

Regulators of G Protein Signaling Attenuate the G Protein-mediated Inhibition of N-Type Ca Channels

KARIM MELLITI,* ULISES MEZA,* RORY FISHER,[†] and BRETT ADAMS*

From the *Department of Physiology and Biophysics and [†]Department of Pharmacology, University of Iowa, College of Medicine, Iowa City, Iowa 52242-1109

ABSTRACT Regulators of G protein signaling (RGS) proteins bind to the α subunits of certain heterotrimeric G proteins and greatly enhance their rate of GTP hydrolysis, thereby determining the time course of interactions among $G\alpha$, $G\beta\gamma$, and their effectors. Voltage-gated N-type Ca channels mediate neurosecretion, and these Ca channels are powerfully inhibited by G proteins. To determine whether RGS proteins could influence Ca channel function, we recorded the activity of N-type Ca channels coexpressed in human embryonic kidney (HEK293) cells with G protein-coupled muscarinic (m2) receptors and various RGS proteins. Coexpression of full-length RGS3T, RGS3, or RGS8 significantly attenuated the magnitude of receptor-mediated Ca channel inhibition. In control cells expressing $\alpha 1B$, $\alpha 2$, and $\beta 3$ Ca channel subunits and m2 receptors, carbachol (1 μ M) inhibited whole-cell currents by $\sim 80\%$ compared with only $\sim 55\%$ inhibition in cells also expressing exogenous RGS protein. A similar effect was produced by expression of the conserved core domain of RGS8. The attenuation of Ca current inhibition resulted primarily from a shift in the steady state dose-response relationship to higher agonist concentrations, with the EC_{50} for carbachol inhibition being ~ 18 nM in control cells vs. ~ 150 nM in RGS-expressing cells. The kinetics of Ca channel inhibition were also modified by RGS. Thus, in cells expressing RGS3T, the decay of prepulse facilitation was slower, and recovery of Ca channels from inhibition after agonist removal was faster than in control cells. The effects of RGS proteins on Ca channel modulation can be explained by their ability to act as GTPase-accelerating proteins for some $G\alpha$ subunits. These results suggest that RGS proteins may play important roles in shaping the magnitude and kinetics of physiological events, such as neurosecretion, that involve G protein-modulated Ca channels.

KEY WORDS: muscarinic receptors • voltage-gated calcium channel • neurosecretion • presynaptic inhibition • regulator of G protein signaling proteins

INTRODUCTION

Voltage-gated N-, P/Q-, and R-type Ca channels mediate the presynaptic Ca influx that triggers exocytosis of neurotransmitters (Dunlap et al., 1995; Wu et al., 1998), and modulation of these Ca channel types through G protein-dependent pathways is a major mechanism of presynaptic inhibition (Wu and Saggau, 1997; Koh and Hille, 1997). G protein-dependent modulation of Ca channels may also be influential in the dynamic regulation of membrane excitability in neuronal soma and dendrites (Kavalali et al., 1997). Inhibition of neuronal Ca channels can occur through a variety of membrane-delimited and cytosolic pathways (Hille, 1994). Inhibition via membrane-delimited pathways develops rapidly, within 1 s of agonist binding to receptors (Jones, 1991; Wilding et al., 1995; Zhou et al., 1997), whereas channel modulation via cytosolic pathways is considerably slower (Nargeot et al., 1983; Beech et al., 1991). An interesting property of membrane-

delimited Ca channel inhibition is its transient relief by brief, strong depolarizations, a phenomenon known as prepulse facilitation (Elmslie et al., 1990; Ikeda, 1991). Facilitation of G protein-inhibited Ca channels can also be induced by trains of action potentials (Brody et al., 1997; Park and Dunlap, 1998), indicating that sustained electrical firing could relieve presynaptic inhibition in vivo (Wu and Saggau, 1997). It was recently demonstrated that membrane-delimited inhibition of Ca channels is effected by $G\beta\gamma$ subunits (Herlitze et al., 1996; Ikeda, 1996), and in vitro biochemical experiments have shown that $G\beta\gamma$ can bind to specific regions of the $\alpha 1$ subunits that compose N-, P/Q-, and R-type Ca channels (De Waard et al., 1997; Qin et al., 1997; Zamponi et al., 1997). Such direct interactions are thought to form the molecular basis for membrane-delimited Ca channel inhibition.

Regulators of G protein signaling (RGS)¹ proteins function as GTPase-accelerating proteins (GAPs) for certain classes of heterotrimeric G protein α subunits

Address correspondence to Brett Adams, Department of Physiology & Biophysics, 5-660 Bowen Science Building, University of Iowa, College of Medicine, Iowa City, IA 52242-1109. Fax: 319-335-7330; E-mail: brett-adams@uiowa.edu

¹Abbreviations used in this paper: CCh, carbachol; EGFP, enhanced green fluorescent protein; GAP, GTPase-accelerating protein; GIRK, G protein-gated inward rectifier K; HEK, human embryonic kidney; RGS, regulator of G protein signaling.

(Dohlman and Thorner, 1997; Berman and Gilman, 1998). Recent studies have demonstrated that RGS proteins bind to G α subunits and increase their GTPase activity up to 100-fold, apparently by stabilizing the transition state for GTP hydrolysis (Berman et al., 1996a; Tessmer et al., 1997; Srinivasa et al., 1998a). All known mammalian RGS proteins contain a highly conserved core domain of ~120 amino acids (De Vries et al., 1995; Tesmer et al., 1997) that is sufficient for binding to certain G α subunits and for stimulating their GTPase activity in vitro (De Vries et al., 1995; Dohlman et al., 1996; Faurobert and Hurley, 1997; Popov et al., 1997). Point mutations or small deletions within this domain can destroy the ability of RGS proteins to function as GAPs (Druey and Kehrl, 1997; Popov et al., 1997; Natochin et al., 1998).

Although the in vitro biochemical properties of RGS proteins have been fairly well characterized, relatively little is known about their functions in living cells. However, given the large number (>19) of distinct mammalian RGS proteins that have been identified to date (Berman and Gilman, 1998), it seems reasonable to expect that RGS proteins participate widely in G protein-dependent pathways. Indeed, because the intrinsic rate of GTP hydrolysis by G α subunits is too slow (two to five per minute; Berman et al., 1996b) to account for the kinetics of some G protein-mediated events in nature, GAPs such as RGS are probably essential components of many signaling processes. As one example, RGS9 was recently identified as the GAP for G α t in retinal rods (He et al., 1998) and cones (Cowan et al., 1998), where it is thought to enable rapid termination of the visual phototransduction cascade (Sagoo and Lagnado, 1997). Many RGS proteins are expressed in tissue-specific fashion within the brain (Gold et al., 1997), suggesting their involvement in specialized neuronal functions.

Previous studies have shown that exogenous RGS proteins can significantly alter the behavior of G protein-gated inward rectifier K (GIRK) channels. Surprisingly, RGS proteins accelerate the activation and deactivation of agonist-dependent GIRK currents without altering current amplitudes or steady state dose-response relationships (Dounnik et al., 1997; Saitoh et al., 1997). These effects of RGS proteins are paradoxical because the mechanism most likely to account for accelerated GIRK current kinetics (i.e., an increased rate of G $\beta\gamma$ sequestration by G α -GDP) would also be expected to lower steady state G $\beta\gamma$ concentrations, thereby reducing GIRK current amplitudes and shifting the dose-response curve to higher agonist concentrations (Dounnik et al., 1997), neither of which is observed. Thus, the mechanisms by which RGS proteins influence ion channel function are not well understood.

Here we examine whether RGS proteins can modulate the receptor-mediated inhibition of N-type Ca

channels. These voltage-gated channels perform essential functions in neurosecretion and other neuronal activities, and they are strongly inhibited by activated G proteins. We find that coexpression of RGS3T, RGS3, or RGS8 shifts the steady state dose-response curve to higher agonist concentrations, thereby attenuating the G protein-dependent inhibition of N-type channels at submaximal agonist concentrations. Coexpression of RGS3T also changes the kinetics of N-type channel modulation by slowing channel reinhibition after a facilitating prepulse and by speeding channel recovery from receptor-mediated inhibition after agonist removal. RGS proteins thus modify the amplitudes as well as the kinetics of N-type Ca channel inhibition. These results suggest that RGS proteins may significantly influence synaptic transmission and other physiological processes involving G protein-modulated, voltage-gated Ca channels.

MATERIALS AND METHODS

Cell Culture and Transfection

Human embryonic kidney (HEK293) cells (American Type Culture Collection) were maintained under standard conditions. The culture medium contained 90% DMEM, 10% fetal bovine serum, and 50 μ g/ml gentamicin. Every 2–3 d, the cells were briefly trypsinized and replated onto 12-mm round glass coverslips. Transfections were by CaPO₄ precipitation (CellPfect Kit, Pharmacia LKB Biotechnology, Inc.). Control cells were transfected with expression plasmids encoding Ca channel α 1B (rabbit brain; Fujita et al., 1993), α 2 (rat brain; Kim et al., 1992), and β 3 (rabbit brain; Witcher et al., 1993) subunits at 0.5 μ g cDNA each per dish, human (m2) muscarinic receptor (Peralta et al., 1987) at 0.025 μ g cDNA per dish, and human CD8 protein (59565; American Type Culture Collection) or jellyfish enhanced green fluorescent protein (EGFP; Clontech) at 0.05 μ g per dish. RGS-expressing cells were transfected with the above plasmids plus expression vectors encoding human RGS3 or RGS3T (Chatterjee et al., 1997) or rat RGS8 (Saitoh et al., 1997) protein at 0.25 μ g cDNA per dish. Successfully transfected cells were visually identified by labeling with 4.5- μ m diameter paramagnetic beads coated with anti-CD8 antibody (Dynal Corp.) or by green fluorescence under ultraviolet illumination. Labeled cells were selected for electrophysiological analysis.

Expression Plasmids and Mutant Construction

α 1B was in the expression vector pKCRH2 (Mishina et al., 1984), α 2 was in pMT2 (Genetics Institute), β 3 was in pcDNA3 (Invitrogen Corp.), RGS3 and RGS3T were in pCR3.1 uni (Invitrogen Corp.), RGS8 was in pCI (Promega Corp.), m2 receptor was in pRK5. RGS3T is generated from an alternative start codon located at nucleotides 940–942 within the RGS3 coding sequence, and it thus lacks the first 313 amino acids of RGS3 (see Fig. 2 B; Druey et al., 1996; Chatterjee et al., 1997). The deletion mutant Δ RGS8 was constructed by digesting pCI-RGS8 with AvrII and TfiI, the over-hanging ends were filled-in using T4 DNA polymerase, and the plasmid was recircularized using T4 DNA ligase. In Δ RGS8, nucleotides 167–478 of the coding sequence of RGS8 have been excised without altering the reading frame, as confirmed by nucleotide sequencing. As a consequence of this dele-

tion, amino acid residues 57–160 are missing from the translated Δ RGS8 protein.

A cDNA construct (“RGS8 core”) encoding the conserved RGS domain was made by PCR-amplifying nucleotides 133–516 from the coding sequence of RGS8 (Saitoh et al., 1997). The forward primer was 5'-GCGCGCAAGCTTACCACCATGCTCTCCACAG-AAGAAGCGACG-3' and the reverse primer was 5'-CGCCGC-GGATCCCAGCAGATCTAAGTACATTTTGGACCT-3'. The amplified product was sequenced and found to be identical to the core domain of RGS8 (Saitoh et al., 1998). The coding sequence of EGFP (nucleotides 616–1329) was amplified from pEGFP-C3 using the forward primer 5'-GCGGCGGGATCCGTCAGCAAG-GGCGAGGAGCTGTTC-3' and the reverse primer 5'-GCGGCG-GAATTCCTACTTGTACAGCTCGTCCATGCCGAG-3'. The RGS8 core and EGFP amplification products were digested with Hind-III/BamHI and BamHI/EcoRI, respectively, and ligated together into the HindIII/EcoRI sites of pcDNA3.1+. The resulting plasmid (RGS8 core-EGFP) encodes methionine followed by the conserved core domain of RGS8 (amino acid residues 45–172; Saitoh et al., 1997), fused to the amino terminus of EGFP. The original start codon of EGFP was mutated (by the forward primer) to prevent its independent translation. The other RGS proteins (RGS3T, RGS3, RGS8, and Δ RGS8) were subcloned into the appropriate version of pEGFP (C1, C2, or C3) such that the RGS protein was fused in-frame to the carboxyl terminus of EGFP. In each case, the composition of the chimeric or mutant plasmid was confirmed by restriction digests and nucleotide sequencing.

Voltage-Clamp Recordings

Large-bore patch pipettes were pulled from 100- μ l borosilicate micropipettes (53432-921; VWR Scientific Products) and filled with a solution containing (mM) 155 CsCl, 10 Cs₂EGTA, 4 Mg-ATP, 0.32 Li-GTP, and 10 HEPES, pH 7.4 with CsOH. Aliquots of the pipette solution were stored at -80°C , kept on ice after thawing, and filtered at 0.22 μm immediately before use. Pipette tips were coated with paraffin to reduce capacitance, and then fire-polished; filled pipettes had d.c. resistances of 1.0–1.5 M Ω . The bath solution contained (mM) 145 NaCl, 40 CaCl₂, 2 KCl, and 10 HEPES, pH 7.4 with NaOH. Residual pipette capacitance was compensated in the cell-attached configuration using the negative capacitance circuit of the amplifier. No corrections were made for liquid junction potentials. Carbachol (CCh) or CdCl₂ was dissolved directly in the bath solution; application of these substances was by bath exchange or by local superfusion using a pressurized macropipette positioning within 3 mm of the cell under study. Temperature (20–22 $^{\circ}\text{C}$) was continuously monitored using a miniature thermocouple placed in the recording chamber.

Ca currents were recorded using the whole-cell patch-clamp technique (Hamill et al., 1981). The steady holding potential was -90 mV. Unless otherwise noted, step depolarizations to various test potentials were delivered every 10 or 15 s; these stimulation rates were sufficiently low that cumulative inactivation of the N-type Ca current was minimized. Currents were filtered at 2–10 kHz using the built-in Bessel filter (four-pole low pass) of an amplifier (Axopatch 200A or 200B; Axon Instruments) and sampled at 10–50 kHz using a Digidata 1200 analogue-to-digital board installed in a Gateway 486 or Pentium computer. The pCLAMP software programs Clampex and Clampfit (v. 6.0.3) were used for data acquisition and analysis, respectively. Curve fits and figures were made using the software program Origin (v. 5.0). Linear cell capacitance (C) was determined by integrating the area under the whole-cell capacity transient, evoked by clamping from -90 to -80 mV with the whole-cell capacitance compensation circuit of the amplifier turned off. The average value of C was 23 \pm

1 pF (mean \pm SEM, $n = 139$ cells). Series resistance (R_s) was calculated as $\tau \times (1/C)$, where τ was the time constant for decay of the whole-cell capacity transient. The average values of τ and R_s , measured before electronic compensation, were 73 ± 4 μs and 3.0 ± 0.1 M Ω , respectively ($n = 139$). When appropriate, τ and R_s were reduced by 35–80% using the series resistance compensation circuit of the amplifier. Maximal Ca current amplitudes, measured at the time of peak inward current, were 1.74 ± 0.17 nA ($n = 139$; test potential +30 mV); thus, voltage errors were typically <5 mV. For example, in 77 cells for which compensated R_s was known, the maximum voltage error was 2.4 ± 0.3 mV. The d.c. resistance of the whole-cell configuration was routinely >1 G Ω . All currents were corrected for linear capacitance and leakage currents using $-P/6$ or $-P/4$ subtraction. Statistical comparisons were by two-tailed, unpaired t tests (two groups) or by one-way analysis of variance (three or more groups), with $P < 0.05$ considered significant.

RESULTS

RGS3T Attenuates the Receptor-mediated Inhibition of N-type Ca Channels

To determine whether RGS proteins can influence the G protein-dependent modulation of voltage-gated Ca channels, we examined the receptor-mediated inhibition of N-type Ca currents in the presence or absence of coexpressed RGS3T protein. Fig. 1 shows whole-cell currents recorded from HEK293 cells expressing cloned N-type Ca channels (composed of transfected $\alpha 1\text{B}$, $\alpha 2$, and $\beta 3$ subunits) and muscarinic (m2) receptors. Exposure to 1 μM carbachol (CCh) produced a rapid (within 1–2 s) and substantial inhibition of the N-type Ca current. This inhibition was mediated by exogenous m2 receptors, as CCh had no effects in cells not transfected with receptor cDNA (data not shown). During inhibition with CCh, N-type currents displayed slowed activation (Marchetti et al., 1986; Wanke et al., 1987) and current-voltage relationships that were shifted to positive potentials by ~ 10 mV compared with currents recorded from the same cells before CCh exposure (Fig. 1 B). The CCh-inhibited currents were facilitated by prepulses to +100 mV (see Fig. 4), demonstrating that a substantial fraction of the inhibition was voltage dependent (Bean, 1989). These characteristics suggest that inhibition of N-type channels by activated m2 receptors occurred, at least in part, through a membrane-delimited pathway (Hille, 1994).

The same concentration of CCh (1 μM) also inhibited the N-type Ca current in cells transfected with RGS3T protein (Fig. 1). However, the magnitude of inhibition was significantly smaller than in control cells. Thus, in RGS3T-expressing cells, 1 μM CCh reduced the N-type current to $36.4 \pm 3.2\%$ ($n = 6$; test potential, +30 mV) of its initial amplitude before CCh exposure, whereas in control cells not transfected with RGS3T the N-type current was reduced to $21.1 \pm 3.6\%$ ($n = 9$) of the initial amplitude ($P \leq 0.01$). These re-

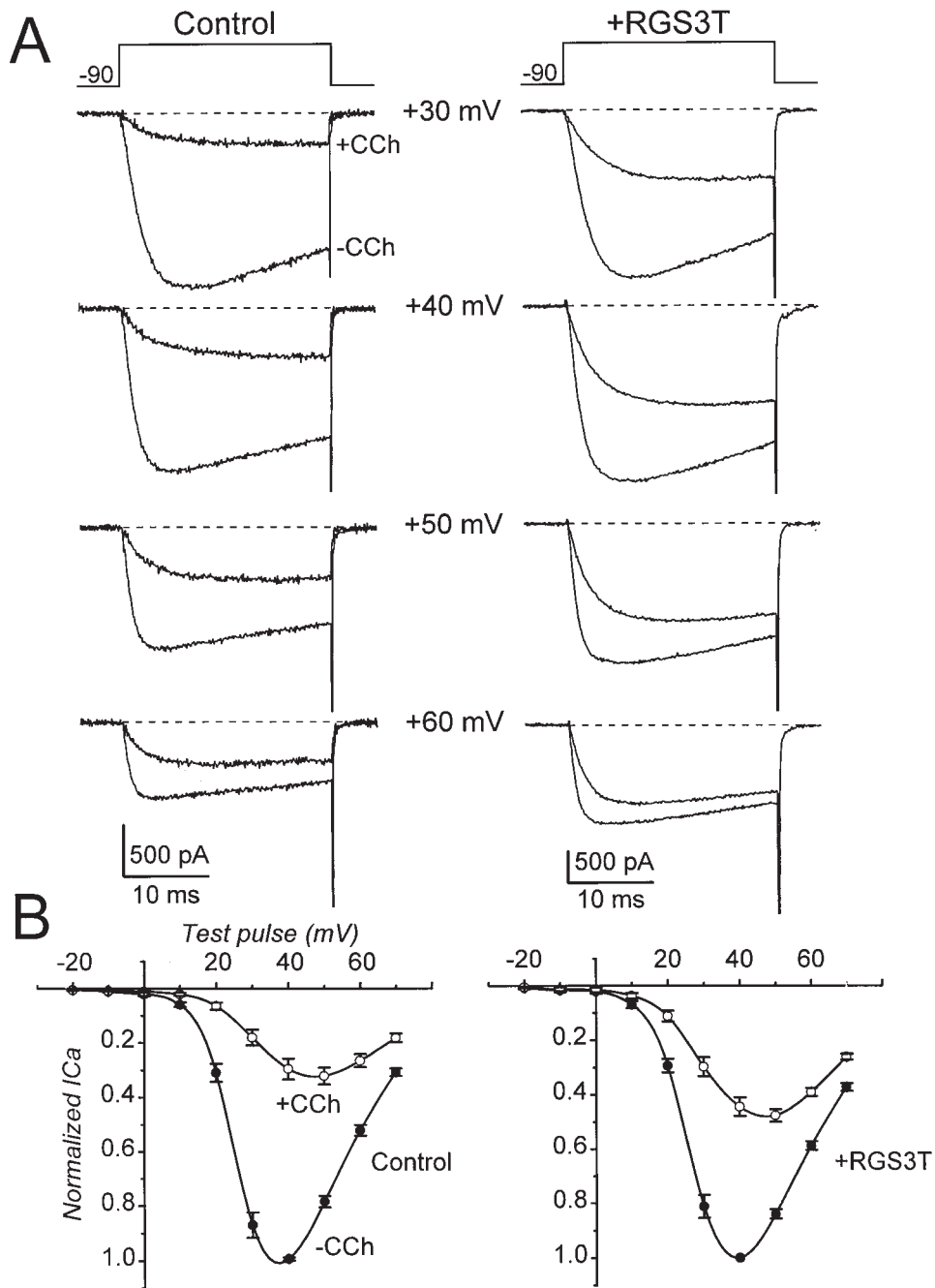


FIGURE 1. Coexpression of RGS3T attenuates Ca channel inhibition. (A) Whole-cell Ca currents were recorded from control (left) and RGS3T-expressing (right) cells before and during exposure to 1 μ M CCh. The steady holding potential was -90 mV; test potentials are indicated. Data files 98422002, 98422003, 98417009, and 98417011. Linear cell capacitances (C) were 19 (Control) and 34 (RGS3T) pF. Series resistances (R_s) were 1.4 (Control) and 1.2 (RGS3T) M Ω . (B) Average I-V relationships of Ca currents recorded before (\bullet) and during (\circ) exposure to 1 μ M CCh in nine control cells (left) and six RGS3T-expressing cells (right).

sults demonstrate that RGS3T can attenuate the receptor-mediated inhibition of N-type Ca channels.

Effects of Other RGS Proteins

To determine whether other RGS proteins could also attenuate Ca channel inhibition, we tested RGS3 and RGS8. Exposure to 1 μ M CCh reduced the Ca current in RGS3-expressing cells to $47.0 \pm 8.8\%$ ($n = 6$; test potential, +30 mV) of the initial amplitude, and in RGS8-expressing cells the Ca current was reduced to $39.3 \pm$

4.6% ($n = 6$). These magnitudes of inhibition are statistically indistinguishable from that measured in cells expressing RGS3T ($P = 0.6$). For comparison, in control cells not transfected with RGS protein, the N-type Ca current was reduced to $21.5 \pm 1.4\%$ ($n = 56$) of its initial amplitude (Fig. 2 A). Thus, expression of RGS3 or RGS8 also attenuated Ca channel inhibition.

The above experiments were performed using cells that had been simultaneously cotransfected with separate expression plasmids encoding Ca channel $\alpha 1B$, $\alpha 2$, and $\beta 3$ subunits, m2 receptors, CD8 or EGFP, and one

kind of RGS protein. In these experiments, we assumed that cells expressing measurable N-type Ca currents and m2 receptors (as evidenced by CCh-induced inhibition of the Ca current) also expressed the transfected RGS protein. Although this assumption is reasonable, the effects of RGS might have been underestimated if our data included measurements from cells not expressing transfected RGS. To address this potential problem, we constructed chimeric cDNAs encoding fusions of EGFP with RGS3T, RGS3, and RGS8, which allowed us to visually confirm their expression in individual cells. As summarized in Fig. 2 A, the magnitude of Ca current inhibition in cells expressing EGFP-RGS fusion proteins was comparable with that in cells transfected with wild-type RGS proteins. These results suggest that cells included in our analysis did indeed express the transfected RGS proteins and also that fusion of EGFP to RGS does not interfere with its biological activity.

The Conserved RGS Core Domain Is Necessary and Sufficient for Attenuation of Ca Channel Inhibition

All mammalian RGS proteins contain a conserved core domain of ~120 amino acids (De Vries et al., 1995; Tesmer et al., 1997) that binds to G α and has GAP activity in vitro (De Vries et al., 1995; Dohlman et al., 1996; Popov et al., 1997; Faurobert and Hurley, 1997). Even small deletions within the core domain of RGS proteins destroy their ability to act as GAPs (Popov et al., 1997). To examine the role of the core domain in N-type Ca channel inhibition, we constructed two mutant RGS proteins (Fig. 2 B). In one mutant (Δ RGS8), the majority of the core domain (amino acid residues 57–160) was deleted from RGS8 without altering the reading frame of the remaining protein. The other mutant (RGS8 core) consisted of the conserved core domain of RGS8 (amino acid residues 45–172). Unambiguous identification of cells expressing the RGS8 core mutant was made possible by fusing it to EGFP.

As shown in Fig. 2 A, expression of Δ RGS8 failed to attenuate the receptor-mediated inhibition of N-type current. Thus, in cells transfected with Δ RGS8, the Ca current was inhibited to $23.8 \pm 2.3\%$ ($n = 12$) of its initial amplitude by $1 \mu\text{M}$ CCh. A similar result was obtained in cells expressing Δ RGS8 fused to EGFP, where $1 \mu\text{M}$ CCh reduced the current to $16.4 \pm 1.2\%$ ($n = 10$) of its initial amplitude. These levels of Ca current inhibition are not different from that observed in control cells ($P = 0.27$), indicating that the core domain of RGS8 is required for its effect on Ca channels.

In contrast, cells expressing the RGS8 core domain exhibited the same levels of Ca current inhibition as cells expressing full-length RGS proteins (Fig. 2 A). Thus, $1 \mu\text{M}$ CCh reduced the N-type current in cells expressing RGS8 core EGFP to $35.1 \pm 2.6\%$ ($n = 10$) of

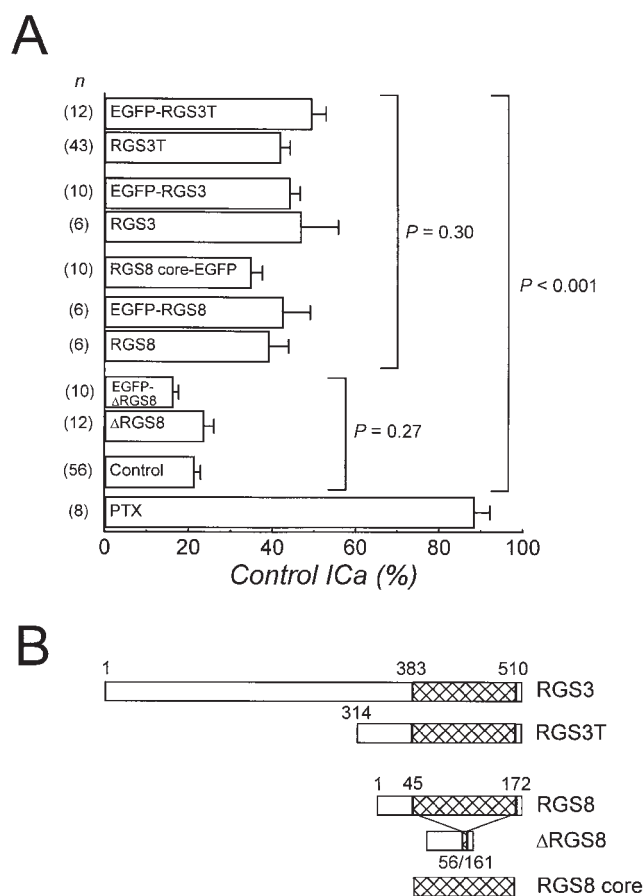


FIGURE 2. Receptor-mediated inhibition of N-type Ca current is attenuated by coexpression of various RGS proteins. (A) Ca currents were recorded during steady state inhibition with $1 \mu\text{M}$ CCh. Test pulses to $+30$ mV were delivered from a steady holding potential of -90 mV. Inhibition is expressed as (current recorded during CCh exposure/current recorded from the same cell immediately before CCh exposure) $\times 100$. EGFP, data obtained exclusively from fluorescent green cells expressing EGFP-tagged RGS proteins. PTX, cells were treated with pertussis toxin (200 ng/ml) in the culture media for 24 h before experiments. The number of cells in each group is indicated in parentheses. P indicates the outcome of analysis of variance performed upon the indicated groups. The maximal Ca current densities (measured at $+30$ mV) were 96 ± 13 pA/pF in control cells ($n = 56$), 76 ± 9 pA/pF in RGS3T-expressing cells ($n = 55$), 41 ± 12 pA/pF in RGS3-expressing cells ($n = 16$), 153 ± 78 pA/pF in RGS8-expressing cells ($n = 12$), 89 ± 27 pA/pF in Δ RGS8-expressing cells ($n = 22$), and 46.5 ± 18.5 pA/pF in cells expressing RGS8 core-EGFP ($n = 10$). There was no correlation between current density and inhibition produced by $1 \mu\text{M}$ CCh, either in control cells ($r^2 = 0.04$, $n = 56$) or in cells expressing RGS3T, RGS3, or RGS8 ($r^2 = 0.009$, $n = 83$). (B) Schematic representation of the expressed RGS proteins. Numbers refer to amino acid residues. The approximate location of the conserved RGS core domain is indicated by dark shading. RGS3T lacks the first 313 amino acids of RGS3, but is otherwise identical. Δ RGS8 is missing amino acid residues 57–160, which compose the major portion of the conserved RGS domain. The RGS8 core is the conserved domain of RGS8 (amino acids 45–172).

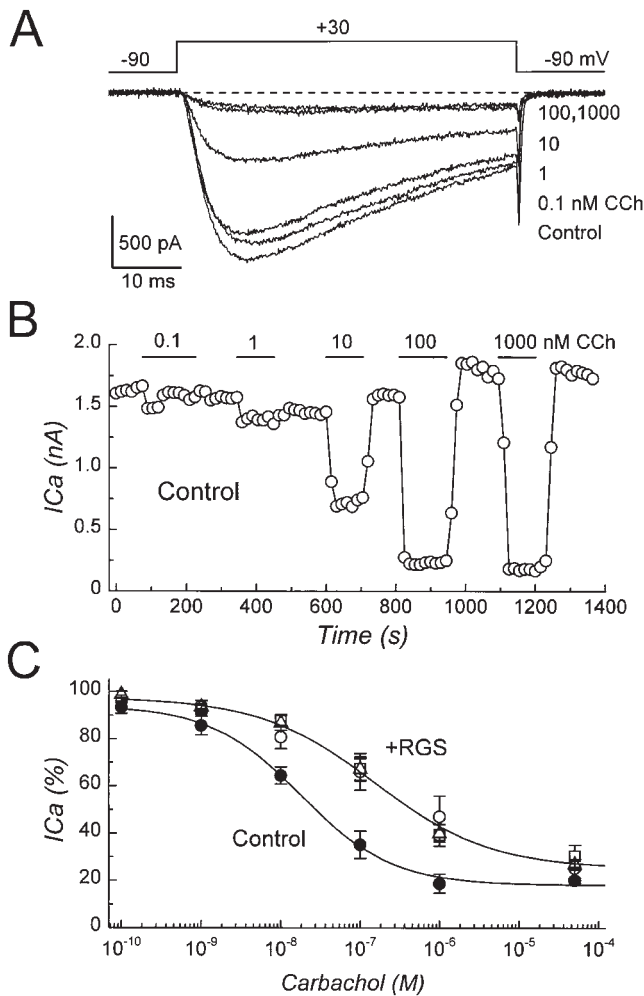


FIGURE 3. Coexpression of RGS proteins shifts the dose–response curve of Ca channel inhibition to higher CCh concentrations. (A) Representative dose–response experiment in a control cell transfected with $\alpha 1B$, $\alpha 2$, and $\beta 3$ Ca channel subunits and m2 receptors. Shown are selected Ca currents recorded before (Control) and during exposure to various concentrations of CCh. Data file 97D15003; $C = 51$ pF; $R_s = 3.0$ M Ω . (B) Plot of peak current amplitudes during a dose–response experiment. Currents were evoked by step depolarizations to +30 mV at 15-s intervals. Applications of various concentrations of CCh are indicated by horizontal bars. Same cell and experiment as in A. (C) Dose–response relationships in control (●) and in RGS-expressing cells (other symbols: ○, RGS3; △, RGS3T; □, RGS8). Error bars represent \pm SEM for 4–11 cells in each group. The highest CCh concentration used was 50 μ M. The dose–response curves represent the following logistic equation: I_{Ca} (% of initial) = $(100 - D) / [1 + ([CCh] / EC_{50})^n]$, where D , the percentage of N-type Ca current that could not be inhibited, is 18.1 (Control) or 25.2 (RGS), and the value of the exponent n is 0.76 (Control) or 0.60 (RGS). The curve for RGS-expressing cells was fit to the average Ca current inhibition at each CCh concentration, using pooled data from cells expressing RGS3, RGS3T, or RGS8. The values of EC_{50} were 18 nM (control cells) and 153 nM (RGS-expressing cells).

its initial amplitude, which is indistinguishable ($P = 0.3$) from the inhibition in cells expressing full-length RGS3T, RGS3, or RGS8. This result is significant because it indicates that the RGS core domain is sufficient for its effects on N-type Ca channel inhibition. Additionally, this finding is consistent with the possibility that the GAP activity of RGS proteins accounts for their influence on G protein–mediated Ca channel modulation.

RGS Proteins Shift the Dose–Response Curve to Higher Agonist Concentrations

The results presented in Figs. 1 and 2 indicate that RGS proteins attenuate receptor-mediated inhibition of N-type Ca channels during exposure to a single concentration (1 μ M) of CCh. To determine whether this effect is due to a shift in the dose–response relationship, we measured Ca current inhibition during exposure to a wide range (0.1 nM–50 μ M) of agonist concentrations. As expected, increasing concentrations of CCh caused a progressive inhibition of N-type Ca current (Fig. 3 A). Importantly, inhibition did not desensitize during the CCh exposures used in our experiments (1–2 min) and it was fully reversible upon CCh washout (Fig. 3 B).

Fig. 3 C shows the steady state dose–response curves for control and RGS-expressing cells. Because the average Ca current inhibition at any given CCh concentration did not differ significantly among cells expressing RGS3T, RGS3, or RGS8 ($P > 0.3$), data from these three groups were pooled to obtain a single average value of Ca current inhibition at each CCh concentration, and these average values were fit with a logistic equation (see Fig. 3, legend) that provided an estimate of the concentration of CCh producing half-maximal inhibition (EC_{50}). In RGS-expressing cells the estimated EC_{50} was 153 nM, nearly 10-fold higher than the EC_{50} in control cells (18 nM). Thus, coexpression of RGS protein shifted the dose–response relationship of N-type Ca channel inhibition to \sim 10-fold higher CCh concentrations. The fact that inhibition in control cells reached maximal levels at 1 μ M CCh, whereas maximal inhibition in RGS-expressing cells required higher concentrations, suggests that Ca current inhibition saturates at lower CCh concentrations than m2 receptor occupancy.

The Decay of Prepulse Facilitation Is Slower in the Presence of RGS

Membrane-delimited inhibition of N-type Ca channels seems to be attributable to $G\beta\gamma$ rather than $G\alpha$ subunits (Herlitze et al., 1996; Ikeda, 1996; but see Diversé-Pierluissi et al., 1997). The RGS-dependent shift in the dose–response curve (Fig. 3 C) suggests that the concentration of inhibiting G proteins, presumably $G\beta\gamma$, was lower in RGS-expressing cells than in control cells

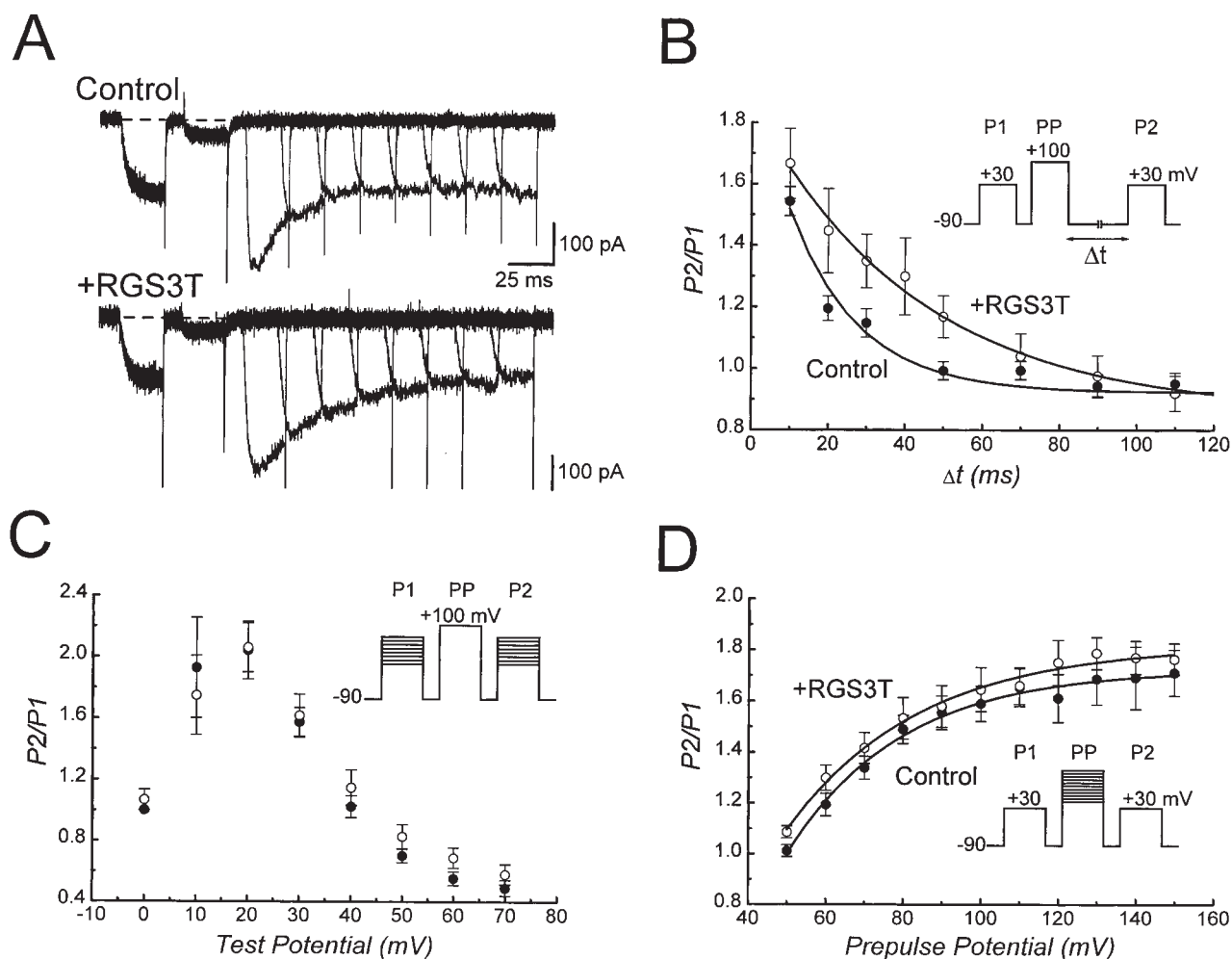


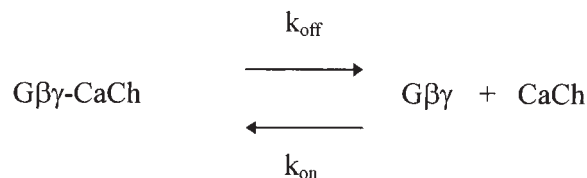
FIGURE 4. RGS proteins slow the decay of prepulse facilitation. (A) Representative experiments with control and RGS3T-expressing cells during inhibition of the N-type Ca current by 1 μ M CCh. Control cell: data file 98528018; $C = 10$ pF; $R_s = 2.5$ M Ω . RGS3T-expressing cell: data file 98522023; $C = 19$ pF; $R_s = 1.4$ M Ω . The voltage protocol is diagrammed in B. The interval between P1 and the prepulse (PP) to +100 mV was fixed at 10 ms; the interval between PP and P2 (Δt) varied from 10 to 110 ms. Successive episodes of this voltage protocol were delivered at 15 s intervals. (B) Slower decay of prepulse facilitation in RGS3T-expressing cells. Symbols represent data averaged from 9–12 cells. The single exponential curves were fit to average values of facilitation (i.e., P2/P1 ratio) at each Δt . The time constant for the decay of facilitation was 17.7 ms for control cells (\bullet) and 42.7 ms for RGS3T-expressing cells (\circ). Ca currents in control cells were inhibited by $78.3 \pm 1.1\%$ ($n = 12$) and those in RGS3T-expressing cells were inhibited by $57.3 \pm 3.4\%$ ($n = 9$); this difference was significant ($P < 0.0001$; t test). (C) The voltage dependence of facilitation is unchanged by expression of RGS3T. The voltage protocol is diagrammed. P1 and P2 varied between 0 and +70 mV; P1, PP, and P2 were each 25 ms in duration; P1-PP and PP-P2 intervals were 10 ms in duration. Symbols represent data averaged from six to nine cells. (D) The dependence of facilitation on prepulse potential is similar in control and RGS3T-transfected cells. Intervals between voltage steps as in C. The amplitude of PP varied from +50 to +150 mV in 10-mV increments. Symbols represent data averaged from six to seven cells. In each panel, error bars indicate \pm SEM.

during a given level of receptor activation. To test this possibility, we measured the decay of prepulse facilitation in both groups. It is thought that prepulse facilitation reflects the voltage-dependent relief of inhibition; G $\beta\gamma$ subunits are thought to dissociate from Ca channels when the channels change their shape in response to depolarization. Upon repolarization, facilitation decays at an exponential rate (Ikeda, 1991), presumably as G $\beta\gamma$ subunits reassociate with channels and reestablish inhibition (Lopez and Brown, 1991; Boland and

Bean, 1993; Golard and Siegelbaum, 1993; Elmslie and Jones, 1994). The decay of facilitation (i.e., reinhibition) can be measured by varying the interval between the facilitating prepulse and a subsequent test depolarization (see Fig. 4 B). The fact that the decay of facilitation can be adequately described by a single exponential function is consistent with the idea that rebinding of a single G $\beta\gamma$ dimer is sufficient to reestablish inhibition (Zamponi and Snutch, 1998). Previous studies have shown that the rate at which facilitation decays is

proportional to the magnitude of Ca current inhibition (Golard and Siegelbaum, 1993; Elmslie and Jones, 1994), suggesting that the rate of reinhibition is proportional to G $\beta\gamma$ concentration. This idea is supported by the dependence of reinhibition rate upon intracellular G $\beta\gamma$ concentration (Zamponi and Snutch, 1998).

Prepulse facilitation and reinhibition can be represented by a simple two-state model (Scheme I). During a prepulse to +100 mV, G $\beta\gamma$ should dissociate from Ca channels (i.e., k_{off} should dominate) and reassociation should be minimal (i.e., k_{on} should be very small). Upon repolarization to -90 mV, G $\beta\gamma$ subunits should overwhelmingly reassociate with Ca channels, and inhibition should be reestablished with a rate of $k_{\text{on}} [\text{G}\beta\gamma] + k_{\text{off}}$. At -90 mV, k_{off} is expected to be very small and can thus be ignored. If k_{on} is similar in control and RGS-expressing cells, any differences in the rates of reinhibition should primarily reflect differences in [G $\beta\gamma$]. By this logic, if G $\beta\gamma$ concentrations are lower in RGS-expressing cells, then reinhibition should proceed more slowly than in control cells. The results presented in Fig. 4, A and B, support this prediction: the time constant for decay of facilitation was 40.5 ± 5.8 ms ($n = 9$) in RGS3T-expressing cells vs. 22.7 ± 1.7 ms ($n = 12$) in control cells ($P < 0.005$). The slower reinhibition in the presence of RGS3T is consistent with lower G $\beta\gamma$ concentrations during a given level of m2 receptor activation.



(SCHEME I)

In contrast to the decay of facilitation, neither the voltage dependence of facilitation (Fig. 4 C) nor the relationship between facilitation and prepulse potential (Fig. 4 D) were altered by coexpression of RGS3T. Because these latter measurements primarily reflect the voltage-dependent dissociation of G $\beta\gamma$ subunits from Ca channels, they are not expected to depend upon G $\beta\gamma$ concentration.

As shown in Fig. 4, similar magnitudes of prepulse facilitation were obtained for N-type Ca currents in control and RGS3T-expressing cells. Upon first consideration, this result may seem at odds with the different magnitudes of receptor-mediated inhibition in control vs. RGS-expressing cells (e.g., Fig. 2 A). However, there are at least two reasons why inhibition and prepulse facilitation might not agree quantitatively. First, rapid reinhibition of Ca channels after the prepulse will tend to reduce the amplitude of P2 currents; reinhibition will be fastest and will cause the largest underestimation of facilitation for the most strongly inhibited currents (i.e., those in

control cells). Second, some of the inhibition produced by CCh is apparently voltage independent and therefore not relieved by a prepulse (Luebke and Dunlap, 1994).

RGS Proteins Speed the Recovery of Ca Channels from Inhibition

In the case of GIRK channels, the most prominent effect of exogenous RGS proteins is to accelerate the kinetics of agonist-dependent macroscopic currents (Doupnik et al., 1997; Saitoh et al., 1997). To investigate whether RGS proteins are capable of altering the kinetics of Ca channel modulation, we monitored the recovery of N-type channels from inhibition. Representative experiments are illustrated in Fig. 5. The time required for recovery from receptor-mediated inhibition was measured as the interval between CCh washout and return of the N-type current to 90% of its final amplitude (t_{90}). After washout of 1 μM CCh, the average value of t_{90} was 15.8 ± 2.5 s ($n = 12$) in control cells, whereas it was only 8.0 ± 1.2 s ($n = 12$) in cells expressing RGS3T ($P = 0.011$). A similar difference was also obtained using a much lower (20 nM) concentration of CCh; under these conditions, t_{90} was 12.3 ± 2.8 s ($n = 6$) in control cells and 4.1 ± 0.4 s ($n = 5$) in RGS3T-expressing cells ($P = 0.027$). These results indicate that coexpression of RGS3T significantly speeds the recovery of N-type Ca channels from receptor-mediated inhibition. As shown in Fig. 5 C, recovery of the N-type current from block by 0.5 mM Cd was complete within ~ 2 s, demonstrating that our measurement of t_{90} was not limited by the rate of solution exchange.

DISCUSSION

RGS Proteins Attenuate the Magnitude of Ca Channel Inhibition

Our motive in performing the present study was to determine whether RGS proteins could play significant roles in the G protein-dependent modulation of neuronal Ca channels. We have found that coexpression of RGS3T, RGS3, or RGS8 significantly attenuates N-type Ca channel inhibition produced by activation of G protein-coupled muscarinic (m2) receptors. This effect of RGS proteins results primarily from a shift in the steady state dose-response relationship of Ca channel inhibition to ~ 10 -fold higher agonist concentrations. That RGS proteins should produce such a shift is perhaps not surprising considering their remarkable GAP activity. Nevertheless, a shift in the dose-response curve is noteworthy because previous studies have found that expression of RGS1, RGS3, RGS4, or RGS8 fails to shift the dose-response relationship of GIRK currents (Doupnik et al., 1997; Saitoh et al., 1997). Interestingly, the three different RGS proteins that we examined (RGS3T, RGS3, and RGS8) shifted the dose-response

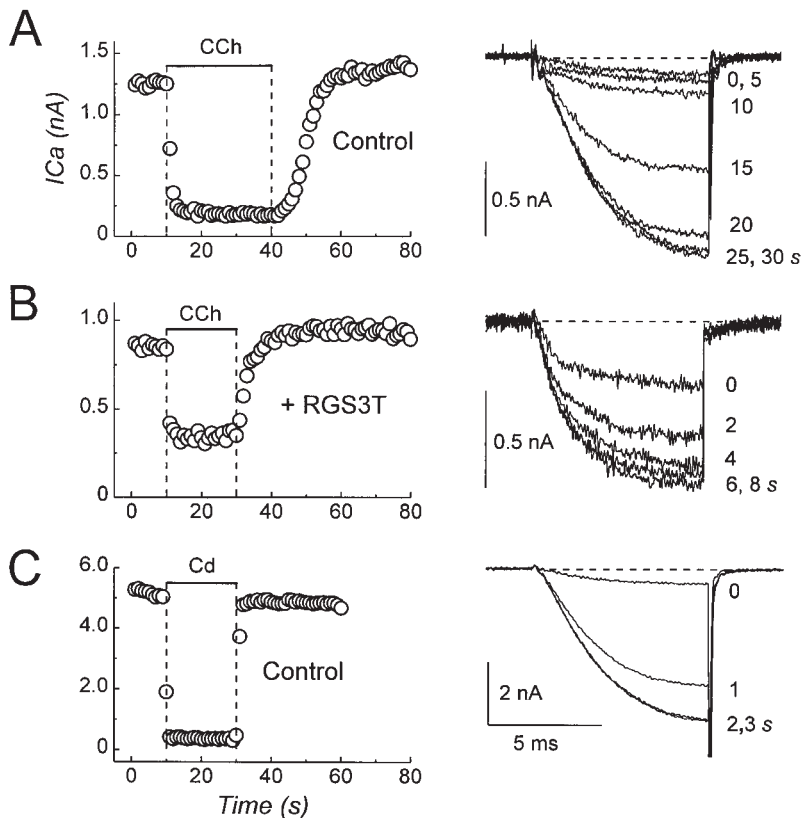


FIGURE 5. RGS3T accelerates the recovery of N-type Ca channels from inhibition. (A) Representative time course for recovery of N-type Ca current in a control cell. Ca currents were evoked by 8-ms step depolarizations to +30 mV, delivered at 1 Hz. The holding potential was -90 mV. The times (in seconds) after CCh (1 μ M) washout are indicated to the right of each Ca current waveform. Data file 98410002; $C = 12$ pF; $R_s = 2.1$ M Ω . (B) Recovery in an RGS3T-expressing cell. Data file 98408013; $C = 30$ pF; $R_s = 1.0$ M Ω . (C) Representative time course for recovery of N-type current from Cd block (0.5 mM) in a control cell. Recovery is complete within 2 s. Data file 98319017; $C = 21$ pF; $R_s = 1.6$ M Ω . In these recovery experiments, a puffer pipette constructed from theta tubing was positioned within 3 mm of the cell under study. One barrel of the theta tubing contained CCh or Cd dissolved directly in external solution. The other barrel contained external solution without CCh or Cd. The inflows to both barrels were under positive pressure. By switching between barrels, CCh or Cd could be rapidly applied or washed from the cell under study. Flow of the bath solution was continuous and orthogonal to the puffer pipette.

curve by the same amount and therefore attenuated Ca current inhibition to very similar degrees (Fig. 2 A). The equivalent effects of RGS3, RGS3T, and RGS8 may indicate that structural differences among these three proteins are not relevant to their function in HEK293 cells. However, subtle differences in the biological activities of RGS3T, RGS3, and RGS8 might not have been apparent if these proteins were each expressed at saturating levels in our experiments.

A rightward shift in the dose-response curve, produced by GTPase-activating proteins, is not without precedent. For example, Berstein et al. (1992) and Bidlecome et al. (1996) found that PLC- β 1, which is a GAP as well as an effector for G α_q , shifted the dose-response curve of GTP hydrolysis to \sim 10-fold higher CCh concentrations. Similarly, Yan et al. (1997) observed that coexpression of RGS4 shifted the dose-response curve for activation of mitogen-activated protein kinase to higher agonist concentrations. Taken together, these results obtained with PLC- β 1 and RGS proteins suggest that GAPs could be expected to produce a rightward shift in the dose-response curve.

RGS Proteins Alter the Kinetics of Ca Channel Modulation

We also find that RGS proteins can change the kinetics of Ca channel modulation. Thus, the facilitation produced by a depolarizing prepulse lasted significantly

longer in RGS-expressing cells than in control cells (Fig. 4 B). Experimentally, the decay of prepulse facilitation can be slowed by reducing the level of receptor activation (Elmslie and Jones, 1994) or by coexpressing a protein (e.g., β ARK1 peptide) that is able to bind and sequester G $\beta\gamma$ subunits (Delmas et al., 1998). Because the decay of facilitation is thought to reflect reassociation of G $\beta\gamma$ with Ca channels, the rate at which facilitation decays should be proportional to the concentration of G $\beta\gamma$ (Golard and Siegelbaum, 1993; Elmslie and Jones, 1994; Zamponi and Snutch, 1998). Our finding that reinhibition of Ca channels is slower in RGS-expressing cells is consistent with the interpretation that G $\beta\gamma$ concentrations were lower in those cells. This interpretation is also in agreement with a previous study demonstrating that the amplitudes of basal, agonist-independent GIRK currents were reduced by coexpression of RGS4 (Saugstad et al., 1998).

Our experiments also indicate that RGS3T speeds the recovery of Ca channels from inhibition (Fig. 5). In principle, the recovery time we measured could be determined by a number of steps including unbinding of CCh from m2 receptors, deactivation of receptors, hydrolysis of bound GTP by G α subunits, and resequestration of G $\beta\gamma$ by GDP-G α . Of these steps, only hydrolysis of GTP is thought to be rate limiting, and this is the step accelerated by RGS proteins (Berman and Gilman, 1998). The faster recovery from inhibition in RGS3T-

expressing cells (Fig. 5) is likely to result from lower concentrations of G $\beta\gamma$ and also from faster resequestration of G $\beta\gamma$ subunits upon receptor deactivation.

RGS Proteins Appear to Have Different Effects on GIRK and N-type Ca Channels

Although GIRK and N-type Ca channels are both modulated by G proteins, the details of their modulation are quite different. For example, GIRK channels are opened and closed by the binding and unbinding, respectively, of G $\beta\gamma$ (Reuveny et al., 1994; Wickman et al., 1994). Consequently, activation and deactivation kinetics of macroscopic GIRK currents are closely related to the kinetics of G $\beta\gamma$ binding to and unbinding from GIRK channels. In contrast, N-type Ca channels are gated by changes in membrane potential. Binding of G $\beta\gamma$ inhibits the activation of N-type Ca channels by slowing the voltage-dependent translocation of gating charges (Jones et al., 1997) and by increasing the first latency of channel opening (Carabelli et al., 1996; Patil et al., 1996). Thus, activation and deactivation of Ca channels are primarily voltage-dependent processes that can be rapidly and reversibly modified by interactions with G $\beta\gamma$.

As previously discussed by Doupnik et al. (1997), the effects of RGS proteins on GIRK macroscopic current kinetics could be explained by an increased rate of dissociation (k_{off}) of G $\beta\gamma$ subunits from GIRK channels. However, an increased k_{off} should also shift the dose-response relationship to higher agonist concentrations and decrease the amplitude of GIRK currents at submaximal agonist concentrations. Because neither of these effects were observed (Doupnik et al., 1997; Saitoh et al., 1997), the mechanisms by which RGS proteins influence GIRK channels remain unclear. In comparison, the effects of RGS on N-type Ca channels appear to be somewhat more straightforward because they are entirely consistent with lower G $\beta\gamma$ concentrations resulting from increased GAP activity.

GAP Activity May Account for the Effects of RGS Proteins on N-type Ca Channel Modulation

In our experiments, N-type Ca channels were inhibited as a consequence of activating G protein-coupled, muscarinic (m2) receptors. When expressed in HEK293 cells, m2 receptors are thought to couple primarily to G α_i (Offermanns et al., 1994). The ability of pertussis toxin to virtually eliminate the response to CCh (Fig. 2 A) indicates that inhibition via m2 receptors is mediated by members of the G α_i subfamily, and our present data therefore indicate that RGS3T and RGS3 can act as GAPs for G α_i . RGS8 is also a GAP for G α_i , as previously demonstrated by Saitoh et al. (1997). Our finding that the core domain of RGS8 produces the same effect

as full-length RGS3T, RGS3, or RGS8 (Fig. 2) is consistent with the idea that the GAP activity of RGS proteins is responsible for their effects on Ca channel modulation. However, the core domains of RGS proteins may have other important functions in addition to stimulating GTPase activity. Interestingly, the amino-terminal regions of RGS4 (Srinivasa et al., 1998b) and RGS16 (Chen and Lin, 1998) are required for these mammalian RGS proteins to substitute for an RGS homologue (Sst2p) in the yeast *Saccharomyces cerevisiae*. Thus, regions of RGS proteins located outside of the conserved core domain may have essential functions in other expression systems, or under cellular conditions different from those of our present experiments.

Relevance of RGS Proteins to Neuronal Ca Channel Function

Our present results with RGS proteins may be applicable to other Ca channels besides N-type. For example, P/Q-type Ca channels encoded by α_1A subunits (Gillard et al., 1997; Ligon et al., 1998; Pinto et al., 1998) and R-type Ca channels encoded by α_1E subunits (Piedras-Rentería and Tsien, 1998) can also be strongly modulated through G protein-dependent pathways (Mintz and Bean, 1993; Yassin et al., 1996; Wu and Saggau, 1997; Meza and Adams, 1998), and it therefore seems likely that these other Ca channel types will also be influenced by RGS proteins. Because RGS proteins exhibit tissue-specific patterns of expression within the brain (Gold et al., 1997), it seems reasonable to postulate that particular RGS proteins might perform specialized roles in specific neuronal pathways. This idea is supported by the recent demonstration that RGS9 is important in visual phototransduction (Cowan et al., 1998; He et al., 1998), a highly specialized neuronal event.

Although our present work does not address the physiological importance of RGS proteins in synaptic transmission or other processes involving neuronal Ca channels, our results clearly demonstrate that RGS proteins can significantly alter the G protein-mediated inhibition of N-type Ca channels. Our results obtained in HEK293 cells may therefore be useful in making predictions about the effects of endogenous RGS proteins in neurons. Previous experiments using rapid perfusion systems (Jones, 1991; Mintz and Bean, 1993; Zhou et al., 1997) have shown that recovery of N- or P-type Ca channels from membrane-delimited inhibition proceeds at a rate very similar to that observed in our HEK293 cells expressing RGS3T (Fig. 5). As previously mentioned by Zhou et al. (1997), these rates are significantly higher than the intrinsic rate of GTP hydrolysis exhibited by purified G α_o subunits in vitro, suggesting the involvement of a GAP. In combination with our present results, these previous observations in neurons suggest the hypothesis that endogenous RGS proteins actively speed the recovery of Ca channels from G pro-

tein-mediated inhibition. One prediction of this hypothesis is that recovery should be slower in neurons lacking appropriate RGS proteins (i.e., RGS capable of acting as a GAP for the G proteins activated by the receptor). Another prediction is that recovery from inhi-

bition should be slowed by experimentally blocking, inactivating, or depleting endogenous RGS proteins. In future studies, it will be important to test these predictions and attempt to gain further insight into the physiological role(s) of RGS proteins.

We thank K. Campbell, S. Ikeda, Y. Mori, E. Peralta, T. Snutch, and D. Yue for generously providing expression plasmids, and N. Artemyev, R. Bannister, and E. Shibata for comments on the manuscript.

This work was supported by a National Institutes of Health (NIH) grant (NS-34423), a Basic Research Grant from the Muscular Dystrophy Association, and a Grant-In-Aid from the American Heart Association to B. Adams, and by NIH grant HL-41071 to R. Fisher. K. Melliti was supported by a University of Iowa Biosciences Initiative Postdoctoral Fellowship. U. Meza was the recipient of a CONAcYT Fellowship.

Original version received 21 July 1998 and accepted version received 12 November 1998.

Note Added in Proof. It was recently shown that expression of RGS10 attenuates N-type Ca channel inhibition in rat SCG neurons, and expression of either RGS4 or RGS10 speeds channel recovery from inhibition in neurons overexpressing G_{α_z} proteins. (Jeong, S.-W., and S.R. Ikeda. 1998. G Protein α subunit G_{α_z} couples neurotransmitter receptors to ion channels in sympathetic neurons. *Neuron*. 21:1201–1212.)

REFERENCES

- Bean, B.P. 1989. Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature*. 340: 153–156.
- Beech, D.J., L. Bernheim, and B. Hille. 1991. Intracellular Ca buffers disrupt muscarinic suppression of Ca current and M current in rat sympathetic neurons. *Proc. Natl. Acad. Sci. USA*. 88:652–656.
- Berman, D.M., and A.G. Gilman. 1998. Mammalian RGS proteins: barbarians at the gate. *J. Biol. Chem.* 273:1269–1272.
- Berman, D.M., T. Kozasa, and A.G. Gilman. 1996a. The GTPase-activating protein RGS4 stabilizes the transition state for nucleotide hydrolysis. *J. Biol. Chem.* 271:27209–27212.
- Berman, D.M., T.M. Wilkie, and A.G. Gilman. 1996b. GAIP and RGS4 are GTPase-activating proteins for the G_i subfamily of G protein α subunits. *Cell*. 86:445–452.
- Berstein, G., J.L. Blank, D.-Y. Jhon, J.H. Exton, S.G. Rhee, and E.M. Ross. 1992. Phospholipase C- β 1 is a GTPase-activating protein for Gq/11, its physiological regulator. *Cell*. 70:411–418.
- Biddlecome, G.H., G. Berstein, and E.M. Ross. 1996. Regulation of phospholipase C- β 1 by Gq and m1 muscarinic cholinergic receptor. *J. Biol. Chem.* 271:7999–8007.
- Boland, L.M., and B.P. Bean. 1993. Modulation of N-type calcium channels in bullfrog sympathetic neurons by luteinizing hormone-releasing hormone: kinetics and voltage dependence. *J. Neurosci.* 13:516–533.
- Brody, D.L., P.G. Patil, J.G. Mulle, T.P. Snutch, and D.T. Yue. 1997. Bursts of action potential waveforms relieve G-protein inhibition of recombinant P/Q-type Ca channels in HEK 293 cells. *J. Physiol. (Camb.)*. 499:637–644.
- Carabelli, V., M. Lovallo, V. Magnelli, H. Zucker, and E. Carbone. 1996. Voltage-dependent modulation of single N-type Ca channel kinetics by receptor agonists in IMR32 cells. *Biophys. J.* 70: 2144–2154.
- Chatterjee, T.K., A.K. Eapen, and R.A. Fisher. 1997. A truncated form of RGS3 negatively regulates G protein-coupled receptor stimulation of adenylyl cyclase and phosphoinositide phospholipase C. *J. Biol. Chem.* 272:15481–15487.
- Chen, C.H., and S.C. Lin. 1998. The core domain of RGS16 retains G-protein binding and GAP activity in vitro, but is not functional in vivo. *FEBS Lett.* 422:359–362.
- Cowan, C.W., R.N. Fariss, I. Sokal, K. Palczewski, and T.G. Wensel. 1998. High expression levels in cones of RGS9, the predominant GTPase accelerating protein of rods. *Proc. Natl. Acad. Sci. USA*. 95:5351–5356.
- Delmas, P., D.A. Brown, M. Dayrell, F.C. Abogadie, M.P. Caulfield, and N.J. Buckley. 1998. On the role of endogenous G-protein $\beta\gamma$ subunits in N-type Ca current inhibition by neurotransmitters in rat sympathetic neurones. *J. Physiol. (Camb.)*. 506:319–329.
- De Waard, M., H.Y. Liu, D. Walker, V.E.S. Scott, C.A. Gurnett, and K.P. Campbell. 1997. Direct binding of G-protein $\beta\gamma$ complex to voltage-dependent calcium channels. *Nature*. 385:446–450.
- De Vries, L., M. Mousli, A. Wurmsler, and M.G. Farquhar. 1995. GAIP, a protein that specifically interacts with the trimeric G protein, $G_{\alpha i3}$, is a member of a protein family with a highly conserved core domain. *Proc. Natl. Acad. Sci. USA*. 92:11916–11920.
- Diversé-Pierluissi, M., A.E. Remmers, R.R. Neubig, and K. Dunlap. 1997. Novel form of cross talk between G protein and tyrosine kinase pathways. *Proc. Natl. Acad. Sci. USA*. 94:5417–5421.
- Dohlman, H.G., J. Song, D. Ma, W.E. Courchesne, and J. Thorner. 1996. Sst2, a negative regulator of pheromone signaling in the yeast *Saccharomyces cerevisiae*: expression, localization, and genetic interaction and physical association with Gpa1 (the G protein α subunit). *Mol. Cell. Biol.* 16:5194–5209.
- Dohlman, H.G., and J. Thorner. 1997. RGS proteins and signaling by heterotrimeric G proteins. *J. Biol. Chem.* 272:3871–3874.
- Douppnik, C.A., N. Davidson, H.A. Lester, and P. Kofuji. 1997. RGS proteins reconstitute the rapid gating kinetics of $G\beta\gamma$ -activated inwardly rectifying K channels. *Proc. Natl. Acad. Sci. USA*. 94: 10461–10466.
- Druey, K.M., and J.H. Kehrl. 1997. Inhibition of regulator of G protein signaling function by two mutant RGS4 proteins. *Proc. Natl. Acad. Sci. USA*. 94:12851–12856.
- Druey, K.M., K.J. Blumer, V.H. Kang, and J.H. Kehrl. 1996. Inhibition of G-protein-mediated MAP kinase activation by a new mammalian gene family. *Nature*. 379:742–746.
- Dunlap, K., J.I. Luebke, and T.J. Turner. 1995. Exocytotic Ca channels in mammalian central neurons. *Trends Neurosci.* 18:89–98.
- Elmslie, K.S., and S.W. Jones. 1994. Concentration dependence of neurotransmitter effects on calcium current kinetics in frog sympathetic neurones. *J. Physiol. (Camb.)*. 481:35–46.
- Elmslie, K.S., W. Zhou, and S.W. Jones. 1990. LHRH and GTP- γ -S modify calcium current activation in bullfrog sympathetic neurons. *Neuron*. 6:75–80.

- Faurobert, E., and J.B. Hurley. 1997. The core domain of a new retina specific RGS protein stimulates the GTPase activity of transducin in vitro. *Proc. Natl. Acad. Sci. USA.* 94:2945–2950.
- Fujita, Y., M. Mynlieff, R.T. Dirksen, M.S. Kim, T. Niidome, J. Nakai, T. Friedrich, N. Iwabe, T. Miyata, T. Furuichi, et al. 1993. Primary structure and functional expression of the ω -conotoxin-sensitive N-type calcium channel from rabbit brain. *Neuron.* 10: 585–598.
- Gillard, S.E., S.G. Volsen, W. Smith, R.E. Beattie, D. Bleakman, and D. Lodge. 1997. Identification of pore-forming subunit of P-type calcium channels: an antisense study on rat cerebellar Purkinje cells in culture. *Neuropharmacology.* 36:405–409.
- Golard, A., and S.A. Siegelbaum. 1993. Kinetic basis for the voltage-dependent inhibition of N-type calcium current by somatostatin and norepinephrine in chick sympathetic neurons. *J. Neurosci.* 13:3884–3894.
- Gold, S.J., Y.G. Ni, H.G. Dohlman, and E.J. Nestler. 1997. Regulators of G-protein signaling (RGS) proteins: region-specific expression of nine subtypes in rat brain. *J. Neurosci.* 17:8024–8037.
- Hamill, O.P., A. Marty, E. Neher, B. Sakmann, and F.J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* 391:85–100.
- He, W., C.W. Cowan, and T.G. Wensel. 1998. RGS9, a GTPase accelerator for phototransduction. *Neuron.* 20:95–102.
- Herlitze, S., D.E. Garcia, K. Mackie, B. Hille, T. Scheuer, and W.A. Catterall. 1996. Modulation of Ca channels by G-protein $\beta\gamma$ subunits. *Nature.* 380:258–262.
- Hille, B. 1994. Modulation of ion-channel function by G-protein-coupled receptors. *Trends Neurosci.* 17:531–536.
- Ikeda, S.R. 1991. Double-pulse calcium channel current facilitation in adult rat sympathetic neurones. *J. Physiol. (Camb.)* 439:181–214.
- Ikeda, S.R. 1996. Voltage-dependent modulation of N-type calcium channels by G-protein $\beta\gamma$ subunits. *Nature.* 380:255–258.
- Jones, S.W. 1991. Time course of receptor-channel coupling in frog sympathetic neurons. *Biophys. J.* 60:502–507.
- Jones, L.P., P.G. Patil, T.P. Snutch, and D.T. Yue. 1997. G-protein modulation of N-type calcium channel gating current in human embryonic kidney cells (HEK 293). *J. Physiol. (Camb.)* 498:601–610.
- Kavalali, E.T., M. Zhuo, H. Bito, and R.W. Tsien. 1997. Dendritic Ca channels characterized by recordings from isolated hippocampal dendritic segments. *Neuron.* 18:651–663.
- Kim, H.L., H. Kim, P. Lee, R.G. King, and H. Chin. 1992. Rat brain expresses an alternatively spliced form of the dihydropyridine-sensitive L-type calcium channel α_2 subunit. *Proc. Natl. Acad. Sci. USA.* 89:3251–3255.
- Koh, D.S., and B. Hille. 1997. Modulation by neurotransmitters of catecholamine secretion from sympathetic ganglion neurons detected by amperometry. *Proc. Natl. Acad. Sci. USA.* 94:1506–1511.
- Ligon, B., A.E. Boyd, and K. Dunlap. 1998. Class A calcium channel variants in pancreatic islets and their role in insulin secretion. *J. Biol. Chem.* 273:13905–13911.
- Lopez, H.S., and A.M. Brown. 1991. Correlation between G protein activation and reblocking kinetics of Ca channel currents in rat sensory neurons. *Neuron.* 7:1061–1068.
- Luebke, J.I., and K. Dunlap. 1994. Sensory neuron N-type calcium currents are inhibited by both voltage-dependent and -independent mechanisms. *Pflügers Arch.* 428:499–507.
- Marchetti, C., E. Carbone, and H.D. Lux. 1986. Effects of dopamine and noradrenaline on Ca channels of cultured sensory and sympathetic neurons of chick. *Pflügers Arch.* 406:104–111.
- Meza, U., and B. Adams. 1998. G-protein-dependent facilitation of neuronal α_{1A} , α_{1B} , and α_{1E} Ca channels. *J. Neurosci.* 18:5240–5252.
- Mintz, I., and B.P. Bean. 1993. GABA_B receptor inhibition of P-type Ca channels in central neurons. *Neuron.* 10:889–898.
- Mishina, M., T. Kurosaki, T. Tobimatsu, Y. Morimoto, M. Noda, T. Yamamoto, M. Terao, J. Lindstrom, T. Takahashi, M. Kuno, and S. Numa. 1984. Expression of functional acetylcholine receptor from cloned cDNAs. *Nature.* 307:604–608.
- Nargeot, J., J.M. Nerbonne, J. Engels, and H.A. Lester. 1983. Time course of the increase in the myocardial slow inward current after a photochemically generated concentration jump of intracellular cAMP. *Proc. Natl. Acad. Sci. USA.* 80:2395–2399.
- Natochin, M., R.L. McEntaffer, and N.O. Artemyev. 1998. Mutational analysis of the Asn residue essential for RGS protein binding to G-proteins. *J. Biol. Chem.* 273:6731–6735.
- Offermanns, S., T. Wieland, D. Homann, J. Sandmann, E. Bombien, K. Spicher, G. Schultz, and K.H. Jakobs. 1994. Transfected muscarinic acetylcholine receptors selectively couple to Gi-type G proteins and Gq/11. *Mol. Pharmacol.* 45:890–898.
- Park, D., and K. Dunlap. 1998. Dynamic regulation of calcium influx by G-proteins, action potential waveform, and neuronal firing frequency. *J. Neurosci.* 18:6757–6766.
- Patil, P.G., M. De Leon, R.R. Reed, S. Dubel, T.P. Snutch, and D.T. Yue. 1996. Elementary events underlying voltage-dependent G-protein inhibition of N-type calcium channels. *Biophys. J.* 71: 2509–2521.
- Peralta, E.G., A. Ashkenazi, J.W. Winslow, D.H. Smith, J. Ramachandran, and D.J. Capon. 1987. Distinct primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3923–3929.
- Piedras-Renteria, E.S., and R.W. Tsien. 1998. Antisense oligonucleotides against α_1E reduce R-type calcium currents in cerebellar granule cells. *Proc. Natl. Acad. Sci. USA.* 95:7760–7765.
- Pinto, A., S. Gillard, F. Moss, K. Whyte, P. Brust, M. Williams, K. Stauderman, M. Harpold, B. Lang, J. Newsom-Davis, et al. 1998. Human autoantibodies specific for the α_1A subunit reduce both P-type and Q-type calcium currents in cerebellar neurons. *Proc. Natl. Acad. Sci. USA.* 95:8328–8333.
- Popov, S., K. Yu, T. Kozasa, and T.M. Wilkie. 1997. The regulators of G protein signaling (RGS) domains of RGS4, RGS10, and GAIP retain GTPase activating protein activity in vitro. *Proc. Natl. Acad. Sci. USA.* 94:7216–7220.
- Qin, N., D. Platano, R. Olcese, E. Stefani, and L. Birnbaumer. 1997. Direct interaction of G $\beta\gamma$ with a C-terminal G $\beta\gamma$ -binding domain of the Ca channel α_1 subunit is responsible for channel inhibition by G protein-coupled receptors. *Proc. Natl. Acad. Sci. USA.* 94:8866–8871.
- Reuveny, E., P.A. Slesinger, J. Inglese, J.M. Morales, J.A. Iniguez-Lluhl, R.J. Lefkowitz, H.R. Bourne, Y.N. Jan, and L.Y. Jan. 1994. Activation of the cloned muscarinic potassium channel by G protein $\beta\gamma$ subunits. *Nature.* 370:143–146.
- Sagoo, M.S., and L. Lagnado. 1997. G-protein deactivation is rate-limiting for shut-off of the phototransduction cascade. *Nature.* 389:392–395.
- Saitoh, O., Y. Kubo, Y. Miyatani, T. Asano, and H. Nakata. 1997. RGS8 accelerates G-protein-mediated modulation of K currents. *Nature.* 390:525–529.
- Saugstad, J.A., M.J. Marino, J.A. Folk, J.R. Hepler, and P.J. Conn. 1998. RGS4 inhibits signaling by group I metabotropic glutamate receptors. *J. Neurosci.* 18:905–913.
- Srinivasa, S.P., N. Watson, M.C. Overton, and K.J. Blumer. 1998a. Mechanism of RGS4, a GTPase-activating protein for G protein α subunits. *J. Biol. Chem.* 273:1529–1533.
- Srinivasa, S.P., L.S. Bernstein, K.J. Blumer, and M.E. Linder. 1998b. Plasma membrane localization is required for RGS4 function in *S. cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 95:5584–5589.
- Tesmer, J.J.G., D.M. Berman, A.G. Gilman, and S.R. Sprang. 1997. Structure of RGS4 bound to AlF₄⁻-activated G α_1 : stabilization of

- the transition state for GTP hydrolysis. *Cell*. 89:251–261.
- Wanke, E., A. Ferroni, A. Malgaroli, A. Ambrosini, T. Pozzan, and J. Meldolesi. 1987. Activation of a muscarinic receptor selectively inhibits a rapidly inactivated Ca current in rat sympathetic neurons. *Proc. Natl. Acad. Sci. USA*. 84:4313–4317.
- Wickman, K.D., J.A. Inñiguez-Lluhi, P.A. Davenport, R. Taussig, G.B. Krapivinsky, M.E. Linder, A.G. Gilman, and D.E. Clapham. 1994. Recombinant G-protein $\beta\gamma$ subunits activate the muscarinic-gated atrial potassium channel. *Nature*. 368:255–257.
- Wilding, T.J., M.D. Womack, and E.W. McCleskey. 1995. Fast local signal transduction between the μ opioid receptor and Ca channels. *J. Neurosci*. 15:4124–4132.
- Witcher, D.R., M. De Waard, J. Sakamoto, C. Franzini-Armstrong, M. Pragnell, S.D. Kahl, and K.P. Campbell. 1993. Subunit identification and reconstitution of the N-type Ca channel complex purified from brain. *Science*. 261:486–489.
- Wu, L.G., and P. Saggau. 1997. Presynaptic inhibition of elicited neurotransmitter release. *Trends Neurosci*. 20:204–212.
- Wu, L.-G., J.G.G. Borst, and B. Sakmann. 1998. R-type Ca currents evoke transmitter release at a rat central synapse. *Proc. Natl. Acad. Sci. USA*. 95:4720–4725.
- Yassin, M., S.Q. Zong, and T. Tanabe. 1996. G-protein modulation of neuronal class E (α_{1E}) calcium channel expressed in GH₃ cells. *Biochem. Biophys. Res. Commun*. 220:453–458.
- Yan, Y., P.P. Chi, and H.R. Bourne. 1997. RGS4 inhibits Gq-mediated activation of mitogen-activated protein kinase and phosphoinositide synthesis. *J. Biol. Chem*. 272:11924–11927.
- Zamponi, G.W., E. Bourinet, D. Nelson, J. Nargeot, and T.P. Snutch. 1997. Crosstalk between G proteins and protein kinase C mediated by the calcium channel α_1 subunit. *Nature*. 385:442–446.
- Zamponi, G.W., and T.P. Snutch. 1998. Decay of prepulse facilitation of N type calcium channels during G protein inhibition is consistent with binding of a single G $\beta\gamma$ subunit. *Proc. Natl. Acad. Sci. USA*. 95:4035–4039.
- Zhou, J.Y., M.S. Shapiro, and B. Hille. 1997. Speed of Ca channel modulation by neurotransmitters in rat sympathetic neurons. *J. Neurophysiol*. 77:2040–2048.

