Regulators of G protein signaling (RGS) constitute a family of proteins with a conserved RGS domain of ~120 amino acids that accelerate the intrinsic GTP hydrolysis of activated Goq and Goα subunits. The phosphorylation-dependent interaction of 14-3-3 proteins with a subset of RGS proteins inhibits their GTPase-accelerating activity in vitro. The inhibitory interaction between 14-3-3 and RGS7 requires phosphorylation of serine 434 of RGS7. We now show that phosphorylation of serine 434 is dynamically regulated by TNF-α. Cellular stimulation by TNF-α transiently decreased the phosphorylation of serine 434 of RGS7, abrogating the inhibitory interaction with 14-3-3. We examined the effect of 14-3-3 on RGS-mediated deactivation kinetics of G protein-coupled inwardly rectifying K+ channels (GIRKs) in Xenopus oocytes. 14-3-3 inhibited the function of wild-type RGS7, but not that of either RSG7P436R or RGS4, two proteins that do not bind 14-3-3. Our findings are the first evidence that extracellular signals can modulate the activity of RGS proteins by regulating their interaction with 14-3-3.

G protein-coupled receptors activate heterotrimeric G proteins by enhancing GDP release and binding of GTP to the Ga subunit. By accelerating the inactivation of GTP-bound Goq and Goα subunits, the “regulator of G protein signaling” (RGS) proteins negatively regulate the strength and duration of G protein signaling. RGS proteins act catalytically; a single molecule binds and inactivates multiple GTP-bound Go subunits. Functional specificity and temporal activity of RGS proteins arise from patterns of expression, transcriptional regulation, subcellular localization, and posttranslational modification (1). Whereas some RGS proteins are ubiquitously expressed, others are temporally and spatially restricted to certain tissues and developmental stages or are induced by cellular activation (reviewed in Refs. 2 and 3). Interaction with other cytoplasmic proteins affects the stability, function, and subcellular localization of RGS proteins (reviewed in Refs. 4 and 5).

We have previously shown that binding to 14-3-3 modulates the activity of certain RGS proteins, including RGS7, in vitro (6). These RGS proteins contain a conserved SYP motif with upstream basic residues located in one of the three contact sites formed between Goq and the RGS domain. Binding of 14-3-3 to the SYP motif of RGS7 requires the phosphorylation of the serine residue at position 434. In this study, we demonstrate that TNF-α inhibits the phosphorylation of serine 434 and the interaction of RGS7 with 14-3-3. RGS7 is highly expressed in the mouse brain, with a substantial fraction associated with 14-3-3. Treatment of mice with TNF-α completely abrogated the interaction of RGS7 with 14-3-3. We examined the effect of 14-3-3 on RGS-mediated deactivation kinetics of G protein-coupled inwardly rectifying K+ channels (GIRKs) in the presence of wild-type RGS7, but had no effect in the presence of either RSG7P436R or RGS4, two proteins that do not associate with 14-3-3. Our findings demonstrate that the activity of RGS7 is regulated by extracellular signals.

EXPERIMENTAL PROCEDURES

Plasmids—FLAG-tagged versions of human RGS7, RGS3, and 14-3-3 have been described previously (6). FLAG-tagged RGS4 and RGS8 were cloned from human cDNA libraries generated by standard cloning procedures. Site-directed mutagenesis was used to insert mutations in RGS7. Point mutations were verified by sequence analysis. M1 and M2 muscarinic receptors and the GIRK constructs were kind gifts of Dr. Silvio Gutkind, Dr. Melanie Mark, and Dr. Ernest Peralta. Generation of a Phospho-specific Phosphoserine 434 Antiserum—A phosphopeptide corresponding to residues 429–451 of human RGS7 (acetyl-CLMKSdpSYPRFIRS-amide) was conjugated to keyhole limpet hemocyanin and used to generate polyclonal antiserum (Quality Controlled Biochemicals, Hopkinton, MA). The rabbit antisem was preabsorbed three times with the corresponding non-phosphopeptide coupled to an agarose column. Specificity and titer were monitored by Western blotting and enzyme-linked immunosorbent assay, respectively, using phospho- and non-phosphopeptide conjugated to BSA. Phospho-specific antiserum was affinity-purified by passing the flow-through of the third preabsorption over a phosphopeptide column. Immunoprecipitation and Immunoblotting—Immunoprecipitation experiments were performed as described (6). Briefly, HEK 293T cells were transiently transfected by the calcium phosphate method. After incubation for 24 h, untreated cells and cells treated with 10 ng/ml TNF-α were washed twice and lysed in 1% Triton X-100 lysis buffer. After centrifugation (15,000 × g, 15 min, 4 °C), cell lysates containing equal amounts of total protein were incubated for 1 h at 4 °C with the appropriate antibody followed by incubation with 40 μl of protein G-Sepharose (for mouse monoclonal antibodies) or protein A-Sepharose (for rabbit polyclonal antisera) (Amersham Biosciences) for ~3 h. Following extensive washing with lysis buffer, protein complexes were separated by SDS-PAGE, transferred to nitrocellulose membrane, and following primary and secondary antibody incubation, detected by chemiluminescence.

Pull-down Assay—HEK 293T cells were transiently transfected with...
FIG. 1. Generation of a phospho-specific RGS7 antiserum. A, structural elements of RGS7 include a DEP domain (dishevelled/EGL-10/pleckstrin), a Gγ-like (GGL) domain, an RGS (regulator of G protein signaling) domain, and a 14-3-3 binding motif consisting of (K/E)-(R/K)-D-pS-Y-P (6). B, the RGS7 phosphopeptide CLMKSDpSYPRFIRS was used to generate an antiserum that specifically recognizes RGS7 when phosphorylated on serine 434. Western blotting analysis using the phospho-specific RGS7 antiserum against dilutions of phosphorylated RGS7 peptide coupled to BSA, non-phosphorylated RGS7 peptide coupled to BSA, and BSA alone. C, PKCα catalyzes the incorporation of phosphate into a truncation of RGS7 fused to maltose-binding protein (MBP.RGS7315–469). D, GST, lacking potential PKC phosphorylation sites, is not phosphorylated by PKCα in vitro. Since the RGS7315–469 truncation contains several potential PKC sites (Ser434, Ser442, Ser456, Thr458, and Ser468), both wild-type GST.RGS7315–469 and the S434D mutant of GST.RGS7315–469 are phosphorylated by PKCα with no discernible difference by autoradiography. The faint band at 83 kDa represents autophosphorylated PKCα. Equal loading of recombinant proteins was verified by staining the protein gel with Coomassie Blue, as depicted in the lower panel. E, the phospho-specific RGS7 antiserum recognizes wild-type MBP.RGS7315–469, but not the S434D mutation of MBP.RGS7315–469 after phosphorylation with PKCα. Equal loading of recombinant proteins was verified by staining the protein gel with Coomassie Blue, as depicted in the lower panel. F, the phospho-specific RGS7 antiserum immunoprecipitates phosphorylated, but not non-phosphorylated, GST.RGS7315–469 nor GST alone. Precipitated proteins were detected with anti-GST antisera (Amersham Biosciences).
TNF-α regulates phosphorylation of serine 434 of RGS7 and binding to 14-3-3. A, TNF-α reduces serine 434 phosphorylation of RGS7. HEK 293T cells were transfected with FLAG-tagged RGS7 or RGS3. Despite an increase of F.RGS7 protein levels after a 1-h incubation with TNF-α (left panel), virtually no RGS7 was precipitated by the phospho-specific RGS7 antiserum (right panel), demonstrating that TNF-α reduces phosphorylation of RGS7 on serine 434. Precipitated proteins were detected with the FLAG-specific M2 antibody. The phospho-specific RGS7 antiserum is highly specific for RGS7 and did not precipitate FLAG-tagged RGS3, a control protein that contains the SYP motif and binds 14-3-3 (right panel). B, TNF-α abrogates binding of 14-3-3 to RGS7. HEK 293T cells were transfected with FLAG-tagged RGS7 and Myc-tagged 14-3-3. Despite progressively increasing F.RGS7 protein levels (lysates, top panel), the amount of F.RGS7 precipitated by the phospho-specific RGS7 antiserum drastically declines after 30 min of TNF-α stimulation (IP, middle panel); precipitated proteins were detected with the FLAG-specific M2 antibody. The diminished serine 434 phosphorylation observed at 30 min coincides with decreased co-precipitation of 14-3-3 with RGS7 (IP, bottom panel). Following precipitation of F.RGS7 by FLAG-specific M2 antibody, co-precipitating Myc.14-3-3 was detected using an anti-Myc antibody. C, binding of RGS7 to recombinant GST.14-3-3 is diminished by TNF-α. HEK 293T cells were transfected with a vector control or FLAG-tagged RGS7. Cell lysates were incubated with GST.14-3-3 immobilized on glutathione-Sepharose. Bound FLAG-tagged RGS7 was detected by Western blot analysis, using the FLAG-specific M2 antibody. GST.14-3-3 immobilized RGS7 from resting cells, but failed to bind significant amounts of RGS7 from cells stimulated with TNF-α. D, TNF-α transiently decreases association of 14-3-3 to RGS7. HEK 293T cells, transfected with FLAG-tagged RGS7 and Myc-tagged 14-3-3, were stimulated with TNF-α for the indicated times. Following precipitation of F.RGS7 by FLAG-specific M2 antibody, co-precipitating Myc.14-3-3 was detected using an anti-Myc antibody. Decreased binding of 14-3-3 to RGS7 is evident at 30 and 60 min. E, TNF-α decreases serine 434 phosphorylation of RGS7. HEK 293T cells were transfected with RGS7 tagged with YFP. Cellular lysates were assessed by Western blot analysis with phosphoserine-specific antiserum to detect dephosphorylation at serine 434 induced by TNF-α. A longer form of RGS7 was used to avoid interference from a nonspecific 60-kDa band arising from immunoblotting with the phosphoserine-specific antiserum.

plasmid DNA as indicated. After stimulation, cells were lysed in 1% Triton X-100, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM NaF, 15 mM Na₃PO₄, 2 mM Na₂VO₃, 1 mM EDTA, and protease inhibitors for 15 min on ice. Following centrifugation, the supernatant was incubated for 1 h at 4 °C with 4–8 μg of recombinant purified GST.14-3-3γ prebound to glutathione-Sepharose (Amersham Biosciences). Bound proteins were separated by SDS-PAGE, and precipitated proteins were visualized by immunoblotting with anti-FLAG antibody (Eastman Kodak Co.). Equal
loading of GST.14-3-3 γ was confirmed by Coomassie Blue staining of the gels.

Immunoprecipitation of Mouse Brain—For preparation of brain protein extracts, female BALB/c mice (20 g in body weight, Charles River) were injected intravenously in the tail vein with solvent or 20 μg of murine TNF-α. After 4 h, the mice were sacrificed and brains were removed and homogenized essentially as described (1). Following centrifugation and ultracentrifugation (100,000 × g, 4 °C, 30 min), the supernatant was subjected to several preclamping steps, divided into two fractions, and immunoprecipitated with specific anti-RGS7 antisera (rabbit polyclonal antisera generated against MBP-RGS7170–469 and affinity-purified with GST-RGS7315–469) and control antibody, followed by incubation with protein G-Sepharose. Resulting precipitates were subjected to immunoblot analysis with anti-14-3-3 monoclonal antibody (Santa Cruz) followed by incubation with horseradish peroxidase-coupled secondary antisera and enhanced chemiluminescence.

In Vitro Phosphorylation—One microgram of purified GST. RGS7170–469 or MBP.RGS7315–469 was incubated with 0.5 unit of recombinant protein kinase C (α (1850 units/mg, Panvera) in enzyme dilution buffer or enzyme dilution buffer alone (control) for 30 min or the indicated times at 37 °C in a 100-μl reaction. Final concentrations of reagents were 20 mM HEPES, pH 7.4, 10 mM MgCl2, 0.1 mM CaCl2, 100 μM ATP, 20 μg/ml dacylglycerol, 100 μg/ml phosphatidylserine, 0.03% Triton X-100. To monitor the incorporation of phosphate, the unlabeled ATP was supplemented with 10 μCl of [γ32P]ATP. Radiolabeled MBP.RGS7170–469 or GST.RGS7315–469 was detected by autoradiography following SDS-PAGE or by scintillation counting after binding to nitrocellulose filter.

Preparation and Injection of Oocytes—Xenopus laevis frogs were anesthetized (4 mM aminobenzoic acid ethyl ester for 25 min), and oocytes were isolated by partial ovarectomy. Subsequently, oocytes were manually dissected from ovarian lobes and defolliculated by treatment with 1 mg/ml collagenase type A (Roche Molecular Biochemicals, Mannheim, Germany) in a Ca2+-free hypotonic solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM HEPES, pH 7.4). Oocytes were washed and transferred to ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, 2.5 mM sodium pyruvate, pH 7.4, supplemented with 50 μM gentamicin and 0.5 mM theophylline. cRNAs were synthesized in vitro using the mMessage Machine kit (Ambion). 12–24 h after isolation, stage V-VI oocytes were injected with 30 nl of water containing 1 ng of cRNA for the M2 receptor, 0.1 ng for GIRK1 and GIRK2, and 10 ng for the different RGS proteins. Control oocytes were injected with water instead of RGS-cRNAs. Voltage clamp experiments were performed 2–4 days after injection of cRNAs. Recombinant proteins were injected into the oocytes 12 h prior to the experiments. 30 nl of GST (0.5 mg/ml) and GST.14-3-3 γ (1 mg/ml) were injected resulting in a final cytosolic concentration of ~500 nM.

Electrophysiology—Standard two-electrode voltage clamp was used to assess the RGS protein function in vivo. Whole cell currents of oocytes were recorded using the Turbo Tec O3X voltage/current clamp amplifier (NPI Electronic, Tamm, Germany). Microelectrodes were pulled on a vertical puller (Physiologisches Institut, Freiburg, Germany) from borosilicate glass capillaries (Clark Instruments, Reading, United Kingdom) and had resistances of 0.5–2 MΩ when filled with 2 mM KCl solution. Experiments were performed at 20 °C. All whole cell voltage clamp experiments were conducted in ND96 solution or a modified ND96-K80 solution in which 80 mM of NaCl were replaced with KCl. Acetycholine (1 μM) and atropine (1 μM) were added to the ND96-K80 solution to activate and antagonize the M2 receptor. Oocytes were clamped at 0 mV, and voltage ramps from −100 to +50 mV were applied every 1.2 s. Time constants for current decrease after removal of acetylcholine were fitted with a single exponential (Origin, Additive, Germany). Data are presented as original recordings and as mean ± S.E. (n = number of experiments). Unpaired Student’s t test was used for statistical analysis. A p value of <0.05 was accepted to indicate statistical significance.

RESULTS

Generation of a Phosphoserine 434-specific Antiserum—The serine at position 434 of RGS7 constitutes a 14-3-3 binding site when phosphorylated (Fig. 1A) (6). To monitor the phosphorylation status of serine 434, a phosphopeptide containing phosphorylated serine 434 was used to generate polyonal anti-
Fig. 5. 14-3-3 inhibits the RGS7-mediated acceleration of GIRK deactivation kinetics in Xenopus oocytes. Xenopus oocytes were microinjected with cRNA encoding for the G protein-activated inwardly rectifying K+ channels subunits GIRK1 and GIRK2, the M2 acetylcholine receptor (M2 AChR), and RGS7 (or water in control oocytes) as indicated. GIRK currents were recorded using the two-electrode voltage-clamp technique. A, current-voltage relations of an oocyte expressing GIRK1/2 and the M2 AChR in the presence of different extracellular solutions: control, ND96; 80 mM K+, ND96-K80; and ACh 1 μM, acetylcholine (1 μM) added to ND96-K80. B, representative experiment of an oocyte expressing GIRK1/2 and M2 AChR. Oocytes were clamped at 0 mV, and voltage ramps from −100 to +50 mV were applied every 1.2 s. C, deactivation kinetics of control oocytes and oocytes expressing RGS7 (±RGS7). Deactivation time constants were derived from single exponential fits of the GIRK current deactivation phase from control oocytes or oocytes expressing RGS7 following acetylcholine removal (holding potential −90 mV). D, comparison of deactivation time constants of control oocytes and oocytes expressing RGS7. Expression of RGS7 significantly decreased the deactivation time constant of acetylcholine-evoked currents. E, microinjection of GST.14-3-3 protein, but not GST, inhibited the RGS7-mediated acceleration of GIRK1/2 deactivation after withdrawal of acetylcholine.

Dissociation with 14-3-3—We have previously shown that binding to 14-3-3 modulates the activity of certain RGS proteins, including RGS7 (6). To determine whether TNF-α mediates the accumulation of functionally active RGS7, we monitored serine 434 phosphorylation of RGS7 in transfected HEK 293T cells. Although lysates of TNF-treated cells contain more RGS7 than untreated cells (Fig. 2A, left panel), precipitation of RGS7 by the phospho-specific antiserum is substantially decreased (Fig. 2A, right panel). The phospho-specific antiserum was used to precipitate phosphoserine 434-containing RGS7; precipitated protein was then detected using the FLAG-specific M2 antibody. After 30-min stimulation with TNF-α, the accumulation of RGS7 is accompanied by decreased phosphorylation of serine 434 and marked dissociation with 14-3-3 (Fig. 2B). These results are supported by pull-down experiments using a recombinant GST.14-3-3 fusion protein immobilized on glutathione-Sepharose. Treatment of cells with TNF-α both rapidly and greatly reduced the amount of RGS7 retained on a GST.14-3-3 column, compared with untreated cells (Fig. 2C). Fig. 2D demonstrates the transient capacity of TNF-α to dissociate RGS7 and 14-3-3. In transfected HEK 293T cells, binding of RGS7 to 14-3-3 is inhibited after 30- and 60-min treatment with TNF-α, but reassociation is evident at 120 min. The dephosphorylation of RGS7 induced by TNF-α occurs rapidly. As shown in Fig. 2E, treatment with TNF-α reduced serine 434 phosphorylation of RGS7 at 15 and 30 min. These results indicate that TNF-α induces dephosphorylation of serine 434 and transiently re-

serum. To affinity-purify antiserum that specifically recognized phosphorylated serine 434, phosphorylated and non-phosphorylated peptides were covalently coupled to agarose. After several rounds of positive and negative selection, the phospho-specific antisera recognized less than 1 pmol of phosphorylated serine 434, phosphorylated and non-phosphorylated RGS7-derived peptide, but did not bind non-phosphorylated peptide nor BSA (Fig. 1B). Serine 434 can be phosphorylated by several protein kinase C isozymes (PKC α, β, γ, ε, μ, γ; scansite.mit.edu), Motif Scanner at low stringency. To test whether phosphorylated RGS7 was specifically recognized by the phospho-specific antiserum, the RGS7 truncation containing the RGS domain (RGS7Δ115–469) was fused to either maltose-binding protein (MBP) or GST and phosphorylated with PKC α (Fig. 1, C–E). Due to the presence of multiple potential PKC phosphorylation sites (Ser442, Ser464, Thr458, Ser468), the overall level of phosphorylation is unchanged by the mutation of serine 434 to aspartic acid (Fig. 1D). However, the phospho-specific antiserum specifically recognized phosphorylated, but not non-phosphorylated RGS7Δ115–469 nor the S434D mutation of RGS7Δ115–469 (Fig. 1E). Furthermore, the antiserum specifically immunoprecipitates phosphorylated RGS7Δ115–469 fused to GST, but not a non-phosphorylated GST-RGS7Δ115–469 fusion protein nor GST alone (Fig. 1F). These experiments demonstrate the specificity of the antiserum for phosphorylated serine 434 of recombinant RGS7 in both immunoblotting and immunoprecipitation.

TNF-α Causes D Dephosphorylation of Serine 434 of RGS7 and...
leaves RGS7 from its inhibitory interaction with 14-3-3.

**TNF-α Inhibits Binding of 14-3-3 to RGS3**—To examine whether TNF-α modulates the association between 14-3-3 and other SYP-containing RGS members, we assessed the interaction between 14-3-3 and RGS3 after stimulation with TNF-α. RGS3 belongs to a subset of RGS family members that bind 14-3-3 (6). It is a stable protein that does not accumulate in response to TNF-α (1). As shown in Fig. 3 (left panel), binding between 14-3-3r and RGS3 is decreased after 30 min of TNF-α treatment. Binding to 14-3-3β is virtually abrogated by treatment with TNF-α (Fig. 3, right panel). These results indicate that TNF-α activates a signaling cascade that regulates the interaction between several 14-3-3 and RGS family members.

**TNF-α Blocks 14-3-3 Binding to RGS7 in Vivo**—We previously reported that TNF-α inhibits the degradation of RGS7 (1), resulting in the accumulation of RGS7 in the brains of mice treated with TNF-α or endotoxin, a stimulator of TNF-α release and formation. In the brain, a significant fraction of RGS7 appears to be phosphorylated on serine 434 and complexed and formation. In the brain, a significant fraction of RGS7 (1), resulting in the accumulation of RGS7 in the brains of mice treated with TNF-α or endotoxin, a stimulator of TNF-α release and formation. In the brain, a significant fraction of RGS7 appears to be phosphorylated on serine 434 and complexed with 14-3-3, conditions known to inhibit the in vitro GAP activity of RGS7 (6). To examine the dual effects of TNF-α in vivo, we examined the interaction of RGS7 with 14-3-3 in mice treated with TNF-α. As shown in Fig. 4A, co-immunoprecipitation of 14-3-3 with RGS7 was evident in brains of solvent-treated animals. This interaction was completely abrogated in mice treated with TNF-α (Fig. 4A, lower panel), although levels of 14-3-3 expression remained unchanged (Fig. 4A, upper panel). Binding of the adaptor protein 14-3-3 strictly depends upon a phosphorylated 14-3-3 binding site. As demonstrated in Fig. 4B, the effect of TNF-α can be mimicked by calf intestine alkaline phosphatase. Treatment of immunoprecipitated RGS7 with calf intestine alkaline phosphatase markedly decreased 14-3-3 binding, indicating that the interaction between RGS7 and 14-3-3 can be abrogated by phosphatases. It is likely that TNF-α triggers the activation of a currently unknown phosphatase that is absent or inactive in resting cells.

14-3-3 Modulates the Deactivation Kinetics of GIRKs Mediated by RGS7—GIRKs open upon binding to Gβγ subunits released by the activation of pertussis toxin-sensitive Goi/Goi-coupled receptors (9). Activation and deactivation kinetics of GIRK channels control the onset and termination of postsynaptic hyperpolarizing currents in neurons and cardiac atrial cells. The kinetics of neuronal and atrial GIRK channel activation and deactivation are markedly faster than those observed in heterologously expressed channels. Several RGS proteins, including RGS1, RGS3, RGS4, RGS7, RGS8, and RGS9, increase the rate at which GIRK channels close following agonist removal, presumably by accelerating the GTPase activity of Goi and thereby sequestering Gβγ (7, 8, 10–14). In the present study, we examined the effect of 14-3-3 on RGS7-mediated changes in deactivation kinetics of heterologously expressed GIRK1/2 channels. Oocytes were injected with water or RGS7 mRNA in combination with the muscarinic M2 acetylcholine receptor (M2 AChR) and GIRK1/GIRK2 to mimic the heteromultimeric state of native neuronal GIRK channels. Since the deactivation kinetics of GIRK channels are not affected by the ratio of G protein-coupled receptors to ion channel, this experimental setting permits the quantification of RGS protein function (15). As reported previously (7), ACh-evoked GIRK currents recorded from Xenopus oocytes in the absence of RGS proteins are deactivated with a time course best described by a single time constant of ~20 s (Fig. 5). In the presence of RGS7, the deactivation kinetics after removal of acetylcholine was significantly accelerated with a time constant of 13 s (Fig. 5, C and D). Microinjection of bacterially expressed and affinity purified recombinant GST 14-3-3 protein, but not of GST alone, significantly reduced the RGS7-mediated deactivation of GIRK currents (Fig. 5E). However, 14-3-3 did not influence GIRK channels kinetics in the absence of RGS proteins (data not shown). These findings indicate that interaction with 14-3-3 inhibits the activity of RGS7 in vivo.

A Conserved SYP/T/S Motif within the RGS Domain Interacts with 14-3-3—To confirm the specificity of the 14-3-3-induced inhibition of RGS7 activity in oocytes, we examined the effect of 14-3-3 on RGS4, a known opener of GIRK current deactivation that does not interact with 14-3-3. RGS4 accelerated the deactivation of GIRK1/2 in microinjected oocytes (Fig. 6, A and B). This RGS4-induced acceleration of GIRK channel deactivation was unaffected by co-injection with GST or GST 14-3-3 (Fig. 6C). The RGS domain of RGS4 contains an SYR motif that does not bind 14-3-3 (Fig. 7A). This region, which is highly conserved across RGS proteins, contains a 14-3-3 binding site in a subset of RGS proteins, including RGS3, RGS7, and RGS8 (Fig. 7, A and B). Alignment of several representative RGS proteins (ClustalW, www.ebi.ac.uk/ clustalw/) reveals a highly conserved SYF/L motif followed by either a proline (or serine/threonine) or arginine/alanine/lysine. A mutation of Pro-to-Arg at position 436 changed RGS7 from a 14-3-3 binding protein to an RGS4-type protein that failed to bind 14-3-3 (Fig. 7B). The ability of RGS7 to accelerate the deactivation of GIRK1/2 (Fig. 7C) was no longer inhibited by 14-3-3 (Fig. 7D). These findings demonstrate that the activity of RGS7 is regulated in vivo by binding of 14-3-3 to a conserved SYP motif. The mutational analysis of RGS4 and RGS7 is consistent with the 14-3-3 binding motifs defined by
Fig. 7. Binding of RGS proteins to 14-3-3 is mediated by a SYP motif. A, sequence comparison of RGS3, RGS4, RGS7, and RGS8 reveals that binding of 14-3-3 requires a proline at the position +2 (red) relative to serine 434 in RGS7. Substitution by arginine, present in either wild-type RGS4 or the RGS7P436R mutation, completely abolishes binding of 14-3-3. Highly conserved amino acids contributing to 14-3-3 binding are shaded (yellow). B, the interaction of 14-3-3 with RGS4, RGS7, RGS7P436R, and RGS8. HEK 293T cells were transfected with FLAG-tagged RGS proteins and hemagglutinin-tagged 14-3-3 as indicated. RGS proteins were precipitated using the FLAG-specific M2 antibody; interacting 14-3-3 was detected using a 14-3-3-specific antibody. As shown in the top panel, only wild-type RGS7, RGS8, and RGS3, all containing the SYP motif, but neither RGS4 nor RGS7P436R, bound 14-3-3. Equal expression of 14-3-3 was verified by Western blot analysis of cellular lysates (middle panel). Expression of the FLAG-tagged RGS proteins is shown in the bottom panel. C, RGS7P436R accelerates the deactivation kinetics of GIRK1/2. Oocytes were injected with cRNA encoding for GIRK1/2, M2 AChR, and RGS7P436R as indicated. RGS7P436R significantly decreased the deactivation time constant of GIRK1/2, indicating that the GTPase activating properties of RGS7 are not affected by this mutation. D, microinjection of recombinant GST or GST.14-3-3 protein does not alter the decrease of the time constant mediated by RGS7P436R. n.s., not significant.
peptide library screening: RGS proteins regulated by 14-3-3 contain an SY/S/T motif, whereas 14-3-3-resistant RGS4 contains an SYR motif (30).

**DISCUSSION**

RGS proteins modulate G protein signaling through their ability to accelerate the intrinsic GTPase activity of Go_i and Go_s subunits. Covalent modifications control the GTPase activity of most RGS proteins (reviewed in Refs. 4 and 5). For example, palmitoylation of a cysteine residue in helix 4, conserved in the RGS domain of all RGS proteins except RGS6 and RGS7, interferes with GTPase-accelerating activity (16). Several RGS proteins are phosphorylated, including RGS2, RGS3, RGS4, RGS7, RGS9, GAIP, and Ser216 (17, 18–22). Phosphorylation appears to affect the subcellular localization of several RGS proteins (17, 19) and inhibits the GTPase activity of RGS2 and RGS7 (6, 21).

RGS7 belongs to a subfamily of RGS proteins that contain a characteristic GY-like domain, which forms obligate heterodimers with the Gβ subunit Gβδ (23–25). Gβδ co-expression dramatically enhances the activity of RGS7; in oocytes, co-expression accelerates the activation and deactivation of GIRK channels (11). *In vitro*, the RGS domain of RGS7 efficiently accelerates GTP hydrolysis of either Go_i or Go_s (26); *in vivo*, the RGS-Gβδ complex appears to predominantly inhibit Go_s-dependent signaling (27). RGS7 exists as both unmodified cytosolic and palmitoylated membrane-derived forms in the brain (28). Both forms of RGS7, when complexed with Gβδ, are equally effective stimulators of Go_s GTPase activity, suggesting that palmitoylation of RGS7 occurs outside of the RGS domain and does not prevent RGS7/Go_s interactions. RGS7 is rapidly degraded by the ubiquitin-dependent proteasome (29), a process that is inhibited by TNF-α (1). The TNF-mediated accumulation of RGS7 requires activation of the MAP kinase p38 and the presence of a short serine/threonine-rich motif (Ser241, Thr245, Thr247) located upstream of the GY-like domain of RGS7 (1), phosphorylation of which inhibits the degradation of RGS7. In contrast, phosphorylation of serine 434 mediates association of RGS7 with 14-3-3, which inhibits the GAP activity of RGS7. While retaining its GAP activity, was no longer regulated by 14-3-3. Our data suggest that most RGS proteins are regulated by phosphorylation of serine 434, which interferes with binding to Go subunits. In a subgroup of RGS proteins, phosphorylation of this serine residue transforms this motif to an active 14-3-3 binding site with pronounced inhibition of GTPase activity *in vitro* (6) and *in vivo*. This is the first evidence that this interaction, and hence the activity of RGS proteins, can be regulated by extracellular signals. It remains to be examined how accumulation of active RGS7, following activation of p38 MAP kinase and release from its inhibitory interaction with 14-3-3, alters G protein-mediated signaling in native tissues. Since RGS7 is highly expressed in the brain, we postulate that transient accumulation of functionally active RGS7 has an important role in modulating the activation and deactivation kinetics of neuronal GIRKs and thus in determining how neuronal cells react to repetitive signals and participate in complex neuronal networks.

**Acknowledgments**—We thank Christina Engel, Stefanie Keller, Birgit Schilling, Helga Rittahler, and Robert Tauber for expert technical assistance; the members of the Walz laboratory for helpful discussions; and Dr. Silvio Gutkind, Dr. Melanie Mark, and members of the Peralta laboratory for providing plasmids.

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