The N-terminal Domain of RGS4 Confers Receptor-selective Inhibition of G Protein Signaling*

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Regulators of heterotrimeric G protein signaling (RGS) proteins are GTPase-activating proteins (GAPs) that accelerate GTP hydrolysis by Gαq and Gα11 subunits, thus attenuating signaling. Mechanisms that provide more precise regulatory specificity have been elusive. We report here that an N-terminal domain of RGS4 discriminated among receptor signaling complexes coupled via Gαq. Accordingly, deletion of the N-terminal domain of RGS4 eliminated receptor selectivity and reduced potency by 10-fold. Receptor selectivity and potency of inhibition were partially restored when the RGS4 box was added together with an N-terminal peptide. In vitro reconstitution experiments also indicated that sequences flanking the RGS4 box were essential for high potency GAP activity. Thus, RGS4 regulates Gαq class signaling by the combined action of two domains: 1) the RGS box accelerates GTP hydrolysis by Gαq and 2) the N terminus conveys high affinity and receptor-selective inhibition. These activities are each required for receptor selectivity and high potency inhibition of receptor-coupled Gαq signaling.

MATERIALS AND METHODS

Expression and Purification of Recombinant RGS4 Proteins—All recombinant RGS proteins were His6-tagged at the N terminus. Protein expression and GAP assays were performed as described (10). Peptide Synthesis—Synthetic peptides were purified by reverse-phase high pressure liquid chromatography and confirmed by amino acid analysis and fast atom bombardment/mass spectroscopy.

Measurement of Ca2+ Release—Measurement of Gαq2-activated Cl− current and Ca2+ release in permeabilized cells was exactly as described.

GTPase Measurements—The agonist-stimulated steady-state GTPase activity of reconstituted phospholipid vesicles that contained m1 muscarinic acetylcholine receptors and heterotrimeric Gαq was assayed in the presence or absence of RGS proteins or peptides as described (14). Single-turnover measurements of the hydrolysis of Gαq-GTP were performed as described (15) using [γ-32P]GTP bound to the R183C mutant of Gαq (chosen to slow hydrolysis and thus facilitate loading).

RESULTS AND DISCUSSION

The N Terminus of RGS4 Is Required for High Potency Inhibition—To determine the domains of RGS4 that conveyed high potency and receptor-selective inhibition, rat pancreatic acinar cells were diazylized with different recombinant RGS4 proteins through a patch pipette and exposed to 100 μM carbachol, the minimal concentration of carbachol needed to generate a maximal cellular response (16, 17). Responses were

Many cells express several Gq-coupled receptors that regulate the location, intensity, and propagation of intracellular Ca2+ waves. For example, pancreatic acini respond to acetylcholine, bombesin, and cholecystokinin by activating the same set of Gq class proteins and mobilizing the same Ca2+ pool, but each receptor evokes distinct patterns of Ca2+ waves (3). Ca2+ release may be regulated by intracellular proteins that interact with guanine nucleotide binding proteins, such as regulators of G protein signaling (RGS) proteins.2

RGS proteins are GTPase-activating proteins (GAPs) that accelerate GTP hydrolysis by Gαq and Gα11 subunits, thus attenuating signaling (5–8). Mammals express over 20 different RGS proteins, of which RGS4 has received the most extensive biochemical characterization (5, 7–12). RGS4 is composed of a central domain of 120 amino acids that is homologous to other RGS class proteins and mobilizing the same Ca2+ waves. For example, pancreatic acini respond to acetylcholine, bombesin, and cholecystokinin by activating the same set of Gq class proteins and mobilizing the same Ca2+ pool, but each receptor evokes distinct patterns of Ca2+ waves (3). Ca2+ release may be regulated by intracellular proteins that interact with guanine nucleotide binding proteins, such as regulators of G protein signaling (RGS) proteins.2

RGS4 preferentially inhibited Gq/11-mediated signaling evoked by carbachol relative to bombesin and cholecystokinin regardless of the identity of the Gq class α subunit.2 Regulatory specificity was apparently conferred by direct or indirect interaction between RGS4 and the receptor.

In the present study, we used deletion mutations to identify two domains in RGS4 that regulate agonist-dependent Ca2+ signaling. The RGS box accelerates GTP hydrolysis by Gαq whereas the N terminus conveys high affinity and receptor-selective inhibition. These combined activities are required for receptor selectivity and high potency inhibition of receptor-coupled Gαq signaling.

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The abbreviations used are: PLC, phospholipase C; IP3, inositol triphosphate; RGS, regulators of G protein signaling; GAP, GTPase-activating protein; 4Box, RGS domain of RGS4; P13–33, N-terminal 33 amino acids of RGS4; Car, carbachol; CCK, cholecystokinin; GTP, guanosine 5′-O-(thio)triphosphate.

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2 Xu, X., Zeng, W., Popov, S., Berman, D. M., Davignon, I., Yu, K., Yowe, D., Offermanns, S., and Wilkie, T. M. (1999) J. Biol. Chem., in press.

3 Popov, S., MCKGLAGLPCLASLRASKDKMHRLHGFILQKSDSC (Ro27-3948); P13–32, MCKGLAGLPCLASLRASKDKMHRLHGFILQKSDSA (Ro27-3949); P13–32, 5-LRASKDKMHRLHGFILQKSDS (Ro27-3950); HT-31, DLIEEASAHRDVDAEVQRAAY (Ro27-1970).

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FIG. 1. Deletion of the RGS4 N terminus destroys high potency inhibition of Ca2+ release. Cells were dialyzed for at least 7 min with the pipette solution before the first stimulation. a, a standard response to carbachol (Car). Inhibition of Ca2+ signaling by recombinant proteins was assayed with: b and c, RGS4; d–f, 4Box, 58–177; g and h, RGS4AC, 1–177; i and j, RGS4ACN, 58–205. Data for RGS4 and 4Box are representative of at least 20 experiments each (RGS4ACN, n = 6; RGS4AC; n = 3).

detected as changes in current carried by a Ca2+-activated Cl− channel. In the control (Fig. 1a), carbachol evoked a typical biphasic Ca2+ response consisting of an initial spike caused by Ca2+ release from internal stores followed by a plateau current. Infusion of full-length RGS4 (10 pm) suppressed the initial Ca2+ release and caused subsequent oscillations in response to maximal carbachol stimulation. Increasing the RGS4 concentration to 100 pm further reduced the Ca2+ response to a low frequency oscillation. A similar transition from a sustained to a weak oscillatory response occurred when the carbachol concentration was reduced from 100 to 1 pm (16, 17). This indicates that carbachol-evoked signaling in intact cells dialyzed with 100 pm RGS4 is functionally equivalent to a 100-fold decrease in the potency of carbachol stimulation. Exposing cells to higher concentrations of carbachol did not change the inhibitory effect of RGS4 on Ca2+ release. Two other proteins related to RGS4, RGS1, and RGS16 (13, 18, 19) also inhibit carbachol-evoked Ca2+ release, but RGS10, a GAP for the G11 class (6, 10), has no effect on Gq-mediated signaling (data not shown).

We next tested the effect of the RGS domain of RGS4 (4Box) (Fig. 1, d–f). Although full-length RGS4 and 4Box accelerated GTP hydrolysis by purified Ga1 with equal potency in vitro (10), 4Box was approximately 104-fold less potent than full-length RGS4 in inhibiting in vitro Ca2+ signaling. For example, 10 pm RGS4 conferred partial inhibition of signaling whereas 100 nm 4Box was required before partial inhibition was apparent (Fig. 1, b and d). Increasing the cellular concentration of full-length RGS4 gradually inhibited signaling, decreasing both the amplitude of the initial current spike and the frequency and amplitude of the subsequent current oscillations (Fig. 1, a–c). By contrast, dialysis with as much as 100 nm 4Box did not significantly reduce the initial current spike but caused rapid termination of signaling and obliterated subsequent oscillations in the continued presence of agonist (Fig. 1d). These data also indicated that the mechanisms of inhibition by RGS4 and 4Box were qualitatively different, suggesting that inhibition by intact RGS4 involves more than just Gq GAP activity.

To identify the flanking structure in full-length RGS4 that conveys high potency inhibition, we tested the activities of other deletion mutants. C-terminal truncation of RGS4 (RGS4C) reduced its potency by about 100-fold (Fig. 1, D–h). Increasing the cellular concentration of 4Box also lost selectivity among different receptors. RGS4 inhibited signaling preferentially through the m3 muscarinic receptor. For example, 10 nm RGS4C inhibited the initial current spike to the same extent as did 100 pm full-length RGS4 and caused similar subsequent oscillations in response to 100 pm carbachol (compare Fig. 1, c and h). This effect was not further characterized because deletion of the N-terminal sequence flanking the RGS box (RGS4N) caused an even more dramatic reduction in potency and altered the mechanism of RGS4 inhibition to resemble that observed with 4Box (Fig. 1, i and j). Hence, the N terminus of RGS4 is essential for high potency inhibition and contributes significantly to RGS4 interaction with the receptor-Gq protein complex.

Besides diminishing the effect and potency of RGS4 action, 4Box also lost selectivity among different receptors. RGS4 inhibits signaling preferentially through the m3 muscarinic receptor compared with the CCK receptor, assayed either separately or sequentially within the same cell (Fig. 2). By contrast, 4Box inhibited Ca2+ signaling by carbachol and CCK equivalently over a wide range of concentrations (Fig. 2, c–f). These data suggest that terminal regions of RGS4 both enhance potency and mediate recognition of receptor.

Receptor-selective Inhibition by the RGS4 N-terminal Domain—Next, we tested whether the 33-amino acid N-terminal peptide (P1–33) of RGS4 (P1–33) can itself alter Gq-mediated signaling. P1–33 (100 nm) blocked the initial spike and converted the following sustained current to an oscillatory response (Fig. 2, g–i). Higher concentrations of P1–33 completely blocked signaling by carbachol. In contrast, these concentrations of P1–33 had no effect on CCK signaling. Only at 1 pm P1–33 was the sustained response evoked by CCK converted to an oscillatory current. These data indicate both that P1–33 effectively inhibited Gq-mediated Ca2+ signaling and that inhibition retained...
the selectivity for the m3 muscarinic receptor over the CCK receptor that is characteristic of RGS4.

**RGS4 Flanking Sequences Enhance Goq-GAP Activity in Vitro**—We then tested the relative GAP activity of RGS4 and 4Box toward purified, recombinant Goq proteins in vitro. In a solution phase, single-turnover Goq, GAP assay, RGS4 was about 10-fold more active than 4Box (Fig. 3, a and c), in contrast to their equal potency when assayed with Goq1 (10). We then compared the GAP activity of full-length RGS4 and 4Box on wild-type Goq in a steady-state, receptor-coupled in vitro assay. Phospholipid vesicles were reconstituted with m1 muscarinic cholinergic receptor and trimeric Goqαβγ, wherein steady-state GDP-GTP exchange on Goqα was agonist-dependent (14). In this assay, RGS4 was about 125-fold more potent than 4Box in stimulating steady-state receptor-dependent GTase activity (Fig. 3, b and c). Phospholipid vesicles reconstituted with m2 muscarinic cholinergic receptor and trimeric Goq1βγ revealed an even more pronounced difference between the relative activities of full-length RGS4 and 4Box (data not shown). These findings provide additional evidence that sequences flanking the RGS box are essential for optimal GAP activity in receptor-G protein-coupled signaling.

**N Terminus and 4Box Act Synergistically to Inhibit Ca2+ Signaling**—The results in Figs. 1–3 clearly show that 4Box has Goq-GAP activity but that the N-terminal domain of RGS4 both conferred receptor selectivity upon RGS4 action and increased its potency in intact cells. To address the mechanism of RGS4 action, Ca2+ release from intracellular stores was measured in stably transfected O2-permeabilized cells. These cells sequester Ca2+ from the incubation medium into cellular organelles and retain Gq-coupled signaling in response to all agonists that act on acinar cells (3, 20). This experimental approach facilitated addition of GTPγS to reverse inhibition by RGS4 as a test of its Goq GAP activity.

As shown in Fig. 4a, muscarinic stimulation released about 75–80% of the IP3-sensitive Ca2+ pool. Addition of 125 nM RGS4 inhibited more than 95% of the normal carbachol-stimulated Ca2+ release (Fig. 4f). Permeabilized cells were less sensitive to RGS4 than patch-clamped cells, probably because of a slower diffusion of proteins from the extracellular media into the cytosol. RGS4 inhibition was fully reversible by addition of GTPγS (Fig. 4g), suggesting that inhibition of signaling reflects the GAP activity of RGS4 under these conditions. By contrast, addition of up to 1.4 μM 4Box to the incubation medium had no effect on carbachol-stimulated Ca2+ release (Fig. 4e) further demonstrating that full-length RGS4 inhibits Gq-coupled signaling far more potently than does 4Box.

Consistent with its effects in intact cells, P1–33 inhibited Ca2+ release in permeabilized cells in a receptor-selective manner (Fig. 4, c–e). Carbachol-evoked Ca2+ release was inhibited completely by 62 μM P1–33, with an IC50 of 25 ± 2 μM (n = 5). Similar measurements with CCK showed an IC50 for P1–33 of 62 ± 5 μM (n = 3). Thus, in permeabilized cells, P1–33 inhibited Ca2+ release evoked by carbachol 2.5-fold better than that evoked by CCK (Fig. 4c). Several control experiments further indicated that inhibition by P1–33 was specific. First, cells responded normally to addition of IP3s, even in the presence of 62 μM P1–33 (Fig. 4, c and d). Second, HT-31, another amphipathic peptide that disrupts protein kinase A anchoring to AKAPs (21), had no effect on signaling in response to carbachol. Third, amino acid substitutions within P1–33 dramatically lowered its inhibitory activity (see legend to Fig. 4). Fourth, P1–33 preferentially inhibited signaling via the muscarinic receptor compared with the CCK receptor.

An important distinction between P1–33 and RGS4 is that the inhibitory effect of P1–33 was not reversed by GTPγS (Fig. 4, c and d), indicating that its effect does not depend on Goq GAP activity. Sequential addition of P1–33 and RGS4 blocked GTPγS-insensitive inhibition (Fig. 4g), indicating the peptide and full-length RGS4 compete for binding to a subsite of the RGS4 binding site in the receptor-Gq complex. To further investigate P1–33 inhibition of Ca2+ release, we changed the order
of addition of inhibitors and activators. If cells were exposed to P1–33, then carbachol, subsequent addition of either RGS4 or 4Box did not restore the ability of GTPγS to stimulate Ca\(^{2+}\) release (similar to Fig. 4d). RGS4 and 4Box only worked when added prior to carbachol stimulation (Fig. 4, f–h). P1–33 does not prevent receptor-catalyzed GTP exchange on Goq, nor does P1–33 act as a Goq GAP (data not shown). This overall pattern of activities suggests that P1–33 binds at a site normally occupied by the N terminus of RGS4, and when added without 4Box, P1–33 blocks interaction of Goq with its effector protein PLCβ.

A final and distinctive activity of P1–33 was that exposure to both 4Box and P1–33 inhibited Ca\(^{2+}\) release with greater potency than either added alone (compare Fig. 4, b, c, and h). Importantly, this inhibition was reversed by GTPγS, indicating that the GAP activity of 4Box was functional in permeabilized cells (Fig. 4, h and i). In cells dialyzed with P1–33 (10 nM) and 4Box (1 nM) through a patch pipette, at concentrations at which neither had activity on its own, the combination fully inhibited carbachol-evoked Ca\(^{2+}\) signaling (data not shown). Thus, the peptide P1–33 and 4Box mutually influenced the activity of each other in cells. We propose the P1–33 peptide facilitates 4Box interaction with the receptor complex (reflected by enhanced potency) and the 4Box alters the P1–33 binding site to relieve GTPγS-resistant inhibition. The analysis in Fig. 4 suggests two separate regions of RGS4 interact with the receptor complex to regulate Ca\(^{2+}\) signaling. A combination of the N-terminal peptide P1–33 and 4Box reconstituted the essential functions of RGS4, those of receptor specificity and GAP activity. Sequence divergence in the N terminus of different RGS proteins may convey regulatory specificity toward other receptor complexes, and this specificity may be retained in peptides analogous with P1–33.

Conclusions—The N-terminal domain and RGS box of RGS4 cooperate via at least two discrete mechanisms to convey receptor-selective inhibition of G protein signaling. Inhibition of Ca\(^{2+}\) signaling by RGS4 was receptor-selective whereas inhibition by 4Box was not. Receptor selectivity of RGS4 inhibition was not influenced by the identity of the Goq class a subunit. Such selectivity suggests intact RGS4 interacts directly or indirectly with receptors, most likely through its N terminus. This is supported by the finding that RGS4 in patch-clamped cells was 10,000-fold more potent than 4Box and RGS4 had 125-fold higher Goq-GAP activity than did 4Box in an agonist-dependent in vitro assay. Finally, partial inhibition by RGS4 simultaneously decreased both the amplitude of the initial Ca\(^{2+}\) signal and the frequency of subsequent oscillations, suggesting recombinant RGS4 protein is active when dialyzed into patch-clamped cells and can preassemble with the receptor-Goq

PLCβ signaling complex. By contrast, 4Box preferentially inhibited signaling following the initial Ca\(^{2+}\) release evoked by carbachol and CCK. This suggests that 4Box is recruited only to active receptor complexes when production of the Goq-GTP substrate would be greatest, and thereby 4Box GAP activity would be most pronounced.

Interaction of the N terminus with components of the receptor-Goq-PLCβ signaling complex is further supported by the ability of the N-terminal peptide P1–33 to inhibit Ca\(^{2+}\) signaling in the absence of 4Box. Inhibition by P1–33 displayed the same selectivity among receptors as displayed by full-length RGS4. In contrast to RGS4, however, inhibition by P1–33 was not overcome by GTPγS, possibly because P1–33 blocked access of Goq-GTPγS to its effector protein PLCβ. Cooperation between the N terminus and 4Box is most apparent in that a combination of both P1–33 and 4Box restored the regulatory activity of intact RGS4 in cells, including its reversibility by GTPγS. Thus, the N terminus provides anchorage (4, 22) and receptor selectivity whereas the RGS box acts as a GAP, and both domains combine to yield the behavior of the intact protein.

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