Regulators of G protein signaling (RGS) proteins accelerate GTP hydrolysis by Gα subunits, thereby attenuating signaling. RGS4 is a GTPase-activating protein for Gq and Gq class α subunits. In the present study, we used knockouts of Gq class genes in mice to evaluate the potency and selectivity of RGS4 in modulating Ca2+ signaling transduced by different Gq-coupled receptors. RGS4 inhibited phospholipase C activity and Ca2+ signaling in a receptor-selective manner in both permeabilized cells and cells dialyzed with RGS4 through a patch pipette. Receptor-dependent inhibition of Ca2+ signaling by RGS4 was observed in acini prepared from the rat and mouse pancreas. The response of mouse pancreatic acini to carbachol was about 4- and 33-fold more sensitive to RGS4 than that of bombesin and cholecystokinin (CCK), respectively. RGS1 and RGS16 were also potent inhibitors of Gq-dependent Ca2+ signaling and acted in a receptor-selective manner. RGS1 showed approximately 1000-fold higher potency in inhibiting carbachol than CCK-dependent signaling. RGS16 was as effective as RGS1 in inhibiting carbachol-dependent signaling but only partially inhibited the response to CCK. By contrast, RGS2 inhibited the response to carbachol and CCK with equal potency. The same pattern of receptor-selective inhibition by RGS4 was observed in acinar cells from wild type and several single and double Gq class knockout mice. Thus, these receptors appear to couple Gq class α subunit isoforms equally. Difference in receptor selectivity of RGS proteins action indicates that regulatory specificity is conferred by interaction of RGS proteins with receptor complexes.

Heterotrimeric G proteins of the Gq class transduce Ca2+ signaling by coupling heptahelical transmembrane receptors to the β isomers of phospholipase C (PLC)1 (1). Many cells express multiple receptors that each activates the Gq signaling pathway (2). For example, pancreatic acinar cells respond to acetylcholine, bombesin and cholecystokinin (CCK) by intense activation of PLC to generate IP3 and mobilize Ca2+ from internal stores. In a recent study we showed that, although these three agonists activate the same Gq-regulated signaling pathway to mobilize the same Ca2+ pool, each agonist evokes a distinct pattern of Ca2+ wave propagation (3). Ca2+ signaling in pancreatic acini triggers the exocytotic release of digestive enzymes from granules adjacent to the secretory membrane (4).

An intriguing question in cell signaling is how different agonists can stimulate the same Gq-coupled pathway to generate distinct spatial and/or temporal patterns of Ca2+ response within the same cell. Although signaling in pancreatic acinar cells is dependent on agonist binding to Gq-coupled receptors, several experiments suggested that Ca2+ release was also regulated by a novel mechanism that controlled G protein signaling. First, Ca2+ release evoked by carbachol, bombesin, or CCK was equally inhibited by titration with proteins that sequestered Gαi3 and with antisera that specifically recognized Gq class α subunits (3, 5). Second, these receptors promiscuously coupled to members of the Gq class α subunits (6). Third, maximal stimulation with each of these agonists generated roughly equal levels of IP3. These results suggest that all three agonists stimulate their receptors to activate the same amount of Gαq11. Nevertheless, we found that GTPγS differentially activated, and GDPβS differentially inhibited, signaling by the various agonists acting in these cells (3, 7). These effects of guanine nucleotides suggested that G protein activity was differentially regulated by an unknown intracellular protein(s) that acted in a receptor-dependent manner.

G protein activity is regulated both by receptor-catalyzed GTP binding to the α subunit and by the rate of GTP hydrolysis. Regulators of G protein signaling (RGS) proteins are a recently identified family of intracellular GTPase-activating proteins (GAPs) (8, 9) that accelerate GTP hydrolysis by Gα subunits (10–15), thus limiting the duration of G protein activation. RGS proteins may regulate signaling by uncoupling the cycle of GTP binding and hydrolysis from effector protein activation by the Gα subunit, even in the presence of persistent agonist stimulation.

RGS proteins were first identified by genetic techniques to be inhibitors of G protein signaling (8, 16, 17). Recent work has identified over 20 RGS proteins expressed in mammals (12, 16, 18–22). In vitro studies suggest that several mammalian RGS proteins, including RGS1, RGS4, and RGS16, have GAP activity toward different Gα and/or Gq class α subunits but not Gαs or Gα12 (10, 11, 15, 23–25). RGS4 inhibited Gαq-dependent PLCβ activation in Xenopus oocytes and transfected COS and HEK293 cells (24–27). Furthermore, addition of recombinant
RGS4 protein to NG-108 cell membranes inhibited Gq-dependent PLCβ activity (28, 29). RGS2 was reported as a specific GAP for Goq in an in vitro assay (30), although it inhibited Gq-dependent signaling when expressed in transfected cells (31). Because RGS proteins with similar GAP activities are co-expressed in cells within a single tissue (16, 18, 23, 29–31), the mechanisms that may provide more precise regulatory specificity have been enigmatic. To date there is no information on the potency or selectivity with which mammalian RGS proteins modulate the same G protein α subunit during its response to different receptors. This highlights the need to analyze RGS proteins in intact cells under controlled conditions to assess their potency and specificity of action.

In the present study, we examined the role of RGS4 and other RGS proteins in regulating Ca2+ signaling in pancreatic acinar cells. RGS4 inhibited Ca2+ signaling assayed either by measuring agonist-dependent Ca2+ mobilization in streptolysin O (SLO)-permeabilized cells or Ca2+-activated Cl− current in intact cells. The potency of RGS4 was exceedingly high in intact cells, and GTPyS reversed the inhibitory action of RGS4. This suggests that catalysis of GTPase activity is the dominant mechanism by which RGS4 regulates Ca2+ signaling. Furthermore, we provide the first evidence for receptor selectivity in RGS4 inhibition of PLCβ and Ca2+ signaling. Even more pronounced receptor selectivity was measured with RGS1 and RGS16. On the other hand, RGS2 showed similar potency in inhibiting m3- and CCK-dependent Ca2+ signaling. The potential role of the α subunits in determining differential sensitivity to RGS4 was analyzed using knockout mice. Deletion of individual Gq class α subunit genes or combinations thereof had no effect on the receptor-specific action of RGS4. Thus, specificity of RGS protein actions depends on their interaction with the receptor complex rather than their interaction with a specific Gq class α subunit.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of RGS Proteins—Recombinant RGS1, RGS2, RGS4, and RGS16 proteins were tagged at the N terminus with the sequence MH6MG using the pET19b and a modified pQE60 expression vector, respectively (Qiagen), expressed in Escherichia coli, and purified as described (10, 14, 15). Briefly, a 10-ml overnight culture of E. coli was grown in Luria–Bertani (LB) medium pre-equilibrated with TBP buffer. The column was washed with 10 ml of TBP and 0.2 M NaCl. The final wash was performed using 10 ml of TBP containing 200 mM imidazole, pH 8.0, and DNA digestion. The total lysate was centrifuged (12,000 × g) at 4 °C, and the supernatant was loaded onto a 2-ml nickel-NTA column pre-equilibrated with TBP buffer. The column was washed with 30 ml of TBP and 0.2 M NaCl. The final wash was performed using 10 ml of TBP containing 200 mM imidazole, pH 8.0, and DNA digestion. The total lysate was centrifuged (12,000 × g) at 4 °C, and the supernatant was loaded onto a 2-ml nickel-NTA column pre-equilibrated with TBP buffer. The column was washed with 30 ml of TBP and 0.2 M NaCl. The final wash was performed using 10 ml of TBP containing 200 mM imidazole, pH 8.0, and DNA digestion. The whole cell configuration of the patch clamp technique (37) was used to measure the effect of agonists on Cl− current. The pipette solution and recording conditions were set to optimize detection of the Ca2+-activated Cl− current as a reporter of changes in [Ca2+]i (for details, see Ref. 5). All experiments were performed at room temperature. The patch clamp output was filtered at 20 Hz. Recording was performed with pClamp 6 and a Digidata 1200 interface (Axon Instruments). In all experiments, the Cl− and cation equilibrium potentials were about 0 mV. Cl− current was recorded at a holding potential of −40 mV. Current amplitude of stimulated cells was within 15% in a given cell preparation but varied between 50 and 150 pA/pF between cell preparations. Therefore, each stimulated cells was within 15% in a given cell preparation but varied between 50 and 150 pA/pF between cell preparations. Therefore, each experiment included at least one control and the inhibition of signaling by RGS proteins was compared with a control performed with a cell from the same preparation.

**RESULTS**

Inhibition of Ca2+ Signaling by RGS4 in Permeable Cells—Previous studies showed that RGS4 added to isolated membranes (28, 29) or overexpressed in cell lines (24–27) inhibited Gq-dependent PLCβ activation. Fig. 1 shows that addition of RGS4 to permeabilized rat pancreatic acinar cells effectively blocked agonist-dependent, Gq-coupled Ca2+ release from internal stores. The permeabilized cells reduced [Ca2+]i in the absence of extracellular Ca2+ and responded to maximal stimulation with carbachol by releasing about 75% of the Ca2+ that is accessible to IP3 (Fig. 1a). Addition of 50 mM RGS4 to the permeabilization medium inhibited about 85% of Ca2+ release triggered by carbachol (Fig. 1b). Inhibition by RGS4 was fully reversible by addition of GTPγS (2.5 μM), a non-hydrolyzable analog that activates Go subunits, suggesting that inhibition of signaling reflects the GAP activity of RGS4 under these conditions. Consistent with this interpretation, RGS4 at concentrations up to 1.87 μM had no effect on Ca2+ release evoked by IP3 (Fig. 1c), which excludes the possibility that RGS4 inhibited the IP3-activated Ca2+ channel.
Agonist Dependence of RGS4 Action—An important finding shown in Fig. 1 is that RGS4 inhibited Ca\(^{2+}\) release stimulated by three different G\(_q\)-coupled receptors with markedly different potencies. Fig. 1 (b, d, and e) shows that, whereas 0.05 \(\mu\)M RGS4 inhibited 85% of Ca\(^{2+}\) release evoked by carbachol, 0.65 \(\mu\)M RGS4 only partially inhibited Ca\(^{2+}\) release and 1.87 \(\mu\)M RGS4 inhibited 85% of Ca\(^{2+}\) release by CCK. The dependence of inhibition of Ca\(^{2+}\) release on RGS4 concentration is illustrated in Fig. 2. Half-maximal inhibition of Ca\(^{2+}\) mobilization induced by carbachol, bombesin, and CCK occurred at [RGS4]\(_{50}\) of approximately 35, 110, and 380 nM, respectively. Hence, cholinergic receptors were 3- to 10-fold more sensitive to RGS4 than were bombesin and CCK receptors. Addition of higher concentrations of any agonist did not alter the IC\(_{50}\) of RGS4 (data not shown).

The differential sensitivity of the G\(_q\)-coupled receptors to RGS4 inhibition was also reflected in activation of PLC and IP\(_3\) production in SLO-permeabilized cells. Fig. 3 shows that the three agonists stimulated PLC activity to the same extent, indicating similar activation of G\(_q\) class \(\alpha\) subunits by each agonist. Consistent with the concentration dependence of Ca\(^{2+}\) signaling inhibition shown above, 50 nM and 0.2 \(\mu\)M RGS4 was needed to inhibit bombesin-stimulated IP\(_3\) production by 60 ± 4% and 91 ± 7%, respectively. By contrast, 0.1 \(\mu\)M and 0.5 \(\mu\)M RGS4 was needed to inhibit bombesin-stimulated IP\(_3\) production by 41 ± 6% and 94 ± 7%, respectively. Finally, 0.4 \(\mu\)M and 2 \(\mu\)M RGS4 inhibited the effect of CCK on IP\(_3\) production by 43 ± 7% and 77 ± 8%, respectively. Thus, RGS4 inhibition of both PLC activity and Ca\(^{2+}\) signaling were receptor-selective.

Selectivity of RGS4 Inhibition Depends on Receptor, Not G\(_q\) Subunit Identity—The receptor-selective action of RGS4 might reflect preferential coupling of the receptors to different G\(_q\) class \(\alpha\) subunits that respond differentially to RGS4. Alternatively, selectivity might be determined by receptor-specific interactions. We used mice genetically deficient in G\(_q\) class \(\alpha\) subunits that are expressed in pancreatic acinar cells (G\(_{q9}\), G\(_{q11}\), and G\(_{q14}\)) (Fig. 4a; Ref. 6) to distinguish these possibilities. When Ca\(^{2+}\) signaling was measured in intact acinar cells of homozygous knockout mice (G\(_{q9}\)/−/−, G\(_{q11}\)/−/−, and the double knockout line G\(_{q11}\)/−/−; G\(_{q14}\)/−/− and G\(_{q14}\)/−/−; G\(_{q14}\)/−/−), maximal Ca\(^{2+}\) responses were identical in wild type and all mutant mice tested (6).

To evaluate the importance of the \(\alpha\) subunit identity in conferring selectivity to RGS4 action, we compared the inhibition of Ca\(^{2+}\) signaling by RGS4 in permeabilized pancreatic acini from WT and G\(_{q9}\) class knockout mice. Representative experiments are illustrated in Figs. 4 and 5, and the results of all experiments are summarized in Table I. Fig. 4 (a–d, e–h, and i–l) show similar experiments for 50–60% inhibition of Ca\(^{2+}\) signaling by comparable concentrations of RGS4 in acini from the G\(_{q9}\)/−/−; G\(_{q14}\)/−/−, G\(_{q11}\)/−/−, and G\(_{q11}\)/−/−; G\(_{q14}\)/−/− mice, respectively. The relative inhibitory potency of RGS4 was unaltered for each of the three agonists used here (Table I). The differential sensitivity to RGS4 among the different receptors was somewhat higher in mouse than in rat pancreas. In cells from WT mice, the response to carbachol was about 4.5- and 33-fold more sensitive to RGS4 than that of bombesin and CCK, respectively. Similar results were obtained in cells from G\(_{q11}\)/−/−, G\(_{q14}\)/−/−; G\(_{q14}\)/−/−, and G\(_{q14}\)/−/− mice. The findings in Table I and Figs. 4 and 5 suggest that interactions with receptor, rather than the iden-
RGS Proteins and Signaling Specificity

**Fig. 4.** Western blot analysis of Gq class a subunits and receptor-dependent inhibition of Ca\(^{2+}\) signaling by RGS4 in pancreatic acini from WT mice. Membrane proteins isolated from pancreatic acini of WT mice express Go\(_{i}\), Go\(_{11}\), and Go\(_{14}\), but not Go\(_{15}\) (a). The protocol of Fig. 1 was used to test the effect of RGS4 on agonist-evoked Ca\(^{2+}\) signaling. The response in the absence of RGS4 (control) is shown only for carbachol (b).

RGS4 Preferentially Inhibits the Response to Carbachol in Intact Cells—Measurements of Ca\(^{2+}\) activated Cl\(^{-}\) current using the whole cell configuration of the patch clamp technique were used to independently assess the potency and receptor-selective action of RGS4. This Cl\(^{-}\) current faithfully reflects changes in [Ca\(^{2+}\)]\(_i\), in pancreatic acinar cells (5, 38). Fig. 6 establishes a dose-response relationship that identified the minimal concentration of carbachol (100 μM) necessary to evoke a maximal Ca\(^{2+}\) response in acinar cells. After dialysis with the pipette solution (7 min), stimulation by 100 μM carbachol generated a typical biphasic Ca\(^{2+}\) response that consists of an initial spike followed by a plateau of Ca\(^{2+}\)-activated Cl\(^{-}\) current. The absence of a response to subsequent addition of high concentrations of carbachol (1 mM) or CCK (10 nM) did not evoke additional activation of the Cl\(^{-}\) current because internal Ca\(^{2+}\) stores were depleted upon the first stimulation. In contrast, stimulation with 10 μM carbachol also evoked a significant but submaximal Ca\(^{2+}\) response because subsequent addition of 1 mM carbachol further activated the Cl\(^{-}\) current (Fig. 6b). The absence of a response to later addition of CCK indicated that 1 mM carbachol maintained the Ca\(^{2+}\) stores in a depleted state. Ca\(^{2+}\) signaling evoked by 30 μM carbachol was also submaximal (data not shown). Reducing the carbachol concentration to 2.5 μM initiated rapid oscillations in the Cl\(^{-}\) current (Fig. 6c), whereas stimulation with an even lower concentration of carbachol initiated lower frequency oscillations (Fig. 6d). Many cell types, including pancreatic acini, typically respond to submaximal agonist stimulation with oscillations in [Ca\(^{2+}\)]\(_i\) (2, 5, 38). Based on the agonist dose-response relationship, we next tested RGS4 inhibition of Ca\(^{2+}\) signaling.

Fig. 7 shows the inhibitory activity of RGS4 at three concentrations with three different Go\(_{1}\)-coupled receptors. Cells were dialyzed with RGS4 for at least 7 min, which is sufficient to allow equilibration of the protein between pipette solution and the cytosol (39). In general, RGS4 was between 100- and 1000-fold more effective in intact than in permeable cells. This most likely reflects improved access of RGS4 to the inner membrane surface in intact cells. In more than 20 experiments, 100 pM RGS4 either completely or almost completely inhibited the effect of carbachol on Ca\(^{2+}\) signaling. This is the highest potency for RGS4 in inhibiting Go-dependent activity reported to date. The agonist-dependent effect of RGS4 seen in permeable cells could be reproduced in intact cells. Thus, bombesin-stimulated Ca\(^{2+}\) signaling was only partially inhibited by 100 pM RGS4 (Fig. 7e), whereas 500 pM RGS4 was needed for complete inhibition (Fig. 7f). RGS4 at 500 pM only partially inhibited the effect of CCK (Fig. 7i), and 5–10 nM were needed for maximal inhibition of CCK-dependent Ca\(^{2+}\) signaling (Fig. 7j). Control experiments showed that boiling RGS4 prevents the inhibition of agonist-evoked Ca\(^{2+}\) signaling by this protein (Fig. 7, b and h).

To obtain unequivocal evidence for the differential sensitivity of the Go\(_{1}\)-coupled receptors to RGS4, we measured the effect of RGS4 on carbachol and CCK stimulation in the same cells (Fig. 8a). Dialyzing cells with 10 pM RGS4 converted the large initial Cl\(^{-}\) current to an oscillatory response (Fig. 8d). Differences in the peak amplitude of the oscillations, the frequency of oscillations, and the number of oscillations were used to determine the concentration of RGS4 necessary for optimal inhibition of both agonists (Fig. 8a, b, and d). The inhibition of carbachol-evoked Ca\(^{2+}\) signaling by RGS4 was dependent on the concentration of carbachol and RGS4. The inhibition of CCK-evoked Ca\(^{2+}\) signaling by RGS4 was independent of the CCK concentration but dependent on the concentration of RGS4.

After addition of atropine to allow reloading of intracellular Ca\(^{2+}\) stores, cells were challenged with CCK to assess the
Receptor selectivity of RGS4 is unaltered in mice deficient in Gq class genes

| Agonist       | Relative inhibitory potency of RGS4 (agonist/carbachol ratio) |
|---------------|-------------------------------------------------------------|
|               | WT | Gq11/14−/− | Gq11/14−/−; RGS4 | Gq14−/−; RGS4 |
| BS/carbachol  | 4.5 ± 0.3 (3) | 4.3 ± 0.4 (3) | 4.1 ± 0.4 (2) | 4.1 ± 0.3 (3) |
| CCK/carbachol | 33 ± 4 (3)   | 35 ± 5 (3)   | 31 ± 6 (2)    | 25 ± 4 (3)    |

* Fold increase in RGS4 concentration in the incubation medium that is necessary to similarly inhibit Ca2+ release evoked by bombesin or CCK relative to carbachol were measured in SLO-permeabilized pancreatic acinar cells from the indicated mouse lines using the protocols of Figs. 4 and 5. The cells were stimulated with carbachol (2 μM), bombesin (BS, 100 nM), or cholecystokinin (CCK, 50 nM). Results are shown for each mouse strain as the mean ± S.E. (number of experiments given in parentheses).

RGS4 is a potent inhibitor of Ca2+ signaling in intact cells. Single pancreatic acinar cells were dialyzed with the pipette solution for at least 7 min before the first stimulation. As indicated in the figure, the pipette solution also contained 100 nM (b, c, and e), 500 nM (f, h, and i), or 10 nM (j) RGS4. In control experiments like those in b and h, the pipette solution was incubated in a boiling water bath for 15–20 min before use. The experiment in j shows that elevating [Ca2+]i, with AQ1867 activated the current, indicating that RGS4 does not affect responses downstream of the [Ca2+]i increase. Similar sensitivity to RGS4 was observed in at least 20 experiments in which the cells were dialyzed with 10, 100, or 1000 nM RGS4 and stimulated with carbachol, 4 experiments in which the cells were dialyzed with 0.1, 0.5, or 2 nM RGS4 and stimulated with bombesin, and 7 experiments in which the cells were dialyzed with 0.1, 1, or 10 nM RGS4 and stimulated with CCK.

RGS1 and RGS16 Preferentially Inhibit the Response of Pancreatic Acinar Cells to Carbachol—The results obtained with RGS4 raised the question of whether other RGS proteins that interact with Gq class a subunits show similar receptor-selective inhibition of Ca2+ signaling. RGS1 and RGS16 dialyzed into acinar cells inhibited Ca2+ signaling evoked by carbachol and CCK (Fig. 9). Furthermore, both RGS proteins showed similar potency in inhibiting the response to carbachol. At a concentration of 0.1 nM, RGS1 and RGS16 inhibited the response to carbachol by 23 ± 5% (n = 3) and an average of 21% (n = 2), respectively. Increasing the concentration of RGS1 and RGS16 to 1 nM resulted in 81 ± 4% (n = 11) and 72 ± 7% (n = 5) inhibition of the response to carbachol, respectively (see Fig. 9, b and e). By contrast, these RGS proteins were relatively poor inhibitors of the response to CCK stimulation. Increasing RGS1 concentration to between 1 and 3 μM was needed to inhibit the response to CCK by 85 ± 8% (n = 4), whereas RGS16 only partially inhibited the response to CCK. RGS16 (100 nM) inhibited CCK evoked Ca2+ release by 57 ± 4% (n = 3), and increasing the concentration to 1 μM (n = 2) or 5 μM (n = 2) did not cause further inhibition (Fig. 9, compare g and h).

RGS2 Equally Inhibits the Response of Pancreatic Acinar Cells to Carbachol and CCK—RGS2 was reported to specifically accelerate GTPase activity of Goq (30), although when expressed in COS cells, it affected signaling mediated by Ga13 and Gi1 (31). Fig. 10 shows the effect of RGS2 on the response of mouse pancreatic acinar cells to carbachol and CCK stimulation. Equivalent results were obtained in rat pancreatic acini. RGS2 inhibited carbachol-evoked Ca2+ signaling in the same concentration range measured with other RGS proteins. Thus, 1 nM RGS2 inhibited the carbachol-induced response by 57 ± 6% (n = 3) and 100 nM RGS2 inhibited the response by 94 ± 5% (n = 4). Unlike the findings with other RGS proteins, RGS2 inhibited the response to carbachol and CCK stimulation with equal potency. For example, in the same cells, 10 nM RGS2 inhibited the response to carbachol by 83 ± 6% and the response to CCK by 81 ± 7% (n = 6).

Estimations of the relative potency with which the different RGS proteins inhibited the response to stimulation of the m3 and CCK receptors are shown in Fig. 10e. The relative potency of RGS2 was estimated from all concentrations between 1 and 100 nM. The relative potencies of RGS4 and RGS1 were determined from the concentrations needed for 89–95% inhibition because of the large difference in potency in inhibiting carbachol and CCK responses. Since RGS16 only partially inhibits the response to CCK, the potency for inhibition of this response was estimated for the fraction inhibited by RGS16. Fig. 10e shows that the difference in potency for the RGS proteins tested spans 3 log units. Hence, although the studies with
RGS2, RGS1, and RGS16 are not as extensive as those with RGS4, they corroborate the central finding that RGS proteins can discriminate between receptor complexes to regulate Ca\textsuperscript{2+} signaling.

**DISCUSSION**

Like many cell types, pancreatic acini respond to a battery of Ca\textsuperscript{2+} mobilizing agonists. Previous studies showed that at least three agonists, carbachol, bombesin, and CCK, interact with receptors coupled to Gq, stimulate PLC to the same extent and mobilize the same Ca\textsuperscript{2+} pool (40, 41). The Ca\textsuperscript{2+} signals evoked by all agonists are in the form of [Ca\textsuperscript{2+}]\textsubscript{i} waves that initiate in the luminal pole and propagate through the cell periphery to the basal pole (42–44). The [Ca\textsuperscript{2+}]\textsubscript{i} waves exhibit agonist specific initiation sites, speed, and propagation patterns (3). Coupling of receptors to G proteins may play a significant role in conferring signaling specificity. Indeed, Ca\textsuperscript{2+} signaling stimulated by several agonists displayed differential sensitivity to guanine nucleotides (3). The present studies show that differences exist in the interaction of receptor-Gq complexes with four RGS proteins: RGS2, RGS4, RGS1, and RGS16. The sensitivity to RGS4 can be best quantitated in permeabilized cells because an averaged response from many cells is recorded. In permeabilized rat pancreatic acinar cells, cholinergic receptors showed 3- and 10-fold higher apparent affinity to RGS4 than bombesin and CCK receptors, respectively (Figs. 1–3). In mice, differences between the receptors were even greater (Fig. 5). Species differences in CCK-dependent Ca\textsuperscript{2+} signaling are well documented (41). Differential sensitivity to RGS4 was corroborated in intact cells from the rat pancreas (Figs. 7 and 8). The fact that RGS4 was a more potent inhibitor of the response to carbachol relative to CCK within the same cell excludes the possibilities of cell to cell variation and restricted access of RGS4 to the inner leaflet of the plasma membrane. Although it was difficult to accurately quantify inhibition in intact cells, the CCK response was at least 10-fold less sensitive to RGS4 (see Figs. 7 and 8), consistent with experiments in permeable cells (Fig. 2). Therefore, the cumulative results from permeable and intact cells indicated that pancreatic acini could be more readily stimulated with CCK than with bombesin or acetylcholine in the presence of RGS4. This conclusion is reinforced by the findings with RGS2, RGS1, and RGS16, which showed no or greater preferences for inhibition of muscarinic stimulation (Fig. 10). Regulation by RGS proteins may there-
Therefore provide the cell with a mechanism for intense (CCK), intermediate (bombesin), and weak (acetylcholine) stimulation of the same Gq-mediated signaling pathway. Intensity of stimulation affects almost all parameters of the [Ca\(^{2+}\)]\(_i\) signal (2, 38, 40).

Several findings of the present and previous work indicate that differential sensitivity to RGS proteins was not the result of different steady state levels of GTP-bound G\(_q\) class \(\alpha\) subunits generated by the three agonists. First, the three RGS proteins showed similar potency toward carbachol stimulation but highly variable potency toward CCK stimulation. Second, acinar cells express similar number of cholinergic and CCK receptors (45). Third, titrating the level of all activated G\(_q\) class \(\alpha\) subunits with antibody raised against a C-terminal sequence common to G\(_\alpha_6\), G\(_\alpha_{11}\), and G\(_\alpha_{14}\) showed similar activation of G\(_q\) by all agonists (3). Fourth, each agonist activated PLC\(\beta\) to the same extent (Fig. 3).

Receptor specificity of RGS protein action could reflect preferential coupling of the receptors to different members of the G\(_q\) class \(\alpha\) subunits, which, in turn, are differentially regulated by RGS proteins. Alternatively, interaction between RGS proteins and receptors may determine receptor specificity. To distinguish between these possibilities, we studied the role of the G\(_q\) class \(\alpha\) subunits in determining receptor specificity and inhibition by RGS4. Our previous finding, that all three receptor types in pancreatic acini showed similar values of \(K_{\text{app}}\) toward their respective agonist in WT and the various G\(_q\) class knockout mice (6), provides strong evidence that the identity of these \(\alpha\) subunits does not play a role in conferring agonist specificity. More importantly, RGS4 was an equally effective inhibitor of Ca\(^{2+}\) signaling in pancreatic acinar cells isolated from WT and mutant mice. This indicates that, regardless of whether the acetylcholine, bombesin, and CCK receptors normally couple to G\(_\alpha_6\), G\(_\alpha_{11}\), or G\(_\alpha_{14}\), it is the receptors, rather than the identity of the G protein \(\alpha\) subunit, that dictate differential sensitivity to RGS4. This unexpected finding further indicates that signaling specificity is regulated not only by receptor-catalyzed GTP loading to activate G proteins but also by GTP hydrolysis to limit the duration of signaling. Thus, RGS proteins play an important role in conferring signaling specificity.

The finding that these RGS proteins inhibit G\(_q\) proteins coupled to acetylcholine receptors in preference to other receptor types present in the same cell suggests selectivity in RGS protein regulation of G protein signaling. Studies with recombinant proteins in vitro showed that RGS4 similarly stimulated the GTPase activity of several G\(_q\) class \(\alpha\) subunits, as did RGS1, GAIP, and RGS10 (11, 13–16). RGS15 (15, 16) and RGS16 (24) were shown to interact with G\(_\alpha_6\), G\(_\alpha_{11}\), and G\(_\alpha_{14}\) subunits. Overexpression in cells by transient or stable transfection of RGS4 and RGS16 equally inhibited signaling evoked by receptors coupled to G\(_q\) and G\(_i\) (25, 26, 46). Similarly, in vitro RGS2 bound to and activated G\(_q\) but not to G\(_\alpha_11\) (30, 31), but when expressed in COS cells it inhibited G\(_\alpha_6\)- and G\(_\alpha_{11}\)-dependent signaling (31). Our ability to control the concentration of RGS proteins in the cytosol allowed us to demonstrate selectivity for interaction of the RGS proteins with several G\(_q\)-coupled receptors present in the same cell. As discussed above, results in cells from the knockout mouse indicate that the receptors must play a central role in conferring selectivity to RGS4 action. Thus, selectivity of RGS proteins appears to extend beyond classes of G\(_\alpha\) subunits to the level of specific receptor types. With increasing knowledge of the distinct properties of numerous RGS proteins, it is clear that experimental systems similar to those used in the present studies will be instrumental in evaluating regulatory specificity of G protein signaling by RGS proteins.

Another implication of the receptor-specific interaction of the RGS proteins used in the present work is that RGS proteins may also interact with the receptor-G\(_i\) complex, not only with activated G\(_q\) class \(\alpha\) subunits. This may be suggested from the finding that the RGS proteins blocked the initiation of signaling. If RGS proteins interacted only with the activated G\(_i\), we would expect them to preferentially inhibit Ca\(^{2+}\) signaling after the initial activation of PLC and Ca\(^{2+}\) release. This type of inhibition was observed with recombinant protein containing only the N-terminal domain of RGS4, which has G\(_i\)-GAP activity but is not receptor-selective (47). The fact that full-length RGS4 and other RGS proteins inhibit initial activation of PLC and Ca\(^{2+}\) release (Figs. 1, 4, and 5 in permeable cells and Figs. 7–10 in intact cells) suggests that RGS proteins may interact with the receptor-G\(_q\) complex. RGS4 interaction with a receptor-G protein-effector complex was also inferred by the mode of activation and inhibition of GIRK channels via G\(_i\)-coupled receptors in Xenopus oocytes overexpressing RGS4 (25). The structure of the RGS4-G\(_\alpha_{11}\) complex showed that only the RGS core domain was visible in the crystal (48). It is possible that amino acids in RGS4 which flank the core domain directly interact with receptors and/or G proteins complexed with receptors. In agreement with this suggestion, we demonstrated that the N-terminal domain of RGS4 confers receptor selectivity (47).

In summary, the present study shows that regulating specificity of G protein-coupled signaling by RGS proteins extends to single receptor types. The identity of the G\(_q\) class \(\alpha\) subunit is not essential for receptor specificity. Rather, interaction of RGS proteins with the receptor complex appears to confer specificity of action. Our study also provides the first demonstration that RGS1, RGS2, RGS4, and RGS16 are potent regulators of Ca\(^{2+}\) signaling in intact cells, which points to the importance of the catalytic action of RGS proteins in regulating Gq-mediated signaling.

**Acknowledgments**—We thank K. Blumer and S. Heximer (Washington University) for RGS1; D. Forsdyke (Queens University) for RGS2; M. Gosselin (Millennium) for RGS16; C. Gowan, D. Smith (University of Texas Southwestern), and S. Pease (Caltech) for technical assistance; P. Sternweis and J. Hepler for antisera; and E. Ross, S. Mumbey, and our colleagues for comments on the manuscript.

**REFERENCES**

1. Hepler, J. R., and Gilman, A. G. (1992) Trends Biochem. Sci. 17, 383–387
2. Berridge, M. J. (1993) Nature 361, 315–325
3. Xu, X., Zeng, W., Diaz, J., and Muallem, S. (1996) J. Biol. Chem. 271, 24684–24690
4. Muallem, S., and Lee, M. L. (1997) Cell Calcium 22, 1–4
5. Zeng, W., Xu, M., and Muallem, S. (1996) J. Biol. Chem. 271, 18520–18526
6. Xu, X., Croy, J. T., Zeng, W., Zhao, L.-P., Davignon, J., Popov, S., Yu, K., Jiang, H., Hoffmanns, S., Muallem, S., and Wilkin, T. M. (1998) J. Biol. Chem. 273, 27275–27279
7. Xu, X., Zeng, W., and Muallem, S. (1996) J. Biol. Chem. 271, 11737–11744
8. Dolman, H. G., and Thorner, J. (1997) J. Biol. Chem. 272, 3871–3874
9. Berman, D. M., and Gilman, A. G. (1998) J. Biol. Chem. 273, 1269–1272
10. Berman, D. M., Wilkie, T. M., and Gilman, A. G. (1996) J. Biol. Chem. 271, 445–452
11. Berman, D. M., Kozasa, T., and Gilman, A. G. (1996) J. Biol. Chem. 271, 27209–27212
12. Faurobert, E., and Hurley, J. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2945–2950
13. Hunt, T. W., Fields, T. A., Casey, P. J., and Peralta, E. G. (1996) Nature 383, 175–177
14. Popov, S., Yu, K., Kozasa, T., and Wilkie, T. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7216–7220
15. Heximer, S. P., Cristollo, A. D., and Forsdyke, D. R. (1997) DNA Cell Biol. 16, 589–598
16. Watson, N., Linder, M. E., Druey, K. M., Kehrl, J. H., and Blumer, K. J. (1996) Nature 383, 172–175
17. Koelle, M. B., and Horvitz, H. R. (1996) Cell 84, 115–125
18. Yu, J. H., Wieser, J., and Adams, T. H. (1996) EMBO J. 15, 5184–5190
19. Druey, K. M., Blumer, K. J., Kang, V. H., and Kehrl, J. H. (1996) Nature 378, 742–746
20. De Vries, L., Mosuli, M., Wurmser, A., and Farquhar, M. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11916–11920
21. Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T. J., Perry, W. L. R., Lee, J. J., Tilghman, S. M., Gumbiner, B. M., and Costantini, F. (1997) Cell 90, 181–192
22. Chen, C. K., Wieland, T., and Simon, M. I. (1996) Proc. Natl. Acad. Sci. U. S. A.
3556

RGS Proteins and Signaling Specificity

23. Huang, L. J., Durick, K., Weiner, J. A., Chun, J., and Taylor, S. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11184–11189

24. Chen, C., Zheng, B., Han, J., and Lin, S. C. (1997) J. Biol. Chem. 272, 8679–8685

25. Yan, Y., Chi, P. P., and Bourne, H. R. (1997) J. Biol. Chem. 272, 11184–11189

26. Huang, C., Hepler, J. R., Gilman, A. G., and Mumby, S. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11184–11189

27. Chen, C., Zheng, B., Han, J., and Lin, S. C. (1997) J. Biol. Chem. 272, 8679–8685

28. Yan, Y., Chi, P. P., and Bourne, H. R. (1997) J. Biol. Chem. 272, 11924–11927

29. Huang, C., Hepler, J. R., Gilman, A. G., and Mumby, S. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6159–6163

30. Doupnik, C., Davidson, N., Laster, H. A., and Kofugi, P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10461–10466

31. Saugstad, J. A., Marino, J. J., Folk, J. A., Hepler, J. R., and Conn, P. J. (1998) J. Neurosci. 18, 905–913

32. Hepler, J. R., Berman, D. M., Gilman, A. G., and Kozasa, T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 428–432

33. Heximer, S. P., Watson, N., Linder, M. E., Blumer, K. J., and Hepler, J. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14388–14393

34. Ingi, T., Krumins, A. M., Chidiac, P., Brothers, G. M., Chung, S., Snow, B. E., Barnes, C. A., Lanahan, A. A., Siderovski, D. P., Ross, E. M., Gilman, A. G., and Worley, P. F. (1998) J. Neurosci. 18, 7178–7188

35. Gold, S. J., Ni, Y. L. G., Dohlman, H. G., and Nestler, E. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 85–91

36. Offermanns, S., Zhao, L.-P., Gohla, A., Sarosi, I., Simon, M. I., and Wilkie, T. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10461–10466

37. Offermanns, S., Hashimoto, K., Watanabe, M., Sun, W., Kurihara, H., Thompson, R. F., Inoue, Y., Kano, M., and Simon, M. I. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14068–14072

38. Lee, M. G., Xu, X., Zeng, W., Diaz, J., Wojcikiewicz, R. J. H., Kuo, T. H., Wuytack, F., Raymaekers, L., and Muallem, S. (1997) J. Biol. Chem. 272, 15765–15770

39. Jensen, R. T., and Gardner, J. D. (1984) J. Pediatr. Gastroenterol. Nutr. 3, 525–535

40. Buckbinder, L., Velasco-Miguel, S., Chen, Y., Xu, N., Talbott, R., Gelbert, L., Gao, J., Seiziger, B. R., Gutkind, J. S., and Kley, N. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7868–7872

41. Zeng, W., Xu, X., Popov, S., Mukhopadhyay, S., Chidiac, P., Swistok, J., Danho, W., Yagaloff, K. A., Fisher, S. L., Ross, E. M., Muallem, S., and Wilkie, T. M. (1998) J. Biol. Chem. 273, 34687–34690

42. Tesmer, J. J., Berman, D. M., Gilman, A. G., and Sprang, S. R. (1997) Cell 18, 251–261