0026-895X/06/6904-1280–1287$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 69:1280–1287, 2006

Vol. 69, No. 4
19059/3092366
Printed in U.S.A.

Endogenous Regulator of G-Protein Signaling Proteins Regulate the Kinetics of $G\alpha_{q/11}$-Mediated Modulation of Ion Channels in Central Nervous System Neurons

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Received September 16, 2005; accepted December 20, 2005

ABSTRACT

Slow synaptic potentials are generated when metabotropic G-protein-coupled receptors activate heterotrimeric G-proteins, which in turn modulate ion channels. Many neurons generate excitatory postsynaptic potentials mediated by G-proteins of the $G\alpha_{q/11}$ family, which in turn activate phospholipase C-β. Accessory GTPase-activating proteins (GAPs) are thought to be required to accelerate GTP hydrolysis and rapidly turn off G-proteins, but the involvement of GAPs in neuronal $G\alpha_{q/11}$ signaling has not been examined. Here, we show that regulator of G-protein signaling (RGS) proteins provide necessary GAP activity at neuronal $G\alpha_{q/11}$ subunits. We reconstituted inhibition of native 2-pore domain potassium channels in cerebellar granule neurons by expressing chimeric $G\alpha$ subunits that are activated by $G\alpha_{i/o}$-coupled receptors, thus bypassing endogenous $G\alpha_{q/11}$ subunits. RGS-insensitive variants of these chimeras mediated inhibition of potassium channels that developed and recovered more slowly than inhibition mediated by RGS-sensitive (wild-type) chimeras or native $G\alpha_{q/11}$ subunits. These changes were not accompanied by a change in agonist sensitivity, as might be expected if RGS proteins acted primarily as effector antagonists. The slowed recovery from potassium channel inhibition was largely reversed by an additional mutation that mimics the RGS-bound state. These results suggest that endogenous RGS proteins regulate the kinetics of rapid $G\alpha_{q/11}$-mediated signals in central nervous system neurons by providing GAP activity.

Heterotrimeric G-protein signaling is initiated when an activated receptor stimulates the exchange of GDP for GTP by the $G\alpha$ subunit of a $G\alpha\beta\gamma$ heterotrimer. This exchange promotes the dissociation of $G\alpha$-GTP and $G\beta\gamma$ subunits, both of which can directly or indirectly modify the function of ion channels. Many receptors couple exclusively to one family of $G\alpha$ subunits and thereby activate a specific set of effector molecules. For example, $G\alpha_{i/o}$ proteins often activate inwardly rectifying potassium channels, whereas $G\alpha_{q/11}$ proteins often inhibit potassium channels or activate nonselective cation channels (Hille, 1994). In neurons, these transduction pathways are responsible for metabotropic inhibitory and excitatory postsynaptic potentials (EPSPs), respectively.

G-protein signaling terminates when $G\alpha$-GTP is hydrolyzed to $G\alpha$-GDP and the heterotrimer reassociates. Although $G\alpha$ subunits possess intrinsic GTPase activity, this activity is too slow to account for the rapid termination of many G-protein-mediated signals. The timely termination of brief physiological signals thus depends on the activity of GTPase-activating proteins (GAPs), which bind to GTP-bound $G\alpha$ subunits and greatly accelerate the rate of GTP hydrolysis (Ross and Wilkie, 2000). Regulator of G-protein signaling (RGS) proteins are ubiquitous proteins that have GAP activity at $G\alpha_{i/o}$, $G\alpha_t$, and $G\alpha_{q/11}$ proteins (Hepler, 1999; Neubig and Siderovski, 2002). As such, these proteins can regulate the strength and kinetics of a variety of G-protein signals. RGS proteins are the only known GAPs at $G\alpha_{i/o}$ and $G\alpha_t$ proteins, and rapid termination of events mediated by these subunits clearly depends on RGS proteins (Doupnik et al., 1997; Saitoh et al., 1997; Chen et al., 2000). In contrast, the GTPase activity of $G\alpha_{q/11}$ proteins can be accelerated not only by RGS proteins but also by the effector molecule phospholipase C-β (PLCβ) (Berstein et al., 1992; Hepler et al., 1997; Chidiac and Ross, 1999). Thus, it is not clear to what

This work was supported by National Institutes of Health grant NS36455.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.019059.

ABBREVIATIONS: EPSP, excitatory postsynaptic potential; GAP, GTPase-activating protein; RGS, regulator of G-protein signaling; PLCβ, phospholipase C-β; CNS, central nervous system; RGSi, regulator of G-protein signaling-insensitive; α2AR, α2 adrenoreceptor; EGFP, enhanced green fluorescent protein; CGN, cerebellar granule neuron; IK_{SO}, standing outward K^+ current; HEK, human embryonic kidney; ANOVA, analysis of variance; NE, norepinephrine; GS, $G\alpha_{qi9}$ G188S; SD, $G\alpha_{qi9}$ S211D; GS:SD, double mutant $G\alpha_{qi9}$ G188S:S211D; WT, wild-type; RGSm, regulator of G-protein signaling-mimicking.

extent RGS proteins are essential for terminating transient $G\alpha_{q/11}$-mediated signals such as slow EPSPs. In addition, RGS proteins could negatively regulate $G\alpha_{q/11}$ signals by serving as "effector antagonists", competing with the effector PLCβ for binding to active G-proteins (Hepler et al., 1997).

We tested the hypothesis that endogenous RGS proteins regulate $G\alpha_{q/11}$-mediated signals by providing GAP activity in CNS neurons. Several previous studies of endogenous RGS protein function in intact cells have relied on functional replacement of native Gα subunits with RGS-insensitive (RGSi) mutants (Chen and Lambert, 2000; Jeong and Ikeda, 2000, 2001). We adopted a similar strategy by expressing RGS-sensitive and RGSi variants of Gα subunit chimeras consisting of $G\alpha_q$ with nine amino acids at the carboxy terminus substituted for those found in $G\alpha_i$ (Conklin et al., 1993). These chimeric ($G\alpha_{qi9}$) subunits are activated by receptors that normally activate $G\alpha_{i/o}$ subunits but couple to downstream effectors that normally interact with $G\alpha_{q/11}$ subunits. Using this system, we reconstituted inhibition of "leak" two-pore domain potassium (K2P) channels with RGS-sensitive and RGSi Gα subunits. We found that signals mediated by RGSi subunits develop and decay more slowly than those mediated by endogenous $G\alpha_{q/11}$ or RGS-sensitive $G\alpha_{qi9}$ subunits. These results suggest that endogenous RGS proteins are essential for the rapid kinetics of $G\alpha_{q/11}$-mediated synaptic potentials.

Materials and Methods

The coding sequence for the chimeric G-protein α subunit $G\alpha_{qi9}$ (provided by Dr. Bruce R. Conklin, University of California, San Francisco, San Francisco, CA) was subcloned into pcDNA 3.1+ (Invitrogen, Carlsbad, CA). The plasmid coding for the α2 adrenoreceptor (α2AR) was obtained from the Guthrie Research Institute cDNA resource (www.cdna.org). A plasmid coding for the enhanced green fluorescent protein (EGFP)-RGS2 fusion protein was provided by Dr. Peter Chidiac (University of Western Ontario, London, ON, Canada). QuikChange (Stratagene, La Jolla, CA) mutagenesis was carried out according to the manufacturer's instructions, and mutagenesis was confirmed by automated oligonucleotide sequencing.

Dissociated cultures of cerebellar granule neurons (CGNs) were prepared from 5- to 8-day-old Sprague-Dawley rats as described previously (Chen et al., 2004). CGNs were maintained in minimal essential medium supplemented with 5% fetal bovine serum, 25 mM KCl, 2% B-27 (Invitrogen, Carlsbad, CA), 0.1% MITO serum extender (BD Collaborative, San Diego, CA), 0.6% glucose, 1 mM pyruvate, 50 IU/ml penicillin, and 50 μg/ml streptomycin in 5% CO_2 at 37°C.

Neurons were transfected after 6 days in culture using polyethylenimine as described previously (Chen et al., 2004). Neurons were cotransfected with a plasmid coding for pEGFP-N1 (Clontech, Mountain View, CA) and were identified for recording by fluorescence microscopy. Control neurons were transfected with vector (pcDNA 3.1+) and marker (pEGFP-N1) only. Transfection efficiency using this method was low (<5%) but provided ample green fluorescent protein-positive cells for consistent recordings. Control experiments were consistent with the idea that the amount of protein expressed in individual cells was related to the amount of DNA added during transfection, although we made no attempt to quantify the levels of protein expressed in individual cells. Electrophysiological recordings were carried out 14 to 48 h after transfection.

Recordings were carried out at room temperature (24°C) on the stage of an inverted fluorescence microscope. Neurons were constantly perfused with a solution containing 150 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 10 mM glucose, 0.5 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.8; osmolality, ~310 mOsm/kg H_2O). Solution changes were made by switching between perfusion reservoirs using a series of pinch valves (Warner Instruments, Hamden, CT). All recordings were made using the whole-cell perforated patch configuration. Recording pipettes were filled with a solution containing 70 mM K^+-gluconate, 70 mM KCl, 0.2 mM EGTA, 10 mM HEPES, and 0.03% amphotericin B, pH 7.3; ~295 mOsm/kg H_2O). Recordings began 5 min after seal formation, at which time series resistance reached a stable minimum because of patch perforation. Neurons were held in voltage-clamp mode at −20 mV and were stepped to −110 mV for 20 ms every 450 ms. Standing outward K^+ current (IK_{SO}) was measured as the absolute holding current at −20 mV. Currents were digitized and recorded using a multifunction I/O board (National Instruments, Austin, TX) and WinWCP software (provided by Dr. J. Dempster, University of Strathclyde, Glasgow, Scotland).

For imaging EGFP-RGS2 translocation, HEK 293 cells were cotransfected with plasmids coding for EGFP-RGS2 and various $G\alpha_{qi9}$ subunits using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. After at least 16 h, cells were imaged using a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Thornwood, NY). Single optical sections through the center of each cell were analyzed using ImageJ software (http://rsb.info.nih.gov/ij/) by drawing a profile normal to and centered on the plasma membrane and plotting the fluorescence intensity along this profile. Because of the limits of diffraction and imperfect centering of these profiles, some fluorescence seems to originate on the extracellular side of the plasma membrane (Fig. 2A).

Response onset and recovery time courses are reported as time to half-maximal inhibition and time to half-maximal recovery, respectively ($t_{1/2}$). This simple parameter was chosen because the response kinetics changed shape depending on the experimental conditions. For example, although response recovery mediated by native Gα subunits and RGS-sensitive chimeras could be fitted adequately with a single exponential function, recovery mediated by RGSi chimeras began after a lag and an overshoot and thus could not be fitted by a simple function. We presume that different mechanisms rate-limit response recovery with and without RGS function.

Concentration-response curves were fitted to a logistic equation using Origin version 6.1 software (OriginLab Corp., Northampton, MA). Numerical values are expressed as mean ± S.E.M. Statistical comparisons were made by ANOVA and unpaired t tests.

Results

Reconstitution of IK_{SO} Inhibition in Cerebellar Granule Neurons with $G\alpha_{qi9}$ Chimeras. Cerebellar granule neurons express "leak" potassium channels of the tandem- or K2P family (Watkins and Mathie, 1996). These channels are constitutively open and mediate an IK_{SO} that contributes to the resting membrane potential of these cells (Watkins and Mathie, 1996). Activation of $G\alpha_{q/11}$-coupled receptors such as the endogenous m3 muscarinic acetylcholine receptors expressed by these cells inhibits this current. Because inhibition of similar channels underlies slow EPSPs in many neurons (Hille, 1994), we chose to use these cells as a model to study the involvement of endogenous RGS proteins in neuronal $G\alpha_{q/11}$ signaling. As described previously (Watkins and Mathie, 1996), application of the nonselective cholinergic agonist carbachol (50 μM) reversibly inhibited IK_{SO} in cultured CGNs, as indicated by a decrease in the outward holding current at a membrane potential of −20 mV and a decrease in whole-cell conductance (Fig. 1A). Previous work has shown that inhibition of K2P channels in these and other cells is mediated by pertussis toxin-insensitive $G\alpha_{q/11}$-proteins and activation of PLCβ (Czirjak et al., 2001; Chemin et al., 2003). We confirmed these observations by applying

pertussis toxin (100 ng/ml overnight), which had no effect on IK_{SO} inhibition, and by transiently transfecting a constitutively active mutant of $G\alpha_q$ ($G\alpha_q$ Q209L), which tonically inhibited IK_{SO} and occluded inhibition by carbachol (data not shown). After carbachol was removed from the bath, IK_{SO} recovered with a $t_{1/2}$ of 5.6 ± 0.9 s (n = 7). Control experiments in which IK_{SO} was inhibited by application of low pH medium documented that the rate of solution exchange using our perfusion system was considerably faster ($t_{1/2}$ ~2 s) than the rate of IK_{SO} recovery, suggesting that the latter accurately reflected the termination of $G\alpha_{q/11}$ signaling. When recovery from carbachol-induced IK_{SO} inhibition was fitted to a single exponential function the corresponding rate constants ranged from 0.09 to 0.27 s^{-1}, values that are approximately 8- to 30-fold faster than the reported intrinsic rate of GTP hydrolysis by purified $G\alpha_q$ (0.012/s) (Mukhopadhyay and Ross, 1999; Ross and Wilkie, 2000).

To test for the involvement of endogenous RGS proteins in this recovery, we first sought to reconstitute IK_{SO} inhibition in CGNs using exogenous $G\alpha$ subunits. To accomplish this, we used G-protein chimeras consisting largely of $G\alpha_q$ except for the nine amino acids at the carboxy terminus, which were identical to those found in the C terminus of $G\alpha_i$. These $G\alpha_{qi9}$ subunits are activated by receptors that normally activate

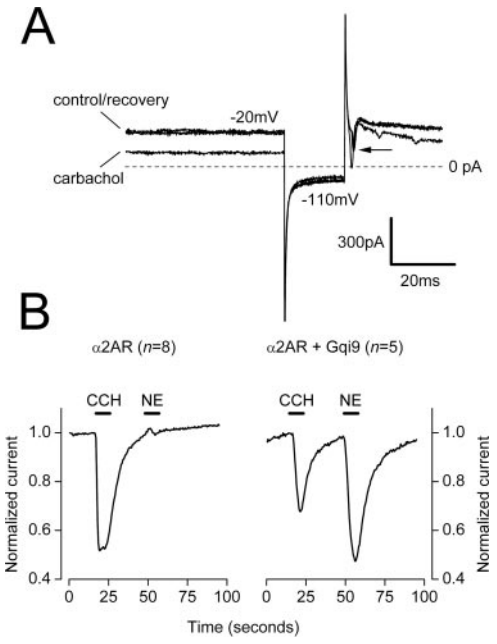

Fig. 1. Reconstitution of IK_{SO} inhibition in cerebellar granule neurons with $G\alpha_{qi9}$ chimeras. A, current traces recorded in voltage-clamp mode using the perforated-patch technique are shown superimposed under control conditions, in the presence of 50 μM carbachol, and after recovery. The holding potential was −20 mV, and the membrane potential was stepped transiently (20 ms) to −110 mV. The sharp upward and downward deflections immediately after the voltage step begins and ends are currents generated by the charging of the membrane capacitance. The transient inward current after the voltage command (arrow) is an unclamped sodium current. Inhibition of the holding current (IK_{SO}) was accompanied by a decrease in whole-cell conductance, as indicated by a decrease in the steady-state current required to induce the voltage change. B, normalized holding current is plotted versus time for neurons transfected with the α2AR and EGFP. Carbachol (50 μM; CCH; applied where indicated by the horizontal line) reversibly inhibited IK_{SO}, whereas NE (10 μM) did not, suggesting that α2ARs do not efficiently activate native $G\alpha_{q/11}$ subunits. The plotted line represents the average response from eight cells. C, cotransfection with α2ARs and $G\alpha_{qi9}$ subunits allows activation of the former to reversibly inhibit IK_{SO}.

$G\alpha_{i/o}$-proteins, but in turn activate effectors that are normally activated by $G\alpha_{q/11}$ subunits such as PLCβ (Conklin et al., 1993). As such, we expected that expressing $G\alpha_{qi9}$ would allow us to inhibit IK_{SO} in CGNs by activating $G\alpha_{i/o}$-coupled receptors, thus bypassing endogenous $G\alpha_{q/11}$ subunits. Indeed, we found that activation of native or transiently transfected $G\alpha_{i/o}$-coupled receptors reversibly inhibited IK_{SO} in neurons that were transfected with $G\alpha_{qi9}$ but not those that were transfected with a control vector or receptor alone. For example, when CGNs were cotransfected with plasmid vectors coding for the α2AR and $G\alpha_{qi9}$ application of 10 μM norepinephrine (NE) reversibly inhibited IK_{SO} in a manner similar to that produced by application of carbachol in untransfected or control cells (Fig. 1B). Inhibition of IK_{SO} by NE under these conditions recovered with a $t_{1/2}$ of 6.7 ± 0.5 s (n = 5), which was not significantly different from recovery from carbachol-induced inhibition in parallel controls (P = 0.37). IK_{SO} was not inhibited by NE in neurons that were transfected only with the α2AR, suggesting that inhibition of IK_{SO} by these receptors was mediated by coexpressed $G\alpha_{qi9}$ rather than endogenous $G\alpha$ subunits. In early experiments, we activated $G\alpha_{qi9}$ with coexpressed A_1 adenosine receptors instead of α2ARs. However, in these experiments, the recovery from adenosine-induced IK_{SO} inhibition was substantially slower than recovery from inhibition by native muscarinic receptors ($t_{1/2}$ ~25 s; data not shown) (Chen et al., 2004). This result is consistent with a recent report by Benians et al. (2003) that ligand unbinding may rate limit the recovery of physiological responses mediated by some receptors, including A_1 adenosine receptors. Because this limitation could mask any effect of endogenous RGS proteins, we used α2ARs for all of our experiments with RGSi $G\alpha_{qi9}$ mutants.

IK_{SO} Inhibition Mediated by RGS-Insensitive $G\alpha_{qi9}$ Chimeras. Having reconstituted IK_{SO} inhibition with exogenous $G\alpha_{qi9}$ subunits, we introduced point mutations to render this subunit insensitive to endogenous RGS proteins (RGSi). In the first set of experiments, we tested two mutations using three different constructs: $G\alpha_{qi9}$ G188S (GS), $G\alpha_{qi9}$ S211D (SD), and a double mutant $G\alpha_{qi9}$ G188S:S211D (GS:SD). These mutations are located in the highly conserved switch regions of the $G\alpha$ subunit and have been shown by several studies to greatly inhibit RGS protein binding without affecting endogenous GTPase activity, nucleotide exchange rates, or effector activation (DiBello et al., 1998; Lan et al., 1998; Natochin and Artemyev, 1998). Although these mutations have been characterized well in previous physiological studies (Chen and Lambert, 2000; Jeong and Ikeda, 2000, 2001), we confirmed the RGSi phenotype of the GS and SD mutants in the context of the $G\alpha_{qi9}$ chimera by testing the ability of constitutively active (R183C mutant) versions of each to translocate EGFP-RGS2 from the nucleus to the plasma membrane of HEK 293 cells (Heximer et al., 2001; Roy et al., 2003). As expected, the RGSi mutants did not recruit EGFP-RGS2 to the plasma membrane, whereas constitutively active $G\alpha_{qi9}$ R183C efficiently translocated EGFP-RGS2 (Fig. 2). The nuclear/cytosolic ratio of EGFP-RGS2 was also unchanged by the RGSi mutants, whereas constitutively active $G\alpha_{qi9}$ R183C recruited EGFP-RGS2 from the nucleus (data not shown).

As was the case with "wild-type" (WT) $G\alpha_{qi9}$, transfection of any of the three RGSi mutants enabled α2ARs to inhibit IK_{SO} in transfected CGNs (Fig. 3A). However, recovery from

NE-induced inhibition was significantly slower for all three of the RGSi mutants compared with parallel WT $G\alpha_{qi9}$ controls ($t_{1/2}$ = 8.6 ± 0.1 s; n = 21). This slowed recovery was consistently more pronounced for the GS single mutant ($t_{1/2}$ = 20.8 ± 1.8 s; n = 21; $P < 0.01$) than the SD single mutant ($t_{1/2}$ = 12.5 ± 1.7 s; n = 16; $P < 0.05$; Fig. 3B). There was a synergistic effect of the two mutations, because inhibition of IK_{SO} mediated by the GS:SD double mutant recovered with a $t_{1/2}$ of 40.9 ± 2.5 s (n = 13; $P < 0.01$). Slow recovery from inhibition mediated by RGSi mutants was accompanied by a change in the shape of the recovery time course. Recovery from IK_{SO} inhibition mediated by WT $G\alpha_{qi9}$ could be fitted well with a single exponential function and began with minimal delay after solution exchange. In contrast, recovery from IK_{SO} inhibition mediated by the most affected RGSi $G\alpha_{qi9}$ mutants (GS and GS:SD) began after a delay of several seconds (Fig. 3D). These results are consistent with the hypothesis that endogenous RGS proteins in CGNs accelerate the termination of $G\alpha_q$ signaling by acting as GAPs.

Several previous studies in heterologous expression systems as well as native cells have shown that RGS proteins participate in the rapid onset of $G\alpha_{i/o}$-mediated events (Doupnik et al., 1997; Saitoh et al., 1997; Jeong and Ikeda, 2000, 2001). We observed a similar effect of endogenous RGS proteins on $G\alpha_{qi9}$-mediated inhibition of IK_{SO} in CGNs. The rate of onset of IK_{SO} inhibition mediated by two of the three RGSi mutants was slower than that mediated by WT $G\alpha_{qi9}$ (Fig. 3B). Changes in onset kinetics paralleled changes in recovery kinetics. Thus, the GS mutant was more affected than the SD mutant, and the double GS:SD mutant was the most affected. The time to reach half-maximal inhibition (onset $t_{1/2}$) of IK_{SO} was 2.3 ± 0.1 s for WT $G\alpha_{qi9}$ and 2.4 ± 0.1 s for the SD mutant. These values were not significantly different ($P > 0.05$) and may have been limited by the speed of solution exchange. However, the onset $t_{1/2}$ values for the GS (5.0 ± 0.3 s) and GS:SD (6.7 ± 0.8 s) mutants were significantly different from that of WT $G\alpha_{qi9}$ ($P < 0.05$). The slow onset of IK_{SO} inhibition mediated by RGSi $G\alpha_{qi9}$ subunits suggests that endogenous RGS proteins may facilitate $G\alpha_{q/11}$ activation by an unknown mechanism, as may also be the case for $G\alpha_{i/o}$ subunits (see *Discussion*).

The maximal extent of IK_{SO} inhibition at steady state was similarly effected by RGSi mutations. IK_{SO} was inhibited 62 ± 2% by WT $G\alpha_{qi9}$, 66 ± 4% by the SD mutant ($P > 0.05$ versus WT $G\alpha_{qi9}$), 46 ± 4% by the GS mutant ($P < 0.05$ versus WT $G\alpha_{qi9}$), and 28 ± 3% by the GS:SD mutant ($P <$

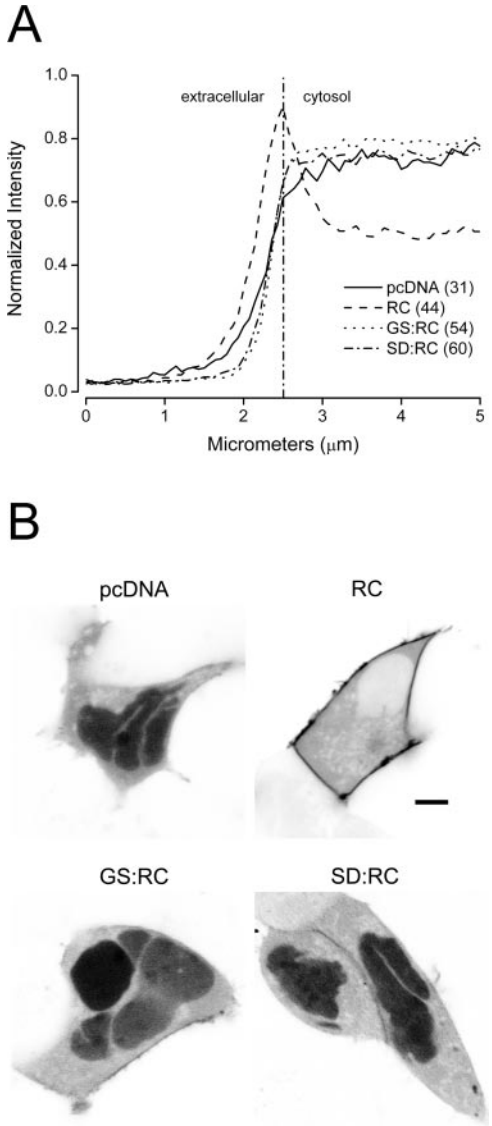

Fig. 2. Constitutively active RGSi $G\alpha_{qi9}$ chimeras fail to recruit EGFP-RGS2 to the plasma membrane. HEK 293 cells were contransfected with EGFP-RGS2 and either the empty vector (pcDNA) or the constitutively active R183C mutants of the WT $G\alpha_{qi9}$ (RC), $G\alpha_{qi9}$ G188S (GS:RC), or $G\alpha_{qi9}$ S211D (SD:RC). A, normalized averaged fluorescence intensity profiles collected over a 5-μm line normal to and centered on the plasma membrane are plotted. WT $G\alpha_{qi9}$ RC recruits EGFP-RGS2 to the plasma membrane, as indicated by the fluorescence intensity peak, whereas no enrichment at the plasma membrane is evident for either of the RGSi mutants. The number of cells (n) is given in parentheses. B, example images from the same cells shown in A. Fluorescence intensity is inverted for clarity. Scale bar, 5 μm.

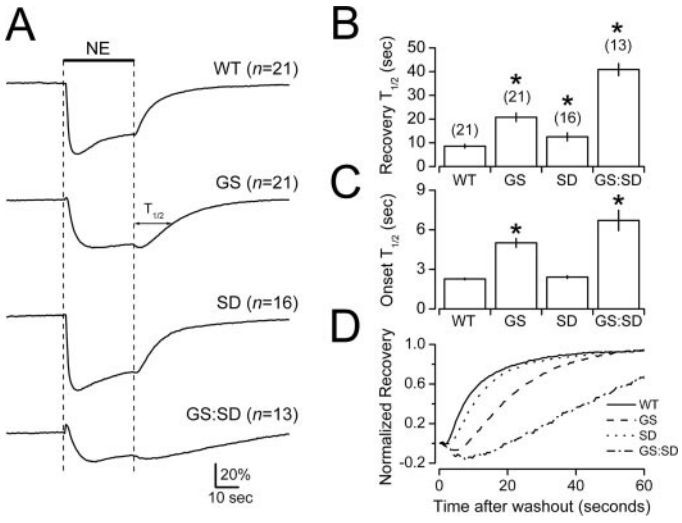

Fig. 3. RGSi $G\alpha_{qi9}$ chimeras mediate inhibition of IK_{SO} with slowed onset and recovery kinetics. A, average normalized current traces recorded from neurons transfected with α2ARs and $G\alpha_{qi9}$ (WT), GS, SD, or GS:SD. NE was applied where indicated by the horizontal bar and vertical dashed lines. The time to 50% recovery ($t_{1/2}$) was measured as indicated by the arrow. B and C, grouped quantitative data for time to 50% recovery (B) and time to 50% maximal inhibition (C). The number of cells represented (n) is shown in parentheses; statistical significance ($P < 0.05$) is indicated above each bar by an asterisk (*). D, expanded normalized current traces during recovery from IK_{SO} inhibition. Recovery from inhibition mediated by the GS and GS:SD RGSi mutants began after a significant delay.

0.05 versus WT $G\alpha_{qi9}$). Increasing the amount of plasmid DNA transfected into CGNs failed to increase the magnitude of steady-state inhibition by RGSi chimeras, even though control experiments using $G\alpha$ subunits to buffer $G\beta\gamma$ subunits indicated more $G\alpha$ protein was expressed (data not shown). One possible explanation for the decreased steady-state inhibition of IK_{SO} by RGSi mutants is that these mutants are constitutively active and thus produce tonic inhibition of IK_{SO}, which would occlude agonist-induced inhibition. However, the amplitude of IK_{SO} before agonist application was 239 ± 20 pA ($n = 9$) in neurons transfected with WT $G\alpha_{qi9}$ compared with 255 ± 42 pA in neurons transfected with $G\alpha_{qi9}$ GS:SD ($n = 10$; $P = 0.69$); thus, no tonic inhibition was evident. Moreover, after partial inhibition mediated by RGSi $G\alpha_{qi9}$, IK_{SO} could be inhibited further by activation of endogenous muscarinic receptors, presumably acting via endogenous $G\alpha_{q/11}$ subunits. These results suggest that RGSi $G\alpha_{qi9}$ subunits are not constitutively active and are unable to fully activate downstream effector molecules.

Because RGS proteins are negative regulators of G-protein signaling, they have been shown to decrease the agonist sensitivity of some $G\alpha_{i/o}$-mediated events (Jeong and Ikeda, 2000). Endogenous RGS proteins could also decrease the agonist sensitivity of $G\alpha_{q/11}$-mediated events by acting either as GAPs or effector antagonists (Hepler et al., 1997). We asked whether endogenous RGS proteins regulated the agonist sensitivity of $G\alpha_{q/11}$-mediated signals in a similar manner by constructing concentration-response curves for NE-induced inhibition of IK_{SO}. If endogenous RGS proteins predominantly enhanced the rate of GTP hydrolysis by $G\alpha_{qi9}$ subunits, it was predicted that RGSi mutants would show a leftward shift in the NE concentration-response curve. However, as shown in Fig. 4 the concentration-response curves for RGSi mutants were slightly right shifted compared with WT $G\alpha_{qi9}$, with EC_{50} values increased by 2- to 5-fold in this series of experiments. A similar shift was not observed in a second, identical series of experiments (see below); thus, we conclude that steady-state agonist sensitivity is not regulated by RGS proteins in these cells.

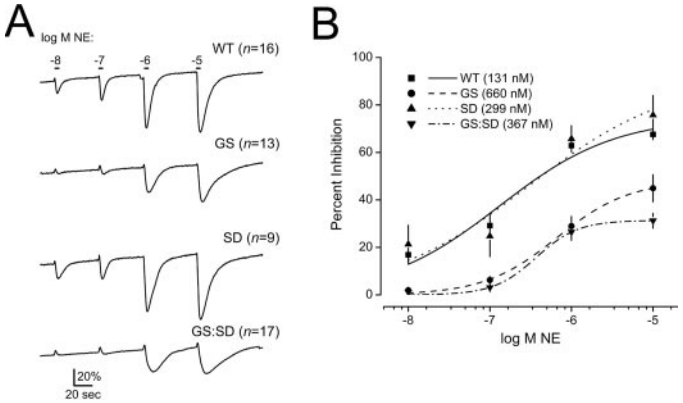

Fig. 4. RGSi $G\alpha_{qi9}$ chimeras mediate inhibition of IK_{SO} with unchanged agonist sensitivity. A, average normalized current traces recorded from neurons transfected with α2ARs and $G\alpha_{qi9}$ (WT), GS, SD, or GS:SD. Increasing concentrations of NE (ranging from 10 nM to 10 μM) were applied where indicated by the horizontal bars. The number of cells represented (n) is shown in parentheses. B, concentration-response plots of percentage of inhibition of IK_{SO} versus NE concentration. Smooth lines represent logistic fits to the data. Calculated EC_{50} values are shown in parentheses.

A Mutation That Mimics the Actions of RGS Proteins Speeds Recovery from IK_{SO} Inhibition Mediated by RGSi $G\alpha_{qi9}$ Chimeras. A recent mutational analysis of the switch regions of $G\alpha_{i1}$ by Thomas et al. (2004) identified a highly conserved glycine residue (G202 in $G\alpha_{i1}$) that when mutated to alanine greatly increased this mutant's rate of GTP hydrolysis. The GTPase activity of $G\alpha_{i1}$ G202A was increased only marginally by added RGS4, even though the affinity of this mutant for RGS4 was increased. Therefore, this mutation seemed to stabilize the pretransition state of the G-protein in a manner similar to RGS protein binding. We reasoned that introducing the corresponding mutation into RGSi $G\alpha_{qi9}$ chimeras could have a similar effect and might thus reverse the changes brought about by disrupting RGS binding. Therefore, we constructed $G\alpha_{qi9}$ chimeras containing this RGS-mimicking (RGSm) mutation (G207A) in the RGSi $G\alpha_{qi9}$ G188S and G188S:S211D backgrounds. As was the case with the other chimeras we tested, $G\alpha_{qi9}$ constructs containing the G207A mutation allowed activation of cotransfected α2ARs to reversibly inhibit IK_{SO}. As shown in Fig. 5, recovery from NE-induced inhibition of IK_{SO} mediated by the RGSi/RGSm $G\alpha_{qi9}$ G188S:G207A (GS:GA) and GS:SD:GA chimeras was not significantly different from that mediated by WT $G\alpha_{qi9}$, whereas recovery from inhibition mediated by their RGSi counterparts was significantly slower, as observed previously ($P < 0.05$; ANOVA followed by Bonferroni comparison of means). Thus, this RGSm mutation was largely able to reverse the effect of the RGSi mutations on IK_{SO} recovery kinetics. In contrast, the RGSm mutation failed to restore rapid onset kinetics, because all RGSi and RGSi/RGSm mutants mediated IK_{SO} inhibition with signifi-

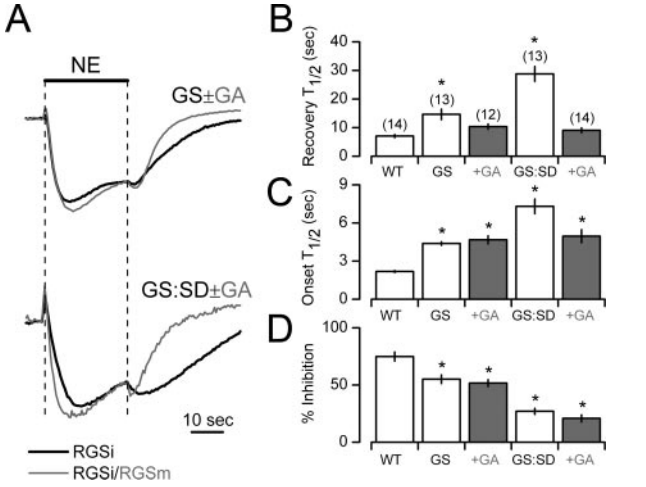

Fig. 5. A mutation that mimics RGS binding accelerates recovery from IK_{SO} inhibition mediated by an RGSi chimera. Neurons were transfected with α2ARs and WT $G\alpha_{qi9}$ (WT), GS, GS:GA, GS:SD, or $G\alpha_{qi9}$ G188S:S211D:G207A (GS:SD:GA). A, average normalized current traces recorded from neurons transfected with α2ARs and RGSi or RGSi/RGSm chimeras are shown superimposed. Superimposed traces are scaled such that the recovery phases are normalized. Grouped quantitative data are plotted for time to 50% recovery (B), time to 50% maximal inhibition (C), and peak steady-state inhibition (D). The number of cells represented (n) is shown in parentheses; statistical significance ($P < 0.05$; ANOVA followed by Bonferroni comparison of means) is indicated above each bar by an asterisk (*). Recovery from inhibition mediated by the GS and GS:SD RGSi mutants was significantly slower than that mediated by WT $G\alpha_{qi9}$, whereas recovery from inhibition mediated by GS:GA and GS:SD:GA RGSi/RGSm mutants was not significantly slower than that mediated by WT $G\alpha_{qi9}$; all other parameters were significantly different from WT $G\alpha_{qi9}$.

cantly slower onset than WT $G\alpha_{qi9}$ (Fig. 5C). However, the onset of IK_{SO} inhibition mediated by the GS:SD:GA chimera was significantly faster than that mediated by the RGSi GS:SD mutant (Fig. 5C; $P < 0.05$), suggesting that this mutation partially restored onset kinetics. Peak steady-state inhibition was decreased for all of the RGSi chimeras, and the addition of the RGSm mutation failed to alter this change (Fig. 5D). However, steady-state agonist sensitivity was unaltered for any of the RGSi or RGSi/RGSm chimeras, as indicated by unchanged EC_{50} values ($n = 6$–13; $P > 0.05$; ANOVA). Together, these results suggest that the slow response recovery characteristic of RGSi chimeras could be counteracted by a mutation that restored GAP activity, and the slow response onset mediated by RGSi chimeras could be partially reversed by restoring GAP activity.

Overexpression of RGS16-EGFP Accelerates Recovery from IK_{SO} Inhibition. The above-mentioned results suggest that slowing the rate of GTP hydrolysis by interfering with endogenous RGS protein GAP activity can slow the termination of $G\alpha_{q/11}$ signals. If timely termination of these signals depends on RGS GAP activity, then it might be possible to accelerate the termination of events mediated by endogenous $G\alpha_{q/11}$ proteins by increasing the availability of RGS proteins. To test this possibility, we transfected cerebellar granule neurons with RGS2-EGFP and RGS16-EGFP, because both of these RGS proteins are known to act as GAPs at $G\alpha_{q/11}$ subunits. However, RGS2-EGFP was not expressed efficiently in these cells, as indicated by the absence of detectable green fluorescence. Cotransfection with dsRed indicated that neurons were actually transfected (data not shown), and transfection of HEK cells under identical conditions resulted in robust RGS2-EGFP expression (Fig. 2). In contrast, RGS16-EGFP expressed well in cerebellar granule neurons, producing substantial fluorescent signal located in the cytosol, extending into neuronal processes (Fig. 6A, inset). We tested the ability of RGS16-EGFP to alter IK_{SO} inhibition mediated by endogenous muscarinic receptors and $G\alpha_{q/11}$ proteins by comparing responses to the agonist carbachol in neurons transfected with RGS16-EGFP and EGFP alone as a control. As shown in Fig. 6, RGS16-EGFP overexpression had no significant effect on the onset or peak magnitude of IK_{SO} inhibition. However, RGS16-EGFP overexpression significantly accelerated recovery from IK_{SO} inhibition from 6.1 ± 0.6 to 3.9 ± 0.3 s ($n = 17$; $P < 0.05$). RGS16-EGFP did not alter the steady-state sensitivity of IK_{SO} inhibition mediated by native receptors and G-proteins, because EC_{50} values were not significantly different (7.0 ± 1.1 μM for EGFP; 8.8 ± 1.8 μM for RGS16-EGFP; $P > 0.05$). This result supports our conclusion that the kinetics of $G\alpha_{q/11}$ signals in these neurons are regulated by RGS GAP activity and suggests that the availability of RGS proteins may limit the degree of this regulation.

Discussion

The GAP activity of RGS proteins has been widely studied using in vitro assays, and the function of these proteins in intact cells has been well established using heterologous expression systems. Genetic approaches have also documented the role of endogenous RGS proteins in physiological events in vivo (Chen et al., 2000; Oliveira-Dos-Santos et al., 2000). However, relatively little is known about the roles of *endogenous* RGS proteins at the cellular level. This deficit largely reflects the absence of effective methods to disable RGS proteins in native cells. Much of what is known about endogenous RGS function has been learned through the use of RGS-insensitive mutants of $G\alpha_{i/o}$ subunits (Chen and Lambert, 2000; Jeong and Ikeda, 2000, 2001). These studies have confirmed that endogenous RGS proteins regulate $G\alpha_{i/o}$-mediated signals much as would be predicted from studies in heterologous systems.

Our aim was to apply this strategy to $G\alpha_{q/11}$-mediated signals in CNS neurons. Endogenous RGS proteins could regulate GTP-bound $G\alpha_{q/11}$ by acting as GAPs or by binding to active $G\alpha_{q/11}$ and blocking access to effectors (Hepler et al., 1997). A significant difference between $G\alpha_{i/o}$ subunits and $G\alpha_{q/11}$ subunits is that RGS proteins are the only known GAPs for the former, whereas both RGS proteins and PLCβ are GAPs at the latter (Berstein et al., 1992). A second difference between $G\alpha_{i/o}$ subunits and $G\alpha_{q/11}$ subunits is that RGS proteins could serve as effector antagonists at the latter by binding to activated $G\alpha_{q/11}$ subunits and thus preventing binding of PLCβ. Rapid signals mediated by $G\alpha_{i/o}$ subunits are generally carried by $G\beta\gamma$ dimers and thus are not susceptible to effector antagonism. The possibility that RGS proteins regulate $G\alpha_{q/11}$ subunits in CNS neurons is an important question, because these subunits mediate synaptic potentials generated by several neurotransmitters.

We found that RGSi mutants of $G\alpha_{qi9}$ mediated inhibition of IK_{SO} that recovered more slowly than inhibition produced by WT $G\alpha_{qi9}$ or native $G\alpha_{q/11}$ subunits. Our results are thus

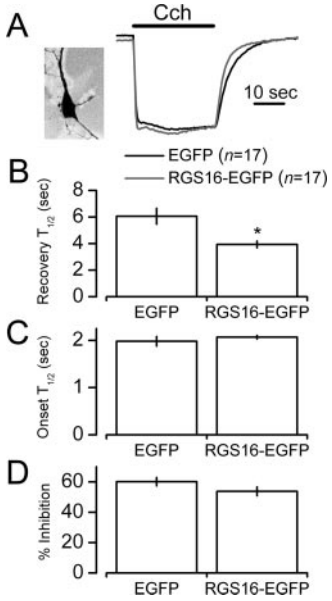

Fig. 6. Overexpression of RGS16-EGFP accelerates recovery from IK_{SO} inhibition mediated by native muscarinic receptors and $G\alpha_{q/11}$ subunits. Neurons were transfected with EGFP alone or RGS16-EGFP. A, average normalized current traces recorded from transfected neurons are shown superimposed, and carbachol (50 μM) was applied where indicated by the horizontal line; superimposed traces are scaled such that the recovery phases are normalized. Inset, example image of a cerebellar granule neuron expressing RGS16-EGFP; fluorescence intensity is inverted for clarity. Grouped quantitative data ($n = 17$ for each condition) are plotted for time to 50% recovery (B), time to 50% maximal inhibition (C), and peak steady-state inhibition (D). Statistical significance ($P < 0.05$; unpaired t test) is indicated above each bar by an asterisk (*). Recovery from inhibition was significantly accelerated by overexpression of RGS16-EGFP.

consistent with the hypothesis that endogenous RGS proteins function as GAPs at GTP-bound $G\alpha_{q/11}$ subunits in CNS neurons, as has been suggested previously in pancreatic acinar cells (Luo et al., 2001). This conclusion depends on the assumption that the RGSi mutations we introduced into $G\alpha_{qi9}$ interfered with RGS GAP activity rather than (for example) the intrinsic rate of GTP hydrolysis. Likewise, interpretation of our experiments using the G207A mutation depends on extrapolating biochemical results obtained with $G\alpha_{i1}$ (Thomas et al., 2004). Previous biochemical characterization of the RGSi mutations has suggested that the intrinsic hydrolysis rate is not changed (DiBello et al., 1998; Lan et al., 1998; Natochin and Artemyev, 1998), but we would emphasize that these mutants have not been biochemically characterized in the $G\alpha_{qi9}$ background, have not been biochemically characterized in combination and have not been challenged with a wide range of RGS proteins. Despite that all of the mutated residues are located in highly conserved regions of the $G\alpha$ sequence, it is possible that functional characteristics other than those intended could have been altered in our experiments (see below). Nonetheless, the results we obtained are entirely consistent with previous studies of endogenous RGS function at $G\alpha_{i/o}$ subunits.

Our results suggest that the kinetics of slow EPSPs can be governed by RGS proteins, and it is therefore possible that changes in the availability of RGS proteins could result in changes in slow synaptic transmission. Indeed, increasing the availability of RGS proteins modestly accelerated recovery from inhibition of IK_{SO}. This result suggests that the rate of RGS-accelerated GTP hydrolysis limits the decay rate of $G\alpha_{q/11}$-mediated signals. Of course, the time course of $G\alpha_{q/11}$-mediated signals might be dictated by different factors in different cells. For example, muscarinic inhibition of the M-current in sympathetic neurons recovers over the course of hundreds of seconds, even though termination of $G\alpha_{q/11}$ signaling in these cells seems to be substantially faster (Delmas et al., 2002; Suh and Hille, 2002). Inhibition of the M-current and K2P channels by $G\alpha_{q/11}$-coupled receptors is thought to be mediated by depletion of phosphatidylinositol 4,5-bisphosphate (Czirjak et al., 2001; Suh and Hille, 2002; Chemin et al., 2003; Zhang et al., 2003); thus, it is possible that slow recovery from M-current inhibition in sympathetic neurons is rate limited by phosphatidylinositol 4,5-bisphosphate resynthesis rather than GTP hydrolysis.

Because PLCβ is also a GAP at $G\alpha_q$ subunits, it is important to consider the possibility that the mutations we introduced into $G\alpha_{qi9}$ altered response kinetics by interfering with the GAP activity of PLCβ. This possibility is unlikely for several reasons. First, there is little structural homology between PLCβ and RGS proteins; thus, the regions of the $G\alpha$ subunit contacted by the two GAPs are likely to be different. This idea is supported by the structure of RGS4 complexed to $G\alpha_{i1}$ (Tesmer et al., 1997), which indicates steric and/or electrostatic interactions between the residues we mutated (G183 and S206 in $G\alpha_{i1}$) and RGS4. In contrast, these residues do not directly contact PLCβ in a structural model of $G\alpha_q$ docked to the PLCβ C-terminal dimer (J. Sondek and A. Singer, personal communication) (Singer et al., 2002). This assertion is consistent with biochemical characterization of $G\alpha_q$ G188S, which indicates that this mutant responds to the GAP activity of PLCβ well, with an unchanged EC_{50} and a maximal effect that reaches 50% of the wild-type protein. In contrast, this mutant responds to the GAP activity of RGS4 very poorly, with a highly right-shifted EC_{50} (E. Ross and S. Nayak, personal communication). Second, RGSi $G\alpha_{qi9}$ subunits mediated responses with slowed onset kinetics. Changes in onset kinetics have been consistently observed in studies where RGS proteins are heterologously overexpressed or with RGSi $G\alpha_{i/o}$ subunits (Chen and Lambert, 2000; Jeong and Ikeda, 2000, 2001). Our observations thus imply there is a common (albeit unknown) mechanism whereby RGS proteins participate in G-protein activation (see below). Finally, Jeong and Ikeda (2000) have previously studied the calcium channel inhibition mediated by the GS, SD, and GS:SD mutants of $G\alpha_{oA}$, which is not subject to PLCβ GAP activity. In that study and the present study, these three mutants differed with respect to the degree of kinetic slowing in an analogous manner: the SD mutant was less effected than the GS mutant, and the double GS:SD mutant was the most effected. Given the structural dissimilarity between RGS proteins and PLCβ, it would be surprising if two point mutations known to interfere with RGS protein binding also interfered with PLCβ GAP activity and did so to the same relative degree. Although we cannot rule out an effect of these RGSi mutations on PLCβ GAP activity, we conclude that the effects we observed largely reflect disruption of endogenous RGS protein function.

Finally, although the slowing of IK_{SO} recovery kinetics is relatively easy to interpret in light of the known GAP activity of RGS proteins at GTP-bound $G\alpha_{q/11}$ subunits, the slowing of the *onset* of IK_{SO} inhibition is more difficult to explain. Because the time for a response to reach equilibrium is inversely proportional to the sum of the activation and deactivation rates, part of the slowed response onset with RGSi $G\alpha_{qi9}$ subunits could be explained by disruption of GAP activity. However, the fact that maximal response amplitude is decreased with RGSi $G\alpha_{qi9}$ subunits implies that there is an additional positive effect of endogenous RGS proteins on $G\alpha_{q/11}$ signaling. This situation mirrors several previous studies of the regulation of $G\alpha_{i/o}$ response kinetics in heterologous and native systems (Doupnik et al., 1997; Saitoh et al., 1997; Chen and Lambert, 2000; Jeong and Ikeda, 2000, 2001). As yet, there is no general agreement regarding the mechanism whereby RGS proteins accelerate $G\alpha_{i/o}$ response onset. One possibility is that RGS proteins serve a scaffold function by binding to receptors, G-proteins, and/or effectors. Interactions between receptors and RGS proteins have been demonstrated and invoked to explain receptor-specific RGS actions (Zeng et al., 1998; Zhang et al., 2002), including receptors that couple to $G\alpha_{q/11}$ (Bernstein et al., 2004). More recently, Tinker and colleagues have suggested that RGS proteins form stable complexes with inactive G-protein heterotrimers and thus participate in a quaternary complex (Benians et al., 2005). A competing hypothesis suggests that RGS proteins serve as kinetic rather than physical scaffolds (Ross and Wilkie, 2000; Zhong et al., 2003) by promoting rapid cycling of active and inactive G-protein. Although our experiments did not directly address this issue, our results are more consistent with the former explanation. A mutation that restored GTPase activity (G207A) to RGSi mutants failed to fully reverse the slow onset of IK_{SO} inhibition. Because kinetic scaffolding would depend entirely on GAP activity, restoring GAP activity should have restored normal onset kinetics if this were the sole mechanism. In either case,

our results indicate that acceleration of response onset is not limited to responses mediated by $G\alpha_{i/o}$ subunits.

In summary, we have shown that RGSi $G\alpha_{qi9}$ chimeras mediate signals with slowed onset and recovery kinetics in primary neurons. These results are consistent with the idea that endogenous RGS proteins provide essential GAP activity for the regulation of slow synaptic events mediated by $G\alpha_{q/11}$ α subunits.

Acknowledgments

We thank John Sondek, Alex Singer, Elliot Ross, and Surendra Nayak for sharing unpublished results. We thank Bruce Conklin and Peter Chidiac for supplying plasmids encoding $G\alpha_{qi9}$ and EGFP-RGS2, respectively. We thank John Dempster for providing WinWCP software.

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