RGS4 Inhibits Gq-mediated Activation of Mitogen-activated Protein Kinase and Phosphoinositide Synthesis*

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Recombinant regulators of G protein-signaling (RGS) proteins stimulate hydrolysis of GTP by α subunits of the Gα family but have not been reported to regulate other G protein α subunits. Expression of recombinant RGS proteins in cultured cells inhibits Gαi-mediated hormonal signals probably by acting as GTPase-activating proteins for Gαi subunits. To ask whether an RGS protein can also regulate cellular responses mediated by G proteins in the Gq/11 family, we compared activation of mitogen-activated protein kinase (MAPK) by a Gq/11-coupled receptor, the bombesin receptor (BR), and a Gαi-coupled receptor, the D2 dopamine receptor, transiently co-expressed with or without recombinant RGS4 in COS-7 cells. Pertussis toxin, which uncouples Gαi from receptors, blocked MAPK activation by the D2 dopamine receptor but not by the BR. Co-expression of RGS4, however, inhibited activation of MAPK by both receptors causing a rightward shift of the concentration-effect curve for both receptor agonists. RGS4 also inhibited BR-stimulated synthesis of inositol phosphates by an effectors of Gq/11, phospholipase C. Moreover, RGS4 inhibited inositol phosphate synthesis activated by addition of AlF4− to cells overexpressing recombinant αq, probably by binding to αq-GDP-AlF4−. These results demonstrate that RGS4 can regulate Gq/11-mediated cellular signals by competing for effector binding as well as by acting as a GTPase-activating protein.

Heterotrimeric G proteins transduce extracellular signals detected by transmembrane receptors into appropriate cellular responses (1, 2). The intensity and duration of these responses depend on the relative rates of biochemical reactions that turn G proteins on and off. The G protein switch turns on when receptors promote replacement of GTP for GDP bound by α subunits of αβγ trimers, leading to dissociation of active GoαGTP from the βγ dimer and consequent regulation of downstream effectors. A GTPase activity intrinsic to α subunits turns off signals by converting αGTP to inactive GoαGDP, which then binds to and inactivates βγ. For pure Goα subunits in vitro the turnoff reaction is slow, ≤4 min−1 (2). In contrast, many G protein-mediated physiological responses must turn off much more rapidly, in fractions of a second.

Two classes of GTPase-activating protein (GAP)1 have been reported to accelerate deactivation of trimeric G proteins. One class includes G protein effectors, such as phospholipase C (PLC) and the cGMP phosphodiesterase γ subunit, which stimulate GTP hydrolysis by αq and αi, respectively (3, 4). Recent investigations have discovered and characterized a second class of Go-GAPs, the RGS (regulators of G protein signaling) proteins. Pure recombinant RGS proteins display GAP activities for certain G protein α subunits (5–9). RGS proteins of mammals (8–12), yeast (13, 14), and Caenorhabditis elegans (11) share a conserved RGS domain and apparently share similar mechanisms of action. Indeed, a mammalian RGS can partially complement yeast mutations that inactivate Sst2p, the RGS of Saccharomyces cerevisiae (12).

Mammalian RGS proteins thus far examined appear to act selectively as GAPs for Gαq proteins in the αq family (5–8), including α5, α6, and αq12, and most recently αq11 (9). Transient expression of RGS4 in HEK293 cells inhibits Gq-mediated activation of MAP kinase (MAPK) in response to stimulation of the interleukin-8 receptor (12). In the yeast two-hybrid system, in vitro binding, and co-immunoprecipitation assays, RGS4 interacts with αi family proteins but not with αq or αi12 (5–8, 10).

Recent experiments indicate that RGS4 can interact with αq11 proteins albeit less efficiently than with αq. A high concentration of αq-GDP bound to AlF4− can inhibit the GAP activity of RGS4 for αq-GTP, presumably because αq-GDP-AlF4− competes against αq-GTP for binding the RGS protein (6). Moreover, RGS4 can stimulate the GTPase activity of αq in reconstituted vesicles (15). It is not known, however, whether RGS proteins can serve in intact cells as αq-GAPs and inhibitors of Gq-mediated cellular signals. Here we use expression of recombinant RGS4 in COS-7 cells to show that RGS4 can inhibit cellular signals mediated by Gq/11.

EXPERIMENTAL PROCEDURES

DNA Constructs and Transfection of COS-7 Cells—FLAG-tagged human RGS4 cDNA was a generous gift from John H. Kehrl at the Laboratory of Immunoregulation, NIAID, National Institutes of Health, Bethesda, MD. cDNA constructs for the bombesin receptor (BR), D2 dopamine receptor (D2R), and the β2-adrenoceptor were as described (16). Chinese hamster cDNA encoding an HA-tagged p44 MAPK was a gift from J. Poupasguer, Nice, France. pcDNA I and pCR3 were from Invitrogen, San Diego.

COS-7 cells were maintained in Dulbecco’s modified Eagle’s H21 medium with 10% calf serum. DNA was transfected with adenovirus and DEAE-dextran as described (17). Transfection efficiencies were determined by co-transfection of the plasmid pON249 encoding β-galactosidase and assayed as described (17). Expression was consistently

1 The abbreviations used are: GAP, GTPase-activating protein; PLC, phospholipase C; G protein, heterotrimeric guanine nucleotide-binding protein; RGS, regulator of G protein signaling; MAPK, mitogen-activated protein kinase; BR, bombesin receptor; D2R, D2 dopamine receptor; PTX, pertussis toxin; MBP, myelin basic protein; IP, inositol phosphates; InsP3, inositol 1,4,5-trisphosphate; HA, hemagglutinin.

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RGS4 inhibits G<sub>i</sub>- and G<sub>Gq/11</sub>-coupled receptors. Cells were transfected with plasmids encoding HA-MAPK (1 μg), BR (1 μg, panel A) or D<sub>R</sub> (1 μg, panel B), and RGS4 (2 μg) or vector plasmid pCR3 (2 μg) and treated for 4 h with or without PTX as indicated. Cells were then exposed to 1 nM bombesin (A) or 10 nM quinpirole (B) for 10 min as indicated, and HA-MAPK activities were determined. HA-MAPK activities are expressed in arbitrary units of MBP phosphofluorescence (see “Experimental Procedures”). Data represent the mean ± S.D. of triplicate determinations; an additional experiment gave similar results.

**RESULTS AND DISCUSSION**

**RGS4 Inhibits G<sub>i</sub>-dependent MAPK Activation by Bombesin**—To assess effects on an RGS protein on cellular responses mediated by G proteins in the G<sub>i</sub> family, we compared MAPK activation by a G<sub>i</sub>-coupled receptor, BR, and a G<sub>i</sub>-coupled receptor, D<sub>R</sub>, that co-expressed with or without recombinant RGS4 in COS-7 cells (Figs. 1 and 2). Expression of RGS4 blocked MAPK activation by the G<sub>i</sub>-coupled receptor agonist, bombesin (1 nM); PTX, which specifically blocks signaling by receptors that activate G<sub>i</sub> proteins, did not inhibit the effect of bombesin (Fig. 1A). These results suggest that RGS4 inhibited bombesin signaling to MAPK by inhibiting the action of a G protein other than G<sub>i</sub>, probably G<sub>i11</sub>. This inference was supported by additional experiments described below. Confirming a previous report (12) that recombinant RGS4 can inhibit G<sub>i</sub>-mediated signals in an intact cultured cell, RGS4 markedly inhibited G<sub>i</sub>-dependent MAPK activation by the D<sub>R</sub> agonist, quinpirole (10 nM, Fig. 1B); PTX also blocked the D<sub>R</sub> effect on MAPK (Fig. 1B). RGS4 did not alter the expression of HA-MAPK in these experiments (data not shown).

To further assess the effectiveness of RGS4 in blocking cellular responses mediated by G<sub>i11</sub> and G<sub>i</sub>, we measured MAPK activation by a range of concentrations of both agonists (Fig. 2). In both cases, a higher concentration of each agonist was required to produce equivalent MAPK activation in cells expressing RGS4, that is expression of RGS4 shifts the agonist concentration–effect curve to the right. In RGS4-expressing cells, the apparent EC<sub>50</sub> for bombesin (Fig. 1A) indicating a relatively greater effectiveness of RGS4 for inhibiting the G<sub>i</sub>-mediated effect in comparison with that mediated by G<sub>i11</sub>. The apparent difference in potency could reflect different mechanisms by which RGS4 inhibits the two effects, but it is also consistent with a simpler interpretation that the RGS protein catalyzes GTP hydrolysis less efficiently with α<sub>i11</sub> than with α<sub>i</sub> proteins.

The RGS-induced rightward shift of signaling concentration–effect curves (Fig. 2) is predictable from the GAP mechanism of RGS action. When a GAP increases the rate of GTP hydrolysis,
Fig. 3. RGS4 inhibits both G<sub>i</sub> and G<sub>q</sub>-mediated activation of MAPK in a dose-dependent manner. Panels A and B, cells were transfected with plasmids encoding HA-MAPK (1 µg), 1 µg each of BR and DR, and the indicated amounts of RGS4. Vector plasmid pCR3 was added to keep the total amount of DNA constant. Cells were exposed for 10 min to 1 nM bombesin or 10 nM quinpirole, and HA-MAPK activities were determined. HA-MAPK activities are expressed in arbitrary units of MBP phosphofluorescence (see “Experimental Procedures”). Data represent the mean ± S.D. of triplicate determinations; two additional experiments gave similar results. Panel C, immunoblots of FLAG-tagged RGS4 and HA-tagged MAPK expressed in cells used in the MAPK assays shown in panels A and B. Total proteins from cell lysates were resolved in 14% polyacrylamide gels and transferred to nitrocellulose membranes. After blotting with M<sub>2</sub> FLAG antibody, the membranes were stripped and probed a second time with the 12CA5 antibody, directed against the HA tag. Blots were developed with an ECL kit (Amersham).

equivalent steady-state concentrations of G<sub>q</sub>-GTP can only be achieved by an increased rate of receptor-catalyzed GTP-for-GDP exchange and thus by higher concentrations of agonist-occupied receptor. The extent of an RGS-induced rightward shift would be limited by the k<sub>d</sub> of the agonist ligand for the relevant receptor. Although a GAP-induced shift in concentration-response curves has not been previously documented, it would be an attractive way to fine tune responsiveness of cells to extracellular stimuli. In phospholipid vesicles reconstituted with a M<sub>2</sub>-muscarnic acetylcholine receptor and G<sub>q</sub>, the EC<sub>50</sub> of carbachol for stimulating GTP hydrolysis was increased by addition of purified PLCβ1, an α<sub>G</sub>-GAP; in this case, addition of the GAP also markedly increased the maximal rate of GTP hydrolysis (3, 20).

To determine whether similar concentrations of cellular RGS4 are required to inhibit G<sub>i</sub>- and G<sub>q</sub>-dependent hormonal signals, we transfected cells with graded amounts of RGS4 plasmid (Fig. 3), which produced graded cellular amounts of RGS4 protein (Fig. 3C). RGS4 inhibited G<sub>i</sub>- and G<sub>q</sub>-mediated elevation of MAPK activity with similar dose-effect curves over a 16-fold range of transfected DNA (Fig. 3, A and B). Although the endogenous amounts of cellular RGS proteins are unknown, this result argues that similar amounts of RGS4 protein are required to produce both inhibitory effects. It is unlikely that all RGS proteins exhibit quantitatively similar abilities to inhibit G<sub>i</sub>- and G<sub>q</sub>-dependent hormonal signals. Indeed, another member of the RGS protein family, GAIP, inhibited G<sub>q</sub>-mediated activation of MAPK much more effectively than that mediated by G<sub>q</sub><sub>11</sub>.

RGS4 inhibits accumulation of second messengers in response to agonists for receptors coupled to G<sub>q</sub><sub>11</sub> and G<sub>q</sub>. A, cells were transfected with plasmids encoding BR (1 µg), plus RGS4 (2 µg), or vector plasmid pCR3 (2 µg) and labeled with myo-[<sup>3</sup>H]inositol. Cells were treated for 45 min with the indicated concentration of bombesin in medium containing 5 mM LiCl, and total cellular IP content was determined. B, cells were transfected with plasmids encoding the β<sub>2</sub>-adrenoreceptor (1 µg), plus RGS4 (2 µg), or vector plasmid pCR3 (2 µg) and labeled with [<sup>3</sup>H]adenine. Cells were treated for 30 min with the indicated concentration of isoproterenol, and CAMP accumulation was determined. Data represent the mean ± S.D. of triplicate determinations; two additional experiments gave similar results.

Fig. 4. RGS4 inhibits accumulation of second messengers in response to agonists for receptors coupled to G<sub>q</sub><sub>11</sub> and G<sub>q</sub>. A, cells were transfected with plasmids encoding BR (1 µg), plus RGS4 (2 µg), or vector plasmid pCR3 (2 µg) and labeled with myo-[<sup>3</sup>H]inositol. Cells were treated for 45 min with the indicated concentration of bombesin in medium containing 5 mM LiCl, and total cellular IP content was determined. B, cells were transfected with plasmids encoding the β<sub>2</sub>-adrenoreceptor (1 µg), plus RGS4 (2 µg), or vector plasmid pCR3 (2 µg) and labeled with [<sup>3</sup>H]adenine. Cells were treated for 30 min with the indicated concentration of isoproterenol, and CAMP accumulation was determined. Data represent the mean ± S.D. of triplicate determinations; two additional experiments gave similar results.

RGS4 reduces accumulation of inositol phosphates stimulated by bombesin—If RGS4 inhibits BR stimulation of MAPK activity by inactivating G<sub>q</sub><sub>11</sub>, the RGS protein should also reduce BR-stimulated synthesis of IP by PLC, the principal effector of G<sub>q</sub><sub>11</sub>. Indeed, expression of RGS4 reduced bombesin-induced IP accumulation by about 50% (Fig. 4A). The inhibitory effect of RGS4 was probably exerted on G<sub>q</sub><sub>11</sub> rather than on G<sub>q</sub>, because PTX failed to inhibit BR-induced IP accumulation (not shown). The BR is likely to stimulate IP accumulation via the α subunit of G<sub>q</sub><sub>11</sub> rather than via its βγ subunit because PLCβ1 and PLCβ3, the G protein-responsive PLC isozymes of COS cells, are sensitive to G<sub>q</sub><sub>11</sub> stimulation but relatively insensitive to stimulation by G<sub>q</sub><sub>11</sub>.2 COS cells lack PLCβ2, the PLC isozyme that is most sensitive to βγ (21).

RGS4 reduced maximal stimulation of IP accumulation by BR stimulation but did not alter the EC<sub>50</sub> for bombesin (Fig. 4A). In contrast, RGS4 expression did not affect maximal activation of MAPK by bombesin but did cause a rightward shift of the bombesin concentration-effect curve (Fig. 2A). How could the relations between agonist concentration and response be different, if as seems likely, both BR responses are mediated by G<sub>q</sub><sub>11</sub> and stimulation of PLC? Although we do not know the reason for this discrepancy, the two assays were performed

2 P. P. Chi, unpublished result.
expression of RGS4 with Goq substantially inhibited IP accumulation in response to AlF4− (Fig. 5). RGS4 presumably inhibited effector stimulation in this case not by accelerating GTP hydrolysis but by binding to and sequestering Goq-GDP-AlF4−. RGS4 also decreased the elevation of MAPK activity seen in untreated cells transfected with Goq (not shown), an effect that probably reflects acceleration of GTP hydrolysis by Goq.

In summary, we present two new sets of observations. While RGS proteins are known to inhibit signals mediated by Goq, we show for the first time that an RGS protein can interact with Goq and inhibit signals transduced by Goq in intact cells. RGS4 probably inhibits bombesin responses by acting as a GAP, that is, by stimulating the intrinsic GTPase activity of Goq. Our experiments also raise the possibility that RGS4 inhibits bombesin responses by sequestering the GTP-bound active conformation of Goq (that is, by the mechanism that probably inhibits the AlF4− response), in addition to stimulating GTP hydrolysis.

Second, we found that RGS4 induces rightward shifts in concentration-effect curves for agonists acting on receptors coupled to either Gq or Goq. It is likely that other RGS proteins modulate hormonal signals mediated by Gq and Goq in much the same way. For each response, the extent of the rightward shift will depend on the local concentration of RGS protein and its relative affinity for the G protein involved. Thus different complements of RGS proteins could allow two cells to mount quantitatively different responses to the same concentration of a physiological agonist even when both cells use the same receptors and G proteins. If the relevant receptor couples to two distinct G proteins (for instance, to Gq and Gq or to Gq and Goq), differing cellular complements of RGS proteins with distinct Go selectivities could even produce qualitatively different responses of two cells to the same agonist.

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