh} than on I_{hK}. However, our data show even greater increase in I_{ACh} than in I_{hK} at all RGS doses (Fig. 1B). This can be explained by the following alternative mechanisms. (i) RGS proteins may promote the formation of complexes between GPCRs and Gα proteins (e.g. [39]), and this may lead to an improved response to agonist. (ii) We have previously reported inhibition of $G_{\beta\gamma}$-activated GIRK by certain GTPγS-activated Gα subunits [40,41]. Binding of RGS to $G\alpha_{GTP}$ can oppose the interaction of Gα with the effector [42] and thus reduce the inhibitory effect of Gα. (iii) Recently, RGS4 was reported to interact with GIRK1/4 channels directly [43]. Such an interaction could in principle improve GIRK activation by $G_{\beta\gamma}$. Understanding why, despite the accelerated deactivation rate, RGS proteins do not induce reduction in signal magnitude is an important challenge for the future.

RGS4 has a stronger maximal effect on deactivation of I_{ACh} than RGS7, with saturation reached at lower levels of injected RNA (Fig. 1), suggesting that RGS4 has a stronger GAP activity, as in the case of RGS8 vs. RGS7 [22]. This is in line with the results of biochemical measurements of GAP activities of RGS4 and RGS7–Gβ5 complex toward $G\alpha_o$ [12]. However, at present it is not clear whether the maximal effect of any of the RGS proteins tested so far in the oocyte reflects the actual limit in GAP activity or is limited by maximally attainable level of their expression.

4.3. Dual regulation of RGS7 by Gβ5

When GIRK is used to assay the effects of RGS proteins, the acceleration of I_{ACh} deactivation is considered to be a straightforward indicator of their GAP activity [19,21]. We find that Gβ5 enhances this effect of RGS7 at all expression levels of the latter. When the level of expression was such that RGS7 produced its maximal physiological effect (10 ng RNA/oocyte), Gβ5 moderately but reproducibly enhanced the RGS7-induced acceleration. Parallel biochemical experiments showed no change in the amount of RGS7 protein in the oocyte, implying that the observed acceleration of deactivation was due to an improvement of GAP activity of RGS7. These data are in full agreement with the results of Kovoor et al. [21], who also used 10 ng RNA/oocyte in their biochemical experiments. However, we found that the enhancement of RGS7-induced acceleration was substantially stronger when the expression level of RGS7 was low, at 0.5 ng RNA/oocyte. At this level, RGS7 alone only marginally increased the rate of deactivation of I_{ACh} (half inactivation time was changed by ~14.5%), and Gβ5 enhanced this action 2-fold. This increase of Gβ5 efficiency appears to be almost completely attributed to the increase in the amount of RGS7 protein (Fig. 4). The latter result is in line with the report of Witherow et al. [10] in mammalian cells. We think that Gβ5 is unable to further increase RGS7 expression at a high RGS7 level because of a certain limiting feature of the oocyte, such as saturation of its RNA translation or protein processing machinery [44]. It is not possible at present to compare the levels of RGS7 expression attained by RNA injection in oocytes vs. DNA transfection in mammalian cells. It is possible that mammalian cells also have an upper limit of RGS7 expression but it was not reached by transient transfection [10].

It is still unclear why in oocytes Gβ5 enhances the GAP action of RGS7 ([21] and this report) whereas pull-down assays with in vitro translated proteins show that Gβ5 inhibits Gα–RGS7 binding [8]. It is likely that additional proteins, e.g. the effector, present in the cell determine the functional outcome of RGS7–Gβ5 interaction. A crucial role for the effector in determining whether $G\beta_{5L}$ would reduce or enhance the GTPase activity of RGS9 has been recently demonstrated [45].

4.4. Mutual effects of Gβ5 and RGS7

It is often observed that proteins that form a stable complex, e.g. subunits of ion channels, increase each other's expression [46]. RGS7 and Gβ5 are found in tightly associated form in neuronal tissue [10,11], and they have been shown to affect the expression of each other in mammalian cells. In accordance with these studies, our current experiments show that Gβ5 can enhance RGS7 expression. Our data also show that RGS7 increases the expression of Gβ5, however this effect was statistically significant only when other participants of the signal transduction pathway, the m2 receptor and the effector, were coexpressed together with RGS7. In the absence of RGS7, m2R and GIRK themselves did not have this effect (Fig. 5). Interestingly, the expression of RGS7 was affected in a similar way by the receptor and effector, with the amount of RGS7 increasing as much as 3-fold upon joint coexpression of Gβ5, GIRK1/2 and m2R. Since in our electrophysiological experiments both GIRK1/2 and m2R were always present, it is very likely that the enhancement of RGS7 levels by Gβ5 contributed a major part of the total physiological effect at low doses of RGS7 RNA.

In all, our results indicate the existence of two separate effects of Gβ5 on RGS7: an enhancement of the GAP activity, and a control of protein level. They also suggest that the

control of protein levels of Gβ5 and RGS7 is reciprocal, a phenomenon not reported previously. A plausible mechanism by which formation of bi- or multiprotein complexes could increase the levels of participating proteins is the prevention of degradation. Using pulse-chase methodology, Witherow et al. demonstrated that in mammalian COS-7 cells separately expressed RGS7 and Gβ5 are degraded more than 10-fold faster than the RGS7–Gβ5 complex [10]. Such a mechanism is also involved in regulation of RGS7 levels by polycystin [47], and in co-regulation of Gβ and Gγ levels whereby non-complexed subunits are proteolytically degraded [48,49]. Finally, the additional increases in RGS7 and Gβ5 levels in the presence of m2R and GIRK support the notion of the existence of a signaling complex containing the GPCR, G protein, RGS and effector [39], which could improve the biosynthesis of participating proteins or protect them from degradation.

4.5. The advantages of Xenopus oocytes for quantitative studies of expressed proteins

It is well known that proteins overexpressed in heterologous systems may have non-specific effects and/or display promiscuous coupling to their targets. One way to assess the specificity of interactions is monitoring and controlling the expression level of the proteins under study. Such a controlled expression is easily accomplished with high accuracy in *Xenopus* oocytes by changing the amount of injected RNA (e.g. [50]). Each RNA-injected cell expresses the protein, unlike in transfection methodologies which leave a large percentage of cells untransfected. In addition, this system provides almost unlimited possibilities for combining various protein messages. The levels of protein in the whole cell, cytosol or in plasma membrane can be monitored biochemically [31,51]. In our hands, the variability of the expressed ion channel currents in oocytes from one batch is low. Variability between batches can be reduced by using oocytes of the same size, same number of days after RNA injection and amount of RNA, and accounted for by repeating the same experiment in several oocyte batches. Utilization of these precautions and advantages allowed us to resolve two recent controversies regarding regulation of GIRK by RGS proteins, one as for the effect of RGS4 on basal GIRK activity, and the other concerning the effect of Gβ5 on expression of RGS7. Both controversies appeared, on the first glance, to arise from the use of different heterologous expression systems: mammalian cell lines vs. *Xenopus* oocytes. Intuitively, because mammalian proteins have been studied, one would gather that the mammalian cells reveal the 'real' effects. However, the present work shows that the differences in both cases were in fact quantitative, and the obtained data in all works reflected fundamental features of the proteins under study, independent of the expression system.

The results of this study further our understanding of the function of RGS7 and RGS4 and of the regulation of RGS proteins containing the GGL domain by Gβ5. A key corollary of our findings is the importance of expression levels of interacting proteins in heterologous expression systems. This dictates both thorough monitoring of expression levels of participating proteins, and testing the functional impact of changes in protein levels.

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