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RGS6, RGS7, RGS9, and RGS11 Stimulate GTPase Activity of G_i Family G-proteins with Differential Selectivity and Maximal Activity*

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Regulator of G-protein signaling (RGS) proteins are GTPase activating proteins (GAPs) of heterotrimeric G-proteins that alter the amplitude and kinetics of receptor-promoted signaling. In this study we defined the G-protein α-subunit selectivity of purified Sf9 cell-derived R7 proteins, a subfamily of RGS proteins (RGS6, -7, -9, and -11) containing a Gγ-like (GGL) domain that mediates dimeric interaction with Gβ_5. Gβ_5/R7 dimers stimulated steady state GTPase activity of Gα-subunits of the G_i family, but not of Gα_q or Gα_{11}, when added to proteoliposomes containing M2 or M1 muscarinic receptor-coupled G-protein heterotrimers. Concentration effect curves of the Gβ_5/R7 proteins revealed differences in potencies and efficacies toward Gα-subunits of the G_i family. Although all four Gβ_5/R7 proteins exhibited similar potencies toward Gα_o, Gβ_5/RGS9 and Gβ_5/RGS11 were more potent GAPs of Gα_{i1}, Gα_{i2}, and Gα_{i3} than were Gβ_5/RGS6 and Gβ_5/RGS7. The maximal GAP activity exhibited by Gβ_5/RGS11 was 2- to 4-fold higher than that of Gβ_5/RGS7 and Gβ_5/RGS9, with Gβ_5/RGS6 exhibiting an intermediate maximal GAP activity. Moreover, the less efficacious Gβ_5/RGS7 and Gβ_5/RGS9 inhibited Gβ_5/RGS11-stimulated GTPase activity of Gα_o. Therefore, R7 family RGS proteins are G_i family-selective GAPs with potentially important differences in activities.

Heterotrimeric guanine nucleotide-binding proteins (G-proteins) act as molecular switches in multiple GPCR[1] signaling pathways via regulation of specific effector molecules such as phospholipase C and adenylyl cyclase. The biological activity of G-protein α-subunits is determined by the identity of the bound guanine nucleotide (GTP or GDP), which in turn is governed by the relative rates of guanine nucleotide exchange and hydrolysis of GTP by the intrinsic GTPase activity of Gα-subunits. These opposing reactions are stimulated by agonist-occupied GPCR and GTPase-activating proteins (GAPs).

Although some effector proteins exhibit GAP activity (1–3), the primary regulators of GTPase activity of Gα-subunits are a diverse family of regulator of G-protein signaling (RGS) proteins that act as GAPs for heterotrimeric G-protein α-subunits (4–7). This family is defined by a conserved RGS domain, which markedly increases the rate of GTP hydrolysis by Gα-subunits and terminates effector activation by both Gα- and G$\beta\gamma$-subunits. More than 30 RGS proteins have been identified and organized into subfamilies based on sequence similarity and domain structure. These families vary in size and complexity, from the R4 family whose structure is largely limited to the RGS domain to the R12 and RhoGEF families whose members are large multifunctional proteins containing several domains (for reviews see Refs. 8–10).

The R7 RGS family is a unique multidomain family, which consists of RGS proteins containing a novel G-γ-like (GGL) domain homologous to the Gγ-subunit of heterotrimeric G-proteins (11). This domain, found in the mammalian proteins RGS6, -7, -9, and -11 and the *Caenorhabditis elegans* proteins EAT16 and EGL10 (7, 12), confers specific binding to Gβ_5-subunits but not to Gβ_{1-4} (11, 13). Heterodimeric association with Gβ_5 appears necessary for stability and biological activity of R7 proteins (14–16). R7 proteins also contain a conserved N-terminal DEP (dishevelled, EGL10, pleckstrin homology) domain of unknown function (17).

RGS proteins may modify GPCR signaling through selective increases in GTP hydrolysis by a subset of G-proteins. For example, RZ family members specifically accelerate GTPase activity of Gα_z (18), and the recently characterized sorting nexin 13 (RGS-PX1) has been reported to increase the rate of GTP hydrolysis by Gα_s but not by Gα_i (19). The G-protein selectivity of the R7 family of RGS proteins has not been clearly defined. In single turnover GTPase assays, Gβ_5/RGS6 and Gβ_5/RGS7 increased GTPase activity of Gα_o (20) and Gβ_5/RGS11 increased GTPase activity of Gα_o and, to a much lesser degree, that of Gα_{i1}, Gα_{i2}, and Gα_{i3} (11). However, the R7 RGS proteins did not affect the single turnover GTPase rates of other Gα-subunits, including Gα_q (R183C), Gα_s, and Gα_{12}. In contrast, when expressed in cultured cell lines, RGS7 inhibited Gα_q-promoted Ca^{2+} responses downstream of M3 receptors (14) and 5-HT_{2c} receptors (21, 22) and inhibited Gα_i-regulated K^+ channel activity in a Gβ_5-dependent manner (23). Therefore, assays of soluble Gα-subunits suggest Gα_o selectivity, while intact cell signaling studies implicate R7 proteins in regulation of G_q as well as G_i pathways.

In face of the uncertainty of the actions of R7 RGS proteins, we purified the four members of the mammalian R7 family.

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[1] The abbreviations used are: GPCR, G-protein coupled receptor; RGS, regulator of G-protein signaling; GAP, GTPase activating protein; GGL, G-γ like; m.o.i., multiplicity of infection; Ni-NTA, nickel-nitrilotriacetic acid; $C_{12}E_{10}$, polyoxyethylene 10-lauryl ether.

The specificities of action of $G\beta_5$/R7 heterodimers were determined in steady state GTPase assays of G_i and G_q family $G\alpha$-subunits reconstituted with GPCR in phospholipid vesicles. $G\beta_5$/RGS6, -7, -9, and -11 increased the GTPase activity of $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ but not $G\alpha_q$ or $G\alpha_{11}$. Notable differences in maximal GAP activities were observed among R7 family proteins, and the maximal activity of the most efficacious RGS protein ($G\beta_5$/RGS11) was inhibited by $G\beta_5$/RGS7 and $G\beta_5$/RGS9.

EXPERIMENTAL PROCEDURES

Protein Purification—The generation of baculoviruses for $G\beta_5$, RGS6, RGS7, and RGS11 have been described previously (11, 20). A baculovirus for RGS9–1 in pFastbac Htb was prepared similarly. Cultures of Sf9 insect cells (1.4×10^6 cells/ml) were co-infected with virus encoding the RGS6, RGS7, or RGS9 gene (m.o.i. of 1) and a hexahistidine-tagged $G\beta_5$ (short isoform) (m.o.i. of 0.5). Forty-eight hours postinfection, 4 liters of cells were collected by centrifugation. Due to lower expression under these conditions, Sf9 cells were infected with the RGS11:$G\beta_5$ viruses at a 10:1 ratio (m.o.i. of 0.5 and 0.05), and cell lysates were obtained 65 h postinfection. Cells were resuspended and lysed in 600 ml of Buffer A (20 mM KPO_4, pH 8, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, protease inhibitors (500 nM aprotinin, 10 μM leupeptin, 200 μM phenylmethylsulfonyl fluoride, 1 nM pepstatin, 10 μM L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone)) with 20 strokes of a Dounce homogenizer at 4 °C. The lysate was cleared by low speed centrifugation, and the supernatant was centrifuged at $100{,}000 \times g$ for 30 min. The soluble protein fraction was loaded on a 3- to 5-ml column of Ni-NTA-agarose resin (Qiagen, Germany) over 3 h. The column was washed with 15 ml of Buffer B (20 mM KPO_4, pH 8, 400 mM NaCl, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, and protease inhibitors) followed by 5 ml of Buffer C (20 mM KPO_4, pH 8, 25 mM NaCl, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, and protease inhibitors). The $G\beta_5$/R7 dimers eluted with 50–150 mM imidazole in Buffer C. This eluate was further purified using 1 ml of HighTrap FPLC columns of either Q-Sepharose ($G\beta_5$/RGS6 and $G\beta_5$/RGS7) or S-Sepharose ($G\beta_5$/RGS9 and $G\beta_5$/RGS11) (Amersham Biosciences). The Ni-NTA eluate was diluted 5:1 in starting buffer for ion exchange chromatography (buffer for Q-Sepharose: 50 mM Tris, pH 8, 2 mM dithiothreitol, protease inhibitors; buffer for S-Sepharose: 50 mM Hepes, pH 8, 1 mM EDTA, 2 mM dithiothreitol, 10% glycerol, protease inhibitors), loaded onto the FPLC column, and eluted in the same buffer with a 0–400 mM NaCl gradient over 30-column volumes. Each dimer eluted at ~200 mM NaCl. Fractions were collected and concentrated using a Centricon centrifugal filter device (Millipore, Bedford, CT). The concentration of purified $G\beta_5$/R7 dimers was determined by Coomassie staining purified product and a standard curve of protein standards resolved by SDS-PAGE. Yield was ~1 mg of $G\beta_5$/R7 dimer per 4 liters except for $G\beta_5$/RGS11, whose purification yielded ~250 μg per 4 liters. $G\alpha$- and $G\beta\gamma$-subunits (24) and muscarinic receptors (25) were purified after expression from baculoviruses in Sf9 insect cells as described.

Vesicle Reconstitution and Characterization—Detergent/phospholipid mixed micelles were prepared by drying 110 μg of phosphatidylethanolamine, 70 μg of phosphatidylserine, and 8 nmol of cholesteryl hemisuccinate under argon and resuspending in detergent buffer (0.4% deoxycholate, 20 mM Hepes, 1 mM EDTA, 100 mM NaCl). Fifty μl of this preparation was combined with 15 pmol of muscarinic receptor, 50 pmol of $G\alpha$, and 150 pmol of $G\beta_1\gamma_2$ in Buffer D (20 mM Hepes, 100 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$) and immediately loaded onto a G-50-Sepharose column equilibrated with Buffer D. The eluate was collected in 200-μl fractions. Fractions were assayed for the presence of muscarinic receptor by incubation of 5 μl per fraction with 20 nM [^{3}H]quinuclidinyl benzilate (~200,000 cpm) in a volume of 100 μl for 90 min at 30 °C and filtration over GF/F filters (Whatman). Peak fractions also were assayed for $G\alpha$ incorporation by incubation of 5 μl of the vesicle preparation with 1 μM ^{35}S-labeled GTPγS (500,000 cpm) in the presence or absence of 0.1% $C_{12}E_{10}$ detergent (total volume = 100 μl) at 30 °C for 90 min. Samples labeled in the absence of $C_{12}E_{10}$ were filtered over GF/F filters, which collect vesicles but not free protein, to quantitate incorporation of $G\alpha$-subunits into vesicles, and $C_{12}E_{10}$-containing samples were filtered over nitrocellulose to quantitate total $G\alpha$.

Steady State GTPase Assays—One microliter (for $G\alpha_{i/o}$-containing vesicles) or 5 μl (for $G\alpha_{q/11}$-containing vesicles) of the vesicle preparations was equilibrated on ice in Buffer D in the presence or absence of 100 μM carbachol and various concentrations of RGS protein. [γ-^{32}P]GTP (2 μM; ~400,000 cpm) was added to each 25 μl of reaction.

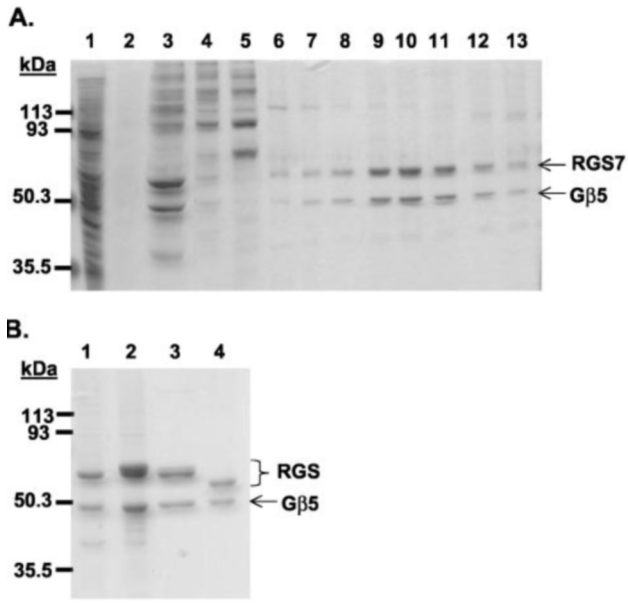

FIG. 1. **$G\beta_5$/R7 purification.** *A*, the purification of $G\beta_5$/RGS7 is shown on a Coomassie Blue-stained SDS-PAGE. The soluble fraction of Sf9 cells infected with RGS7 and hexahistidine-tagged $G\beta_5$ was loaded onto a Ni-NTA column. The column was consecutively washed with 400 mM NaCl (*lane 1*), 25 mM NaCl (*lane 2*), 50 mM imidazole (*lane 3*), 150 mM imidazole (*lane 4*), and 300 mM imidazole (*lane 5*). The 50 mM imidazole elution was further purified on a HighTrap Q anionic exchange column and eluted with a gradient of 0–400 mM NaCl. *Lanes 6–13* show fractions eluting at ~175–225 mM NaCl. Fractions shown in *lanes 9–11* were pooled and concentrated. *B*, the final purified products of all four $G\beta_5$/R7 dimers are shown: $G\beta_5$/RGS6 (*lane 1*), $G\beta_5$/RGS7 (*lane 2*), $G\beta_5$/RGS9 (*lane 3*), and $G\beta_5$/RGS11 (*lane 4*).

The reaction was transferred to a 30 °C water bath for 15 min (for $G\alpha_{i/o}$-containing vesicles) or 30 min (for $G\alpha_{q/11}$-containing vesicles) and quenched on ice with 975 ml of cold 5% activated charcoal in 20 mM NaH_2PO_4. The charcoal was pelleted by centrifugation, and a portion of the supernatant was added to scintillant for $^{32}P_i$ quantitation.

RESULTS

The specificity of RGS proteins for G-protein substrates determines in part their physiological effects on signaling. Previous *in vitro* studies with $G\alpha$-subunits in solution have illustrated specificity of R7-RGS proteins for $G\alpha_o$, whereas *in vivo* observations have suggested broader activities. To more specifically address the selectivity of individual $G\beta_5$/R7 heterodimers for $G\alpha$-subunits, we purified $G\beta_5$/RGS6, $G\beta_5$/RGS7, $G\beta_5$/RGS9, and $G\beta_5$/RGS11 to near homogeneity and directly measured their selectivity in steady state GTPase assays with proteoliposomes reconstituted with M1 or M2 muscarinic receptors and various heterotrimeric G proteins of the G_q and G_i families, respectively.

$G\beta_5$/R7 Protein Purification—Full-length RGS6, -7, -9, and -11 were co-expressed with hexahistidine-tagged $G\beta_5$ in Sf9 insect cells using the baculovirus expression system. Dimers were purified from the soluble fraction using Ni-NTA-agarose and ion exchange chromatography. Twenty-five to seventy-five percent of the total cellular immunoreactive R7 protein was recovered in the soluble fraction, 25–75% of the soluble protein was recovered following Ni-NTA chromatography, and nearly 100% of the Ni-NTA eluate was recovered following the final ion exchange purification. The results of a typical purification ($G\beta_5$/RGS7) are shown in Fig. 1*A*. Purified $G\beta_5$/RGS6, $G\beta_5$/RGS9, and $G\beta_5$/RGS11 dimers are illustrated in Fig. 1*B*.

Vesicle Reconstitution—G-protein α-subunits ($G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_q$, $G\alpha_{11}$) were reconstituted in phospholipid vesicles with $G\beta_1\gamma_2$ and either M1 (G_q family G-proteins) or M2 (G_i family G-proteins) muscarinic receptors under conditions sim-

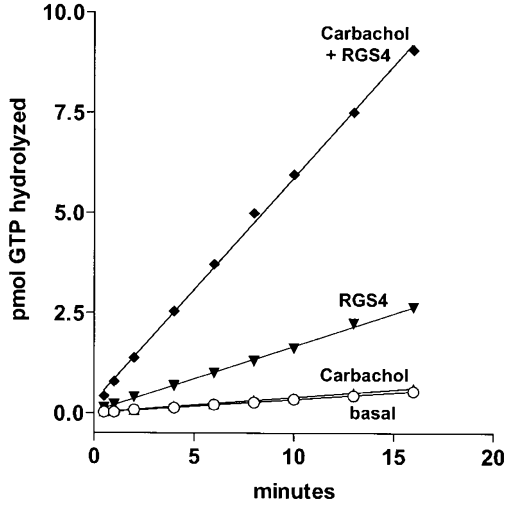

FIG. 2. **Steady state GTPase activity of reconstituted Gα-subunits.** The time course of GTP hydrolysis by $G\alpha_o$ reconstituted in phospholipid vesicles with the M2 muscarinic receptor and $G\beta_1\gamma_2$ under basal conditions (○), in the presence of 100 μM carbachol (▲), 200 nM RGS4 (▼), or carbachol plus RGS4 (♦) is shown.

ilar to those described by Ross and coworkers (26, 27). Recovery of Gα-subunits and M1 or M2 muscarinic receptors in the various vesicle preparations was quantitated as described under "Experimental Procedures." Essentially 100% of added $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, or $G\alpha_{i3}$ was incorporated into vesicles, and receptor recovery in the proteoliposomes was ~50%. We also prepared and resolved $G\alpha_o$-containing vesicles using the higher exclusion limit Sephacryl S-300 gel filtration resin, which separates vesicles from free $G\alpha_o$, and observed nearly all of the $G\alpha_o$ immunoreactivity co-migrating with vesicles in the void volume (data not shown). The four varieties of M2·$G\alpha_{i/o}$ vesicles contained similar Gα protein levels (~100 fmol/μl) and receptor: Gα ratios (1:6) (data not shown). Quantitation of $G\alpha_q$ and $G\alpha_{11}$ is difficult due to the low rates of guanine nucleotide turnover by these Gα-subunits. Therefore, calculations of GTPase activity reported below were made assuming that incorporation of $G\alpha_{q/11}$ into vesicles was equal to that of G_i family Gα-subunits.

Steady State GTPase Assays—The GAP activity of $G\beta_5$/R7 proteins toward Gα-subunits of the G_i and G_q families was assessed in steady state GTPase assays, which measure multiple rounds of GTP hydrolysis and, as such, reflect both guanine nucleotide exchange and GTPase activity. RGS4, an effective GAP against G_q and G_i family Gα-subunits (28), was used as a reference RGS protein in all experiments. Only minor increases were observed in the rate of GTP hydrolysis in the presence of either agonist (100 μM carbachol) or GAP (200 nM RGS4) alone in proteoliposomes formed by reconstitution of M2 muscarinic receptor, $G\alpha_o$, and $G\beta_1\gamma_2$ (Fig. 2). In contrast, the combined presence of carbachol and RGS4 resulted in a markedly synergistic increase in GTPase activity, and the rate of hydrolysis of GTP was linear for at least 15 min.

In the presence of a maximally effective concentration of RGS protein, guanine nucleotide exchange is rate-limiting, and therefore stimulation of GTPase activity by carbachol was observed with a concentration dependence of agonist that approximated its occupancy curve for binding to the M2 muscarinic receptor (data not shown). Similarly, in the presence of a maximally effective concentration of carbachol, guanine nucleotide exchange was no longer rate-limiting, and marked concentration-dependent stimulation of GTPase was observed with RGS4 (data not shown) and $G\beta_5$/R7 RGS proteins (see below).

$G\beta_5$/R7 Proteins Stimulate Steady State GTPase Activity of G_i Family Gα-subunits—To compare the capacity of $G\beta_5$/R7 proteins to accelerate GTPase rates of G_i family Gα-subunits, steady state GTPase activities were determined in the presence and absence of 100 μM carbachol and in the presence and absence of 1 μM RGS protein (either RGS4 or each $G\beta_5$/R7 dimer). RGS4 markedly increased GTPase activity for $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ in the presence of 100 μM carbachol (Fig. 3). Each of the $G\beta_5$/R7 dimers also stimulated to varying degrees GTP hydrolysis by $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ in the presence of agonist (Fig. 3 and Table I). The rate observed with $G\beta_5$/RGS11 was as high or higher than the rate with RGS4 with all four G_i family Gα-subunits, while the GTPase rates in the presence of 1 μM $G\beta_5$/RGS6, $G\beta_5$/RGS7, and $G\beta_5$/RGS9 were significantly lower. Vesicles containing $G\alpha_o$ achieved the highest maximal GTPase rates irrespective of the RGS protein. However, the basal rate of GTP hydrolysis by $G\alpha_o$ in the absence of RGS protein was also higher than that observed in $G\alpha_I$-containing vesicles. Thus, the fold increase in activity (GTPase rate in the presence of RGS and agonist divided by the GTPase rate with agonist alone) of $G\alpha_{i3}$ was as high or higher than that of $G\alpha_o$ in response to $G\beta_5$/RGS6, -7, -9, and -11 stimulation. Further, the effects of R7 proteins on GTPase activity of $G\alpha_o$-subunits reconstituted with purified P2Y12 receptors was also determined (in the presence of the agonist 2-methylthio ADP). Similar to the results observed with M2 receptor-coupled G-proteins, each of the $G\beta_5$/RGS11 dimers stimulated steady state GTPase activity of $G\alpha_o$, and $G\beta_5$/RGS11 stimulated much higher GTPase rates than the other R7 proteins (data not shown).

$G\beta_5$/R7 Proteins Do Not Stimulate Steady State GTPase Activity of G_q Family Gα-subunits—Regulation of GTPase activities of $G\alpha_q$ and $G\alpha_{11}$ was examined in vesicles reconstituted with M1 muscarinic receptor and heterotrimeric G-proteins (Fig. 4). RGS4 increased steady state GTPase activity of $G\alpha_q$ and $G\alpha_{11}$ in the presence of agonist by nearly 5-fold to final GTPase rates of ~200 fmol of GTP/min/pmol of Gα. Consistent with previous observations of guanine nucleotide exchange/GTPase kinetics of G_q (29), these rates are lower than those observed for G_i family α-subunits. In contrast to the activity of RGS4, none of the $G\beta_5$/R7 dimers significantly increased steady state GTPase activity of $G\alpha_q$ or $G\alpha_{11}$ in the presence of carbachol (Fig. 4*A*). Likewise, $G\beta_5$/R7 dimers did not stimulate GTPase activity of $G\alpha_q$- or $G\alpha_{11}$-subunits reconstituted with purified P2Y1 receptors (in the presence of the agonist 2-methylthio ADP) (data not shown). Further, 1 μM $G\beta_5$/R7 dimers had no effect on the GTPase activity of M1 receptor-coupled $G\alpha_q$ and $G\alpha_{11}$ stimulated by RGS4 and carbachol (Fig. 4*B*). The partial inhibition observed with $G\beta_5$/RGS11 was nonspecific as demonstrated by equivalent inhibitory activity observed with boiled $G\beta_5$/RGS11. Therefore, under the conditions of these assays, R7 proteins neither stimulate GTPase activity of $G\alpha_q$ or $G\alpha_{11}$ nor affect GTPase activity stimulated by agonist and RGS4.

$G\beta_5$/R7 Proteins Exhibit Differences in Maximal Activity and Potency toward G_i Family Gα-subunits—To more fully elucidate any selectivity of $G\beta_5$/R7 proteins as GAPs for G_i family subunits, full concentration effect curves of each $G\beta_5$/R7 protein were generated in the presence of a maximally effective concentration of carbachol. Consistent with the data in Fig. 3, maximally effective concentrations of $G\beta_5$/RGS11 produced larger effects than $G\beta_5$/RGS7, $G\beta_5$/RGS6, and $G\beta_5$/RGS9 on the GTPase activity of each Gα-subunit (Fig. 5 and data not shown), and the highest maximal rate observed with each RGS protein was observed with $G\alpha_o$ as substrate (not shown). Each of the $G\beta_5$/RGS dimers produced a near maximal effect at a concentration of 1 μM, and therefore each activation curve was normalized to 100% of maximal activity for comparison of EC_{50} values (Fig. 6). All four $G\beta_5$/R7 dimers

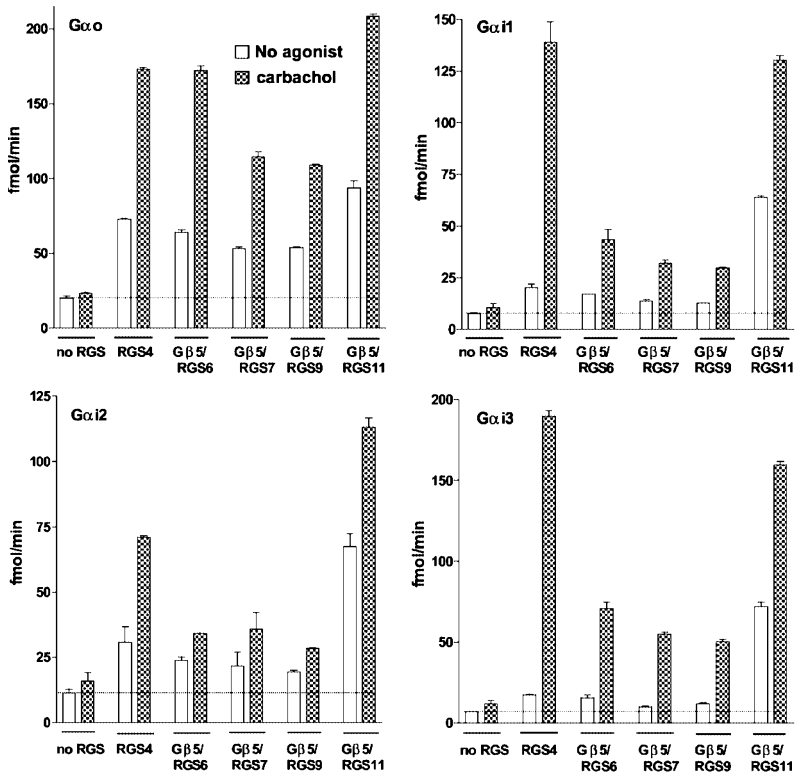

FIG. 3. **$G\beta_5$/R7 proteins stimulate GTPase activity of G_i family Gα-subunits.** The effects of 100 μM carbachol and 1 μM of the indicated RGS protein on the steady state GTPase activity of $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ reconstituted in phospholipid vesicles with the M2 muscarinic receptor and $G\beta_1\gamma_2$ were determined as described under "Experimental Procedures." *Open bars*, no agonist. *Shaded bars*, 100 μM carbachol. Results shown are representative of at least three separate experiments using three independent vesicle preparations.

TABLE I
GTPase rates of G_i family Gα-subunits in the presence of RGS4 and $G\beta_5$/R7 proteins
GTPase rates (min^{-1}) attained in the presence of 100 μM carbachol and 1 μM RGS protein are shown with mean ± S.D.

	$G\beta_5$/RGS6	$G\beta_5$/RGS7	$G\beta_5$/RGS9	$G\beta_5$/RGS11	RGS4
$G\alpha_o$	8.97 ± 0.15	6.10 ± 0.16	5.56 ± 0.02	10.61 ± 0.08	8.59 ± 0.05
$G\alpha_{i1}$	1.86 ± 0.17	1.44 ± 0.06	1.16 ± 0.01	4.82 ± 0.08	4.92 ± 0.35
$G\alpha_{i2}$	1.75 ± 0.01	1.79 ± 0.26	1.28 ± 0.01	4.81 ± 0.14	2.88 ± 0.02
$G\alpha_{i3}$	3.39 ± 0.16	2.69 ± 0.06	2.26 ± 0.06	7.02 ± 0.09	8.06 ± 0.14

exhibited similar potency for $G\alpha_o$ (EC_{50} = 16–47 nM), while $G\beta_5$/RGS9 and $G\beta_5$/RGS11 were more potent (EC_{50} = 25–80 nM) than $G\beta_5$/RGS6 and $G\beta_5$/RGS7 (EC_{50} = 150–350 nM) for $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ (Table II).

$G\beta_5$/RGS7 and $G\beta_5$/RGS9 Inhibit $G\beta_5$/RGS11-stimulated $G\alpha_o$ GTPase Activity—Marked differences in the maximal GTPase rate of Gα-subunits were observed across the $G\beta_5$/R7 protein family. For example, $G\beta_5$/RGS11-stimulated GTPase activity of $G\alpha_o$ was twice that achieved in the presence of $G\beta_5$/RGS9 or $G\beta_5$/RGS7. These results suggest that $G\beta_5$/RGS7 and $G\beta_5$/RGS9 interaction with G-proteins results in a less active Gα conformation with respect to GTPase activity than that promoted by $G\beta_5$/RGS11 interaction. To test this hypothesis, steady state GTPase activity of M2·$G\alpha_o$·$\beta_1\gamma_2$ vesicles was measured in the presence of 100 nM $G\beta_5$/RGS11 or 1 μM $G\beta_5$/RGS7 alone or with 100 nM $G\beta_5$/RGS11 plus 1 μM $G\beta_5$/RGS7. As illustrated in Fig. 7*A*, GTPase activity in the presence of $G\beta_5$/RGS11 was nearly twice that observed with a 10-fold higher concentration of $G\beta_5$/RGS7. However, the combined presence of 1 μM $G\beta_5$/RGS7 and 100 nM $G\beta_5$/RGS11 resulted in activity only slightly greater than that of $G\beta_5$/RGS7 alone, suggesting that $G\beta_5$/RGS7 competitively antagonizes the action of the more efficacious $G\beta_5$/RGS11. Heat-inactivated $G\beta_5$/RGS7 neither stimulated GTPase activity nor inhibited the stimulatory effect of $G\beta_5$/RGS11 on GTPase activity (Fig. 7*A*), demonstrating that both effects of $G\beta_5$/RGS7 are dependent on protein activity. To further characterize the interaction of RGS proteins and $G\alpha_o$, the concentration dependence of the inhibitory effect of $G\beta_5$/RGS7 and $G\beta_5$/RGS9 was determined by varying the concentrations of these proteins in the presence of carbachol and 100 nM $G\beta_5$/RGS11. Both $G\beta_5$/RGS7 and $G\beta_5$/RGS9 significantly inhibited $G\beta_5$/RGS11-stimulated GTPase activity of M2·$G\alpha_o$ vesicles with IC_{50} values of 100–200 nM (Fig. 7*B*). These data indicate that while $G\beta_5$/RGS7 and $G\beta_5$/RGS9 are less efficacious activators of GTPase activity, they interact with a similar region of $G\alpha_o$ as does $G\beta_5$/RGS11.

DISCUSSION

The results of this study demonstrate $G\alpha_{i/o}$ specificity of $G\beta_5$/R7 proteins and found no evidence of regulation of $G\alpha_q$ GTPase activity by these proteins. Further, we demonstrated that differences exist in the potencies and relative efficacies of the $G\beta_5$/R7 proteins for their $G\alpha_{i/o}$ substrates. Finally, we illustrated that $G\beta_5$/RGS7 and $G\beta_5$/RGS9, which are less effective promoters of maximal GTPase activity than is $G\beta_5$/RGS11, inhibit $G\beta_5$/RGS11-stimulated GTPase activity of $G\alpha_o$.

The drug selectivity of G-protein-coupled receptors has been widely exploited therapeutically to manipulate specific cellular processes (30–34). Similarly, selectivities of G-proteins for effector activation and potential selectivities of RGS proteins for deactivation of G-proteins may provide equally rich targets for pharmacological modulation of G-protein-regulated signaling (35, 36). Whereas the role of receptor activity and selectivity in regulating various classes of G-proteins has been studied extensively in the past decades, the roles of proteins exhibiting Gα

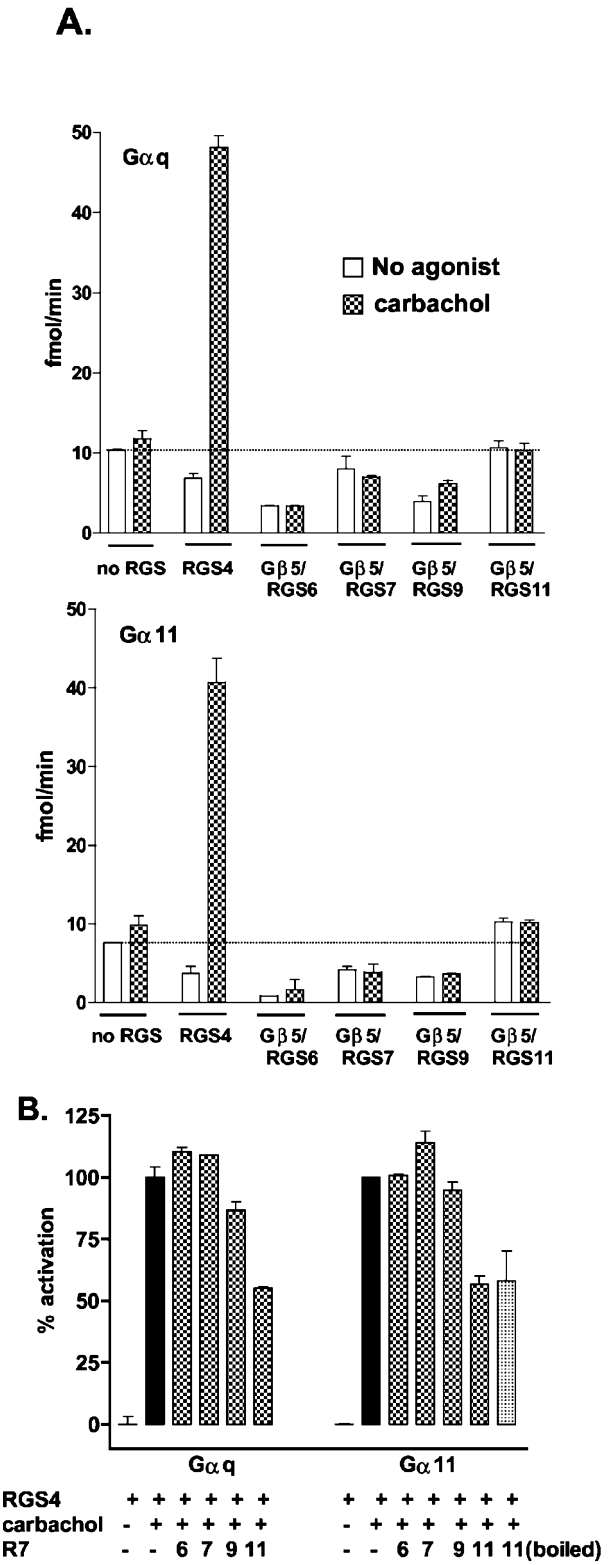

FIG. 4. **$G\beta_5$/R7 proteins do not stimulate GTPase activity of G_q family Gα-subunits.** *A*, the effects of 100 μM carbachol and 1 μM of the indicated RGS protein on the steady state GTPase activity of $G\alpha_q$ and $G\alpha_{11}$ reconstituted in phospholipid vesicles with the M1 muscarinic receptor and $G\beta_1\gamma_2$ were determined as described under "Experimental Procedures." *Open bars*, no agonist. *Shaded bars*, 100 μM carbachol. Results shown are representative of three separate experiments using three independent vesicle preparations. *B*, the effects of 1 μM $G\beta_5$/RGS proteins on agonist-stimulated steady state GTPase activity of M1 receptor-coupled $G\alpha_q$ and $G\alpha_{11}$ were determined in the presence of 100 nM RGS4. The effects of heat inactivated $G\beta_5$/RGS11 were also determined. The results are presented as a percentage of the agonist-stimulated activity.

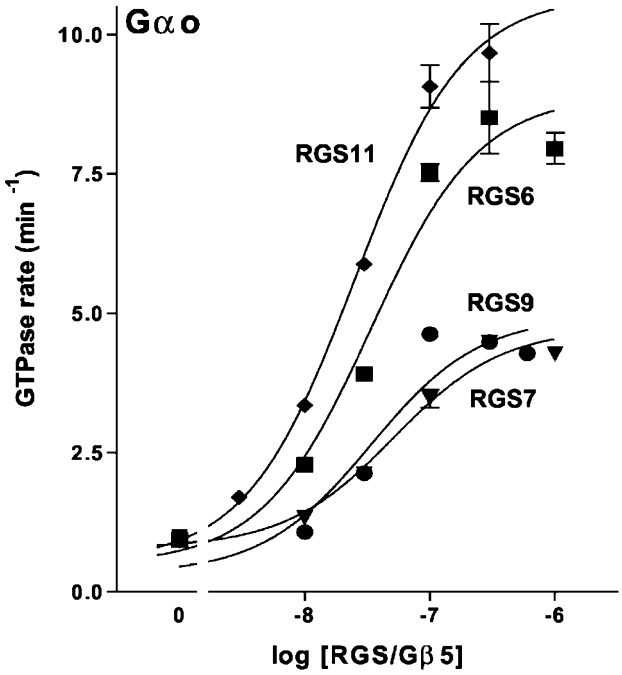

FIG. 5. **$G\beta_5$/R7 proteins stimulate different maximal GTPase activity.** Steady state GTPase rates were determined for M2·$G\alpha_o$·$G\beta_1\gamma_2$ vesicles in the presence of 100 μM carbachol and various concentrations of R7 proteins to generate concentration effect curves. Values observed at each RGS concentration in the absence of vesicles have been subtracted from the data. Data are plotted as GTPase rates (min^{-1}). Symbols are $G\beta_5$/RGS11 (♦), $G\beta_5$/RGS9 (●), $G\beta_5$/RGS7 (▼), and $G\beta_5$/RGS6 (■). Results are representative of at least three independent determinations using three separate vesicle preparations.

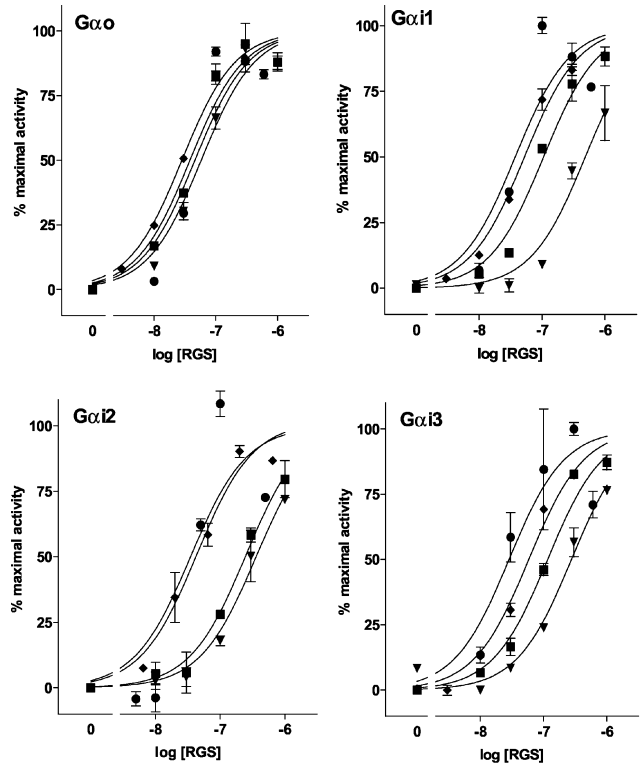

FIG. 6. **$G\beta_5$/R7 proteins exhibit differences in potency toward G_i family Gα-subunits.** Steady state GTPase rates were determined for M2·$G\alpha_{i/o}$·$G\beta_1\gamma_2$ vesicles in the presence of 100 μM carbachol and various concentrations of R7 proteins to generate concentration effect curves. Data are normalized to 100% activity for comparison of EC_{50} values. Symbols are $G\beta_5$/RGS11 (♦), $G\beta_5$/RGS9 (●), $G\beta_5$/RGS7 (▼), and $G\beta_5$/RGS6 (■). Results are representative of at least three independent determinations using three separate vesicle preparations.

TABLE II
EC_{50} values $G\beta_5$/R7 proteins for stimulation of GTPase activity of G_i family Gα-subunits

The mean ± S.D. of EC_{50} values (nM) are shown from three independent determinations of the concentration effect relationship of the R7 proteins as GAPs of G_i family Gα-subunits using three different vesicle preparations.

	Gβ_5/RGS6	Gβ_5/RGS7	Gβ_5/RGS9	Gβ_5/RGS11
Gα_o	34 ± 8.5	40 ± 18	47 ± 22	16 ± 5.1
Gα_{i1}	160 ± 84	240 ± 110	56 ± 23	38 ± 5.0
Gα_{i2}	160 ± 49	305 ± 92	54 ± 21	80 ± 50
Gα_{i3}	180 ± 57	351 ± 45	27 ± 11	43 ± 7.0

GAP activity and selectivities within this class of proteins remain undefined.

Members of the Gβ_5/R7 family previously were reported to be specific for Gα_o in single turnover assays of soluble Gα-subunits (11, 20). These assays may not accurately represent physiological interactions between G-proteins and RGS proteins for several reasons including the lack of a lipid bilayer with which G-proteins and RGS proteins may associate, the lack of a GPCR, which may form a complex with RGS proteins (37–39) and facilitate interaction with Gα-subunits, and the necessity of using GTPase-deficient mutants of G_q family G-proteins given their low rates of exchange. These limitations may explain discrepancies between selectivities for G-protein α-subunits observed in single turnover *versus* cell-based assays. Indeed, RGS2 behaves as a Gα_q-specific GAP in single turnover assays, but exhibits GAP activity toward Gα_i as well as Gα_q in steady state GTPase assays of proteoliposomes reconstituted with GPCR and heterotrimeric G-proteins (28).

In this study, we examined the GAP activity of Gβ_5/R7 proteins using steady state GTPase assays of receptor-coupled G-proteins reconstituted in phospholipid bilayers. Because these assays measure multiple rounds of hydrolysis in the presence of receptor-stimulated guanine nucleotide exchange, wild type Gα_q or Gα_{11} may be used, and the contributions made by agonist, receptor, G$\beta\gamma$-subunits, and the phospholipid bilayer to GTPase activity are likely more representative of a cellular environment. Our results differ from those from single turnover assays (11, 20) with respect to the selectivity within the G$\alpha_{i/o}$ family since all four Gα-subunits of this family are substrates for Gβ_5/R7 proteins.

Our results also differ from published reports that indirectly suggest that Gβ_5/R7 proteins stimulate GTPase activity of Gα_q or Gα_{11} (14, 21, 22) in that we did not observe stimulation of Gα_q or Gα_{11} GTPase activity in response to R7 proteins. A trivial explanation for our observation is that R7 proteins inhibit agonist promoted exchange and thereby mask GAP activity in steady state GTPase assays. However, the lack of an effect of R7 proteins on the steady state GTPase activity achieved in the presence of carbachol and RGS4 demonstrates that Gβ_5/R7 heterodimers do not significantly affect agonist-promoted exchange of guanine nucleotides under the conditions of these assays (Fig. 4*B*). We also observed minimal to no effects of Gβ_5/R7 proteins on agonist-stimulated guanine nucleotide exchange measured directly in GTPγS binding assays (not shown). Therefore, the inability of Gβ_5/R7 proteins to stimulate steady state GTPase activity of Gα_q and Gα_{11} indicates that they do not function as G_q family GAPs under the conditions of our assay. In the absence of GAP activity, the reported effects of RGS7 on G_q family G-protein signaling could reflect direct inhibition of phospholipase enzymes, as observed by Posner *et al.* (20). Although we have observed some inhibition of receptor-stimulated inositol phosphate accumulation in cells cotransfected with R7 RGS proteins and Gβ_5, this inhibition is less pronounced and requires expression to much higher levels than does the marked inhibition of phospholipase C response observed in cells overexpressing RGS2 or RGS4 (data not shown). Thus, the reported effects of R7 proteins on cellular G_q pathways may reflect either loss of GAP selectivity due to protein overexpression or a more complex interaction of Gβ_5/R7 dimers with the G-protein signaling cycle.

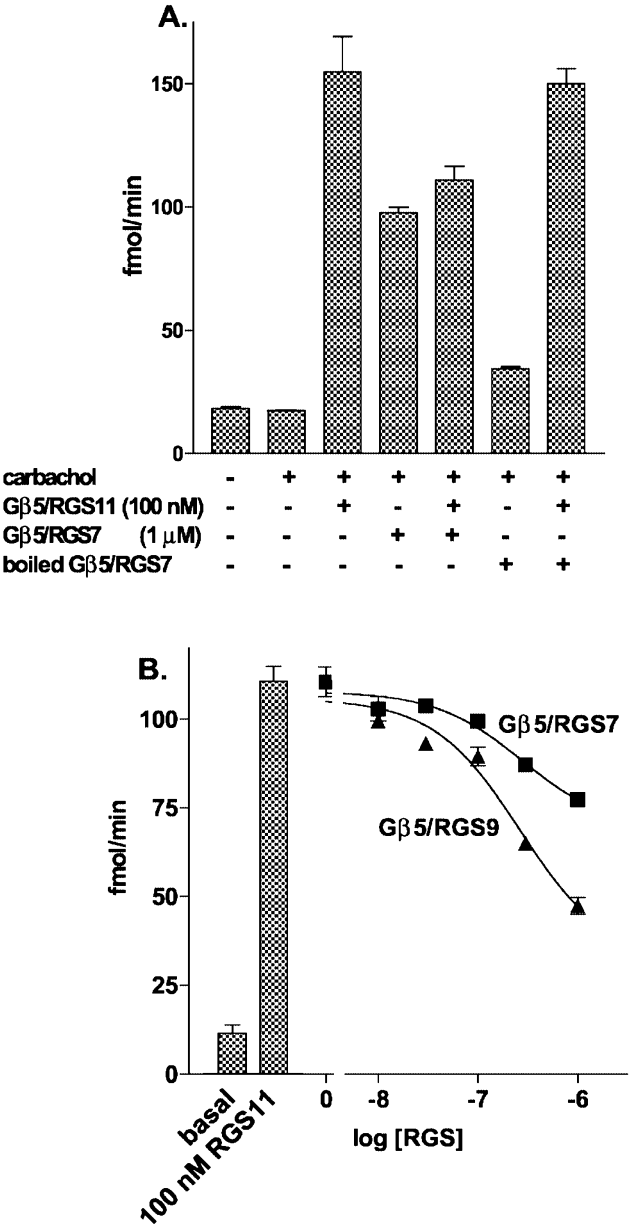

FIG. 7. **Gβ_5/RGS7 and Gβ_5/RGS9 inhibit Gβ_5/RGS11-stimulated GTPase activity of Gα_o.** *A*, steady state GTPase activity of M2·Gα_o·G$\beta_1\gamma_2$ vesicles was determined in the presence of 100 μM carbachol alone or in the presence of Gβ_5/R7 proteins. The effects of 100 nM Gβ_5/RGS11, 1 μM Gβ_5/RGS7, and heat-inactivated Gβ_5/RGS7 (1 μM) were assayed separately and in combination. Results are representative of two independent experiments with two RGS11 preparations. *B*, Gβ_5/R11-stimulated steady state GTPase activity of Gα_o was determined at various concentrations of added Gβ_5/RGS7 and Gβ_5/RGS9.

Gβ_5/R7 proteins exhibited differences in the potency and efficacy of their GAP activity against the G$\alpha_{i/o}$ family. Gβ_5/RGS6 and Gβ_5/RGS7 each exhibited 10-fold lower potency for Gα_i α-subunits than for Gα_o. In contrast, Gβ_5/RGS9 and Gβ_5/RGS11 exhibited similar potency for all four G_i family Gα-subunits. This pattern mirrors the grouping of R7 proteins by sequence similarity; that is, RGS6 and RGS7 have higher sequence identity to each other than to RGS9 and RGS11 and *vice*

versa (13). The R7 proteins group differently with respect to their apparent efficacies for stimulation of GTPase activity. Gβ5/RGS11 exhibited the highest maximal effect, while the maximal effects of Gβ5/RGS7 and Gβ5/RGS9 were much less and Gβ_5/RGS6 exhibited an intermediate maximal effect. These differences in activity inversely correlate with the expression of R7 transcripts in rat brain where RGS11 is expressed at much lower levels than RGS7 and -9, and again RGS6 is intermediate (42). We speculate that expression of the robustly active RGS11 may be regulated differently than the less active proteins.

Gα_{i1}, Gα_{i2}, and Gα_{i3} share high sequence homology, and these signaling proteins are essentially interchangeable in many signaling processes. However, although these proteins often are expressed in the same cell, they may not be entirely functionally redundant. For example, selectivity of coupling of certain G protein-coupled receptors among these three Gα_i proteins has been illustrated (43–46), and several reports suggest selective coupling of receptors to ion channels through specific Gα_i-subunits (for review see Ref. 47). Our observation of selectivity of action of β_5/RGS proteins among Gα_{i1}, Gα_{i2}, Gα_{i3}, and G$\alpha_{i/o}$ likely has physiologically important ramifications. For example, this family of RGS proteins is highly expressed in the central nervous system as are G_i family Gα-subunits. Given the broadly different patterns of expression of RGS6, RGS7, RGS9, and RGS11 in the brain (42), we hypothesize that otherwise functionally redundant Gα_i-subunits may exhibit cell-specific differences in signaling activities as a consequence of the presence of different RGS proteins.

The differences observed in the maximal GAP activity of R7 family RGS proteins may reflect differences in their interactions with Gα. Unlike GAP proteins for the Ras superfamily GTPases, which contribute a catalytic arginine required for GTPase activity, RGS proteins are considered to enhance GTPase activity solely by stabilizing Gα switch regions (48). Presumably, R7 RGS proteins bind Gα-subunits through interactions similar to those observed in the RGS4/Gα_i (48) and the RGS9/G$\alpha_{t/i1}$ crystal structures (49), in which the base of a 4-helix bundle (α_4, α_5, α_6, α_7) of the RGS domain directly contacts portions of the three switch regions of Gα. Martemyanov and Arshavsky recently reported that mutation of RGS9 residues in the base of Gα-interacting helices α_5 and α_6 (L353E/R360P) resulted in markedly higher maximal GAP activity toward Gα transducin than observed in wild type RGS9, consistent with a role for this region in determining maximal GAP activity (50). It will be equally important to determine if regions outside the RGS box play important roles in defining selectivities of β_5-RGS proteins among Gα-subunits as suggested in studies of Gα selectivity of *C. elegans* R7 family members (40, 41).

In summary, we have demonstrated that R7 family RGS proteins selectively stimulate GTPase activity of G_i family Gα-subunits. We have shown differences in potency and efficacy of Gβ_5/R7 dimers as GAPs among the G_i family Gα-subunits. Further, lower efficacy GAPs were shown to inhibit GTPase activity achieved in the presence of a more efficacious GAP, indicating that RGS proteins apparently interacting with the same activating surface of a Gα-subunit promote different maximal rates of catalysis by the Gα GTPase.

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