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Endogenous Regulator of G-Protein Signaling Proteins Modify N-Type Calcium Channel Modulation in Rat Sympathetic Neurons

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Experiments using heterologous overexpression indicate that regulator of G-protein signaling (RGS) proteins play important roles in G$\beta\gamma$-mediated ion channel modulation. However, the roles subserved by endogenous RGS proteins have not been extensively examined because tools for functionally inhibiting natively expressed RGS proteins are lacking. To address this void, we used a strategy in which Gα_{oA} was rendered insensitive to pertussis toxin (PTX) and RGS proteins by site-directed mutagenesis. Either PTX-insensitive (PTX-i) or both PTX- and RGS-insensitive (PTX/RGS-i) mutants of Gα_{oA} were expressed along with Gβ_1 and Gγ_2 subunits in rat sympathetic neurons. After overnight treatment with PTX to suppress natively expressed Gα subunits, voltage-dependent Ca^{2+} current inhibition by norepinephrine (NE) (10 μM) was reconstituted in neurons expressing either PTX-i or PTX/RGS-i Gα_{oA}. When compared with neurons expressing PTX-i Gα_{oA}, the steady-state concentration–response relationships for NE-induced Ca^{2+} current inhibition were shifted to lower concentrations in neurons expressing PTX/RGS-i Gα_{oA}. In addition to an increase in agonist potency, the expression of PTX/RGS-i Gα_{oA} dramatically retarded the current recovery after agonist removal. Interestingly, the alteration in current recovery was accompanied by a slowing in the onset of current inhibition. Together, our data suggest that endogenous RGS proteins contribute to membrane-delimited Ca^{2+} channel modulation by regulating agonist potency and kinetics of G-protein-mediated signaling in neuronal cells.

Key words: calcium channel; G-protein; Gα; G$\beta\gamma$; intranuclear injection; RGS protein; sympathetic neuron; voltage-dependent inhibition

Biochemical studies indicate that members of the regulator of G-protein signaling (RGS) family of proteins serve as GTPase-activating proteins (GAPs) for Gα_i and Gα_q but not for Gα_s (for review, see Dohlman and Thorner, 1997; Koelle, 1997; Berman and Gilman, 1998). Recent evidence implicates RGS proteins as key components in G$\beta\gamma$-mediated modulation (Wickman and Clapham, 1995; Ikeda and Dunlap, 1999) of inwardly rectifying K^+ (GIRK) and N-type Ca^{2+} channels. The potential roles of RGS proteins in G$\beta\gamma$-mediated ion channel modulation were deduced from the discrepancy between the fast kinetics of modulation *in vivo* and the relatively slow intrinsic GTPase activities of G$\alpha_{o/i}$ *in vitro*. For example, after agonist removal, muscarinic (M_2) receptor-activated GIRK currents in atrial myocytes deactivate ~40-fold faster than the GTP hydrolysis rate of pertussis toxin (PTX)-sensitive Gα subunits *in vitro* (Breitwieser and Szabo, 1988; Kurachi, 1995; Shui et al., 1995). Logically, these discrepancies might be reconciled by invoking the ability of natively expressed RGS proteins to stimulate the intrinsic GTPase of Gα subunits thereby accelerating termination of G$\beta\gamma$-mediated signaling by favoring reformation of the heterotrimeric state. In support of this notion, heterologous expression of RGS proteins has been shown to accelerate the activation and deactivation kinetics of GIRK currents in *Xenopus* oocytes (Doupnik et al., 1997; Saitoh et al., 1997, 1999; Kovoor et al., 2000). In addition, heterologous expression of RGS proteins have been shown to alter the magnitude and kinetics of N-type Ca^{2+} channel inhibition (Jeong and Ikeda, 1998; Melliti et al., 1999). To date, however, the ability of natively expressed RGS proteins to influence G$\beta\gamma$-mediated ion channel modulation in neurons has not been established primarily because few tools are available for inhibiting endogenous RGS proteins.

In the present study, we addressed this void in our knowledge by developing a strategy based on a combination of previously identified point mutations in Gα subunits. First, Gα_{oA} was rendered insensitive to the actions of PTX by mutating the C-terminal cysteine in which PTX-mediated ADP ribosylation occurs (Milligan, 1988). In this way, natively expressed Gα subunits could be uncoupled from receptors by PTX treatment, thereby isolating the action of the mutated Gα subunit (Taussig et al., 1992; Hunt et al., 1994; Senogles 1994; Kozasa et al., 1996; Jeong and Ikeda, 2000). Second, point mutations were introduced into the switch I and II regions of Gα_{oA} that have been identified previously to prevent RGS binding and GAP activity without altering GDP release and basal GTP hydrolysis in other Gα subunits (Lan et al., 1998; Natochin and Artemyev, 1998a) (but see DiBello et al., 1998). Using this strategy, we examined alterations in the magnitude and kinetics of voltage-dependent (VD) N-type Ca^{2+} current inhibition reconstituted in PTX-treated rat sympathetic neurons expressing the Gα_{oA} mutants. Our results indicate that endogenous RGS proteins in sympathetic neurons play an integral role in controlling the magnitude and speed of neurotransmitter-induced N-type Ca^{2+} channel modulation.

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MATERIALS AND METHODS

Preparation of sympathetic neurons. Superior cervical ganglion (SCG) neurons were enzymatically dissociated as described previously (Ikeda, 1997). Briefly, adult (200–350 gm) male Wistar rats were decapitated using a laboratory guillotine. The SCG were dissected free of the carotid bifurcation and placed in cold (4°C) HBSS. The ganglia were desheathed, cut into small pieces, and transferred to the oxygenated Earle's balance salt solution (EBSS), pH 7.4, containing 0.6 mg/ml collagenase type D (Boehringer Mannheim, Indianapolis, IN), 0.4 mg/ml trypsin (TRL type; Worthington, Lakewood, NJ), and 0.1 mg/ml DNase Type I (Sigma, St. Louis, MO) in a 25 cm^2 tissue culture flask. The EBSS was modified by adding 3.6 gm/l glucose and 10 mM HEPES. After incubation for 60 min in a water bath shaker at 35°C, neurons were dispersed by vigorous shaking of the flask. After centrifugation twice for 6 min at 50 × *g*, the neurons were resuspended in Minimum Essential Medium (Mediatech, Inc., Herndon, VA) containing 10% fetal calf serum (Atlanta Biologicals, Atlanta, GA) and 1% glutamine/penicillin–streptomycin solution (Life Technologies, Grand Island, NY). Neurons were then plated onto polystyrene culture dishes (35 mm), coated with poly-L-lysine, and maintained in a humidified atmosphere of 95% air–5% CO_2 at 37°C. As appropriate, neurons were incubated overnight (16–20 hr) with 500 ng/ml PTX (List Biologic, Campbell, CA).

Construction and expression of RGS-insensitive mutants of Gα subunits. Previously, we have generated a PTX-insensitive $G\alpha_{oA}$ (PTX-i $G\alpha_{oA}$) by introducing a cysteine (C) to glycine (G) mutation in the residue −4 from the C terminus (C351G) (Jeong and Ikeda, 2000). Using PTX-i $G\alpha_{oA}$ as a template, additional point mutations (DiBello et al., 1998; Lan et al., 1998; Natochin and Artemyev, 1998a) were introduced to render $G\alpha_{oA}$ both PTX- and RGS-insensitive (PTX/RGS-i $G\alpha_{oA}$). The following mutations were introduced into $G\alpha_{oA}$(C351G) using the GeneEditor site-directed mutagenesis kit (Promega, Madison, WI) per instructions of the manufacturer: G184S, S207D, and G184S/S207D. The following primers were used in constructing the mutations: G184S, 5′-GTCAAAACAACTTCCATCGTAGAAACCCAC-3′; and S207D, 5′-TCGGGGGCCAGCGAGATGAACGCAAGAAGTG-3′. The sequence of each construct was verified with an automated DNA sequencer (ABI 310; Applied Biosystems, Foster City, CA). Either PTX-i $G\alpha_{oA}$ or PTX/RGS-i $G\alpha_{oA}$ subunits were coexpressed with $G\beta_1$ and $G\gamma_2$ subunits (denoted $G\beta_1\gamma_2$, hereafter) by intranuclear microinjection as described previously (Ikeda, 1996, 1997). All G-protein subunits were expressed from the cytomegalovirus promoter-driven vector, pCI (Promega). Neurons were used within 24 hr after intranuclear injection of vectors. Injected neurons were identified by fluorescence from coexpressed jellyfish green fluorescent protein (EGFP; Clontech, Palo Alto, CA).

Electrophysiology. Ca^{2+} channel currents were recorded using the whole-cell variant of the patch-clamp technique (Hamill et al., 1981) as described previously (Ikeda, 1991; Ikeda et al., 1995). To isolate Ca^{2+} currents, patch electrodes were filled with a solution containing (in mM); 120 *N*-methyl-D-glucamine methanesulfonate (MS), 20 tetraethylammonium (TEA)-MS, 20 HCl, 11 EGTA, 1 $CaCl_2$, 10 HEPES, 4 MgATP, 0.3 Na_2GTP, and 14 creatine phosphate, pH 7.2 (297 mOsm/kg H_2O). The external recording solution contained (in mM): 145 TEA-MS, 10 HEPES, 10 $CaCl_2$, 15 glucose, and 0.0003 tetrodotoxin, pH 7.4 (318 mOsm/kg H_2O). All experiments were performed at room temperature (21–24°C). Norepinephrine (NE) was applied to single neurons via a gravity-fed fused silica capillary tube connected to an array of seven polyethylene tubes. The outlet of the perfusion system was located within 100 μm of the cell. Drug application was started by switching the control external solution to a drug solution. Complete solution exchange occurred within <1 sec.

Data analysis. The concentration–response curves were plotted as values normalized to the inhibition produced at the maximal [NE] (30 μM). The concentration–response curves were fit to the following Hill equation: $I = 1/[1 + (k/[NE])^n]$, where I, k, [NE], and n are normalized inhibition, a constant, NE concentration, and Hill factor, respectively. The EC_{50} values were calculated using the following relationship: $EC_{50} = k^{-n}$. The "on–off" times of the NE-induced Ca^{2+} current inhibition were approximated by fitting a polynomial function to the time courses and interpolating the appropriate values (i.e., $t_{0.5}$ and t_{rise}). All curve fitting was performed with the IGOR PRO data analysis package (WaveMetrics, Lake Oswego, OR). Data are presented as means ± SEM. Student's *t* test (unpaired) or ANOVA followed by a *post hoc* Dunnett's test, as appropriate, were applied to the data to determine statistical significance. $p < 0.05$ was considered significant.

RESULTS

Reconstitution of NE-induced Ca^{2+} current inhibition after expression of PTX- and RGS-insensitive $G\alpha_{oA}$ subunits

As a first step toward elucidating the potential roles of endogenous RGS proteins in Ca^{2+} channel modulation, we tested whether heterologous expression of PTX/RGS-i $G\alpha_{oA}$ subunits reconstituted NE-induced VD Ca^{2+} current inhibition in SCG neurons after uncoupling of endogenous $G_{o/i}$-proteins by PTX treatment. $G\alpha_{oA}$ was selected to be rendered RGS-insensitive because G_o has been implicated as the primary signaling element coupling α_2-adrenergic receptors (α_2-ARs) to N-type Ca^{2+} channels (Caulfield et al., 1994) in sympathetic neurons.

Figure 1 illustrates the effects of PTX/RGS-i $G\alpha_{oA}$ subunits on NE-induced Ca^{2+} current inhibition when expressed in SCG neurons. Ca^{2+} currents were evoked from a holding potential of −80 mV with a double-pulse protocol consisting of two identical test pulses to +10 mV separated by a large depolarizing conditioning pulse to +80 mV (Fig. 1*A*). In uninjected control neurons, NE (10 μM) inhibited Ca^{2+} currents by 63 ± 2% ($n = 30$) (Figs. 1*A*, 2). NE-induced Ca^{2+} current inhibition displayed the hallmarks of VD inhibition (Ikeda and Dunlap, 1999), i.e., slowed activation kinetics in the prepulse and an enhanced postpulse amplitude (Figs. 1*A*, 2). Prepulse facilitation ratio (PFR), defined as the ratio of the postpulse to prepulse current amplitude, increased from 1.20 ± 0.02 to 2.40 ± 0.10 ($n = 30$) after NE application. This form of N-type Ca^{2+} channel modulation has been shown to be mediated by the $G\beta\gamma$ subunit (Herlitze et al., 1996; Ikeda, 1996).

Overnight treatment of the neurons with PTX (500 ng/ml) greatly attenuated NE-induced Ca^{2+} current inhibition (8 ± 1%, $n = 25$) (Figs. 1*B*, 2), confirming the involvement of a PTX-sensitive G-protein(s) in the signaling pathway (Hille, 1994). To reconstitute NE-induced Ca^{2+} current inhibition in PTX-treated neurons, either PTX-i $G\alpha_{oA}$ or PTX/RGS-i $G\alpha_{oA}$ subunits were coexpressed with $G\beta_1\gamma_2$ in SCG neurons using an intranuclear microinjection technique. As shown previously (Jeong and Ikeda, 2000), "balanced" expression of $G\alpha_{oA}$ mutants and $G\beta_1\gamma_2$ was critical for successful reconstitution on the time scale used in these experiments (<24 hr after injection of cDNA). Briefly, if expression of $G\alpha$ mutants greatly exceeded that of $G\beta\gamma$, basal facilitation and NE-induced Ca^{2+} current inhibition were ablated. This effect likely reflects sequestration of free $G\beta\gamma$ subunits (both endogenous and exogenous) by the GDP-bound $G\alpha$ (Ikeda, 1996; Jeong and Ikeda 1999b). In contrast, if expression of $G\beta\gamma$ exceeded that of $G\alpha$, a significant tonic inhibition (as indicated by increased basal facilitation) was observed. This result presumably reflects interaction of "free" or excess $G\beta\gamma$ with N-type Ca^{2+} channels (Herlitze et al., 1996; Ikeda, 1996; García et al., 1998; Ruiz-Velasco and Ikeda, 2000). Thus, we have defined a basal PFR range of 1.02–1.50 as indicating adequate balance between PTX-i $G\alpha_{oA}$ and $G\beta\gamma$ subunits (Jeong and Ikeda, 2000). In general, we applied the same criteria for PTX/RGS-i $G\alpha_{oA}$ mutants. In a few neurons expressing $G\alpha_{oA}$(G184S:C351G), however, reconstitution of Ca^{2+} current inhibition occurred with basal PFR of <1. NE (10 μM) inhibited Ca^{2+} currents by 52 ± 3% ($n = 9$) when $G\alpha_{oA}$(C351G) was coexpressed with $G\beta_1\gamma_2$ in PTX-treated neurons. The characteristics of the Ca^{2+} current inhibition reconstituted by the PTX-i $G\alpha_{oA}$ were basically similar to those of uninjected controls neurons in terms of voltage dependence of the inhibition (PFR increased from 1.15 ± 0.05 to

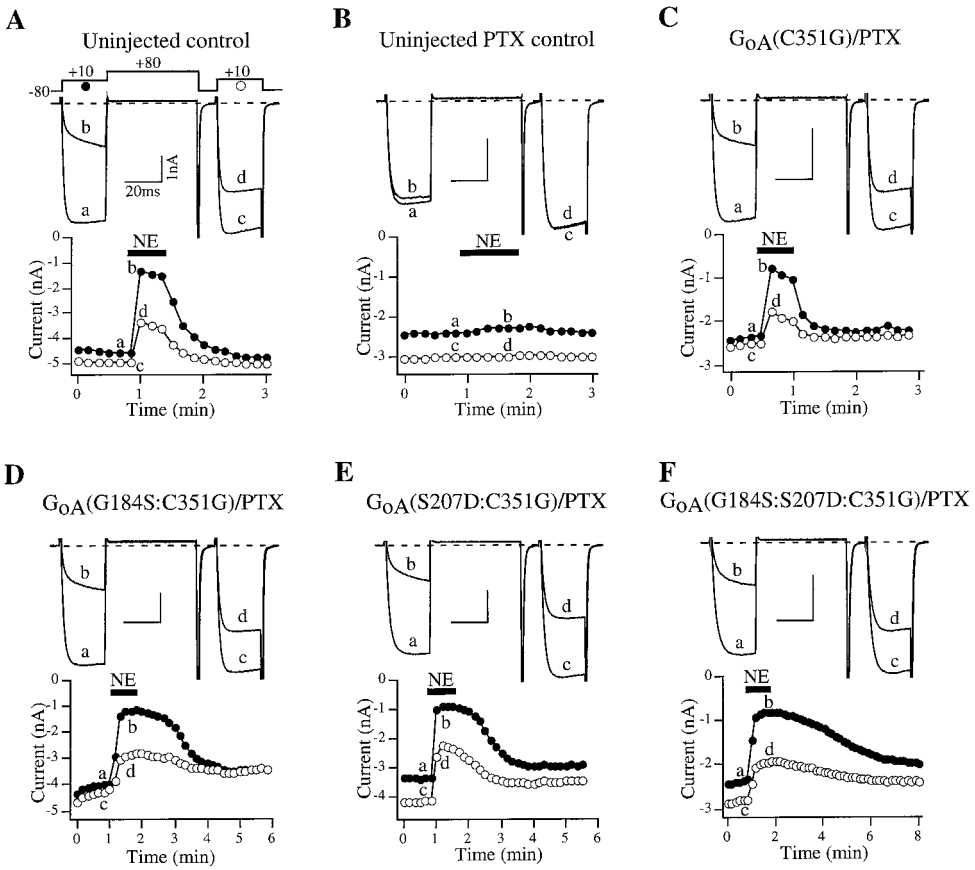

Figure 1. Reconstitution of α_2-AR coupling to N-type Ca^{2+} channels by expression of PTX/RGS-i $G\alpha_{oA}$ mutants. Time courses of current inhibition and superimposed current traces in the absence (*a*, *c*) or presence (*b*, *d*) of 10 μM NE recorded from uninjected control neurons (no PTX) (*A*), uninjected PTX-treated control neurons (500 ng/ml PTX, overnight) (*B*), PTX-treated neurons expressing a PTX-i $G\alpha_{oA}$ mutant, $G\alpha_{oA}$(C351G) (*C*), and PTX-treated neurons expressing PTX/RGS-i $G\alpha_{oA}$ mutants, $G\alpha_{oA}$(G184S:C351G) (*D*), $G\alpha_{oA}$(S207D: C351G) (*E*), or $G\alpha_{oA}$(G184S: S207D: C351G) (*F*). The cDNAs encoding $G\alpha$ mutants and $G\beta_1\gamma_2$ were directly injected into nuclei of SCG neurons. The Ca^{2+} current was evoked every 10 sec by a double-pulse voltage protocol (see *inset* in *A*) consisting of two identical test pulses (+10 mV from a holding potential of −80 mV) separated by a large depolarizing conditioning pulse to +80 mV. The amplitudes of currents generated by prepulses (*filled circles*) and postpulses (*open circles*) were plotted. Note that the expression of RGS-insensitive $G\alpha$ mutants retarded onset of the steady-state current inhibition and the current recovery after agonist withdrawal.

1.90 ± 0.09 after NE application) and relatively rapid on–off kinetics of NE action. These results are consistent with previous findings using the $G\alpha_{oA}$(C351G) mutant (Jeong and Ikeda, 2000). Similarly, coexpression of the PTX/RGS-i $G\alpha_{oA}$ subunits $G\alpha_{oA}$(G184S:C351G) or $G\alpha_{oA}$(S207D:C351G), along with $G\beta_1\gamma_2$, resulted in successful reconstitution of NE-induced Ca^{2+} current inhibition (Fig. 1). These results demonstrate that introduction of the additional point mutations did not affect the ability of $G\alpha_{oA}$(C351G) to form heterotrimers or couple receptors to Ca^{2+} channels. On average, NE (10 μM) inhibited Ca^{2+} currents by 69 ± 1 (n = 6) and 70 ± 2% (n = 8) in neurons expressing $G\alpha_{oA}$(G184S:C351G) and $G\alpha_{oA}$(S207D:C351G), respectively (Fig. 2). It should be noted that these magnitudes of inhibition were significantly larger than those of uninjected or PTX-i $G\alpha_{oA}$-expressing neurons ($p < 0.05$). The voltage dependence of the reconstituted Ca^{2+} current inhibition remained intact as evidenced by the kinetic slowing of current activation and increase in PFR after NE application [1.01 ± 0.02 to 2.23 ± 0.06 for $G\alpha_{oA}$(G184S:C351G); 1.19 ± 0.05 to 2.26 ± 0.07 for $G\alpha_{oA}$(S207D:C351G)] (Fig. 2). However, the most striking feature produced by expression of the PTX/RGS-i $G\alpha_{oA}$ subunits was a sluggish recovery from inhibition after agonist removal (Fig. 1*D*,*E*). Interestingly, the onset of agonist action also appeared slower when compared with control neurons (see Fig. 4). Coexpression of RGS8 (1 ng/μl DNA) with $G\alpha_{oA}$(G184S:C351G) in a limited number of neurons (n = 2) resulted in qualitatively similar results to those obtained in neurons expressing only $G\alpha_{oA}$(S207D:C351G) (data not shown). In particular, the recovery from inhibition after agonist removal remained very slow, indicating an inability of RGS8 to reverse this effect. In addition to $G\alpha_{oA}$(G184S:C351G) and $G\alpha_{oA}$(S207D:C351G), we generated a construct that contained both the G184S and S207D mutations. When expressed along with $G\beta_1\gamma_2$, $G\alpha_{oA}$(G184S:S207D:C351G) reconstituted VD Ca^{2+} current inhibition (62 ± 2%; PFR, 2.37 ± 0.07, n = 7) (Figs. 1*F*, 2). The time course of current recovery after agonist removal was dramatically retarded in neurons expressing this mutant. It is not clear whether this effect reflects an intrinsically greater "resistance" to the effects of RGS proteins or a secondary effect of the combined mutations. Together, these data demonstrate that expression of the PTX/RGS-i $G\alpha_{oA}$ subunits (together with $G\beta_1\gamma_2$) reconstituted functional coupling of α_2-ARs to N-type Ca^{2+} channels in PTX-treated neurons. In addition, the data are consistent with biochemical data showing that the G184S and S207D mutations confer resistance to the GAP effects of exogenous RGS proteins. Thus, additional studies were undertaken to quantify the alterations in Ca^{2+} channel inhibition and time course.

Expression of PTX/RGS-i $G\alpha_{oA}$ subunits produces a leftward shift in concentration–response curves

As noted above, Ca^{2+} current inhibitions produced by a single concentration of NE (10 μM) were modestly enhanced in neurons expressing PTX/RGS-i $G\alpha_{oA}$ subunits when compared with neurons expressing PTX-i $G\alpha_{oA}$. These results are consistent with previous studies demonstrating that increasing levels of RGS proteins attenuate agonist-induced Ca^{2+} current inhibition in SCG neurons (Jeong and Ikeda, 1998) and HEK293 cells (Melliti et al., 1999). In the latter study, rightward shifts in steady-state concentration–response curves were observed after heterologous expression of RGS proteins. Therefore, it was predicted that expression of PTX/RGS-i $G\alpha_{oA}$ subunits would shift concentration–response curves in the opposite direction, i.e., leftward, if

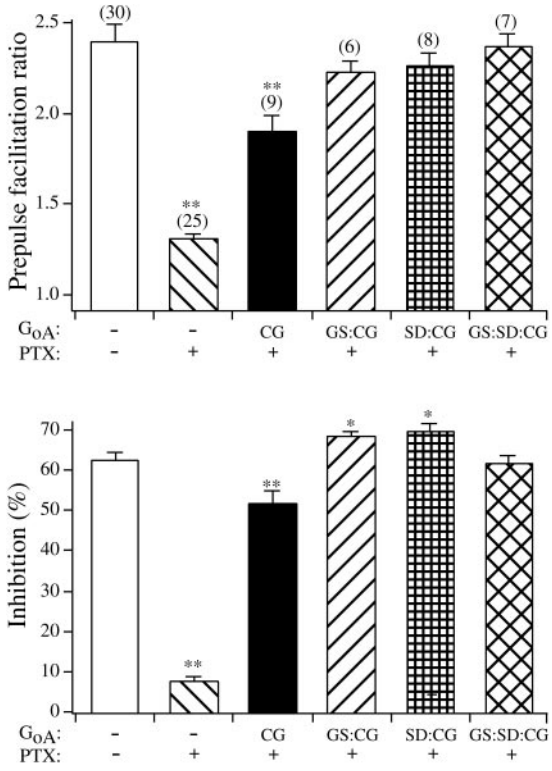

Figure 2. Summary of prepulse facilitation and Ca^{2+} current inhibition in the presence of NE. Prepulse facilitation was calculated by the ratio of the postpulse to prepulse current amplitude measured isochronally at 10 msec after the start of the test pulses. Inhibition (percentage) was calculated using the amplitudes of currents determined isochronally 10 msec after the start of the prepulse. Data are presented as mean ± SEM, and *numbers* in parentheses indicate the number of neurons tested. ANOVA ($p < 0.0001$) followed by Dunnett's *t* test; uninjected control versus PTX control, *CG*, *GS:CG*, *SD:CG*, and *GS:SD:CG*. $*p < 0.05$; $**p < 0.01$.

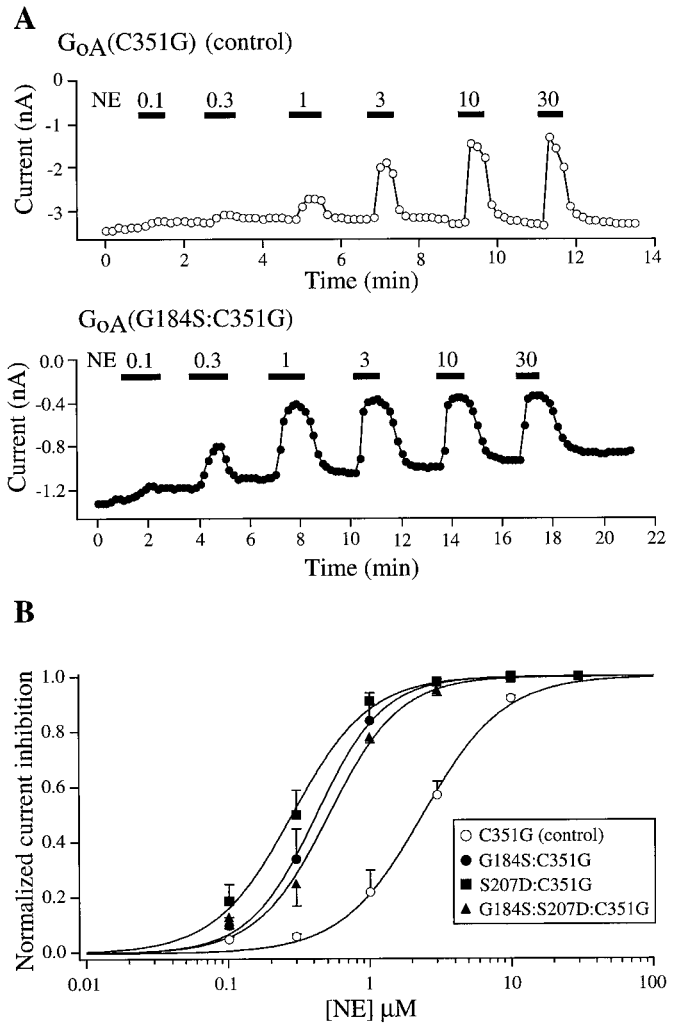

Figure 3. Expression of PTX/RGS-i $G\alpha_{oA}$ mutants shifts concentration–response relationships of NE-induced Ca^{2+} current inhibition to lower concentrations. *A*, Representative time courses of Ca^{2+} current inhibitions acquired from sequential applications of NE over a wide range of concentrations (0.1–30 μM) in neurons expressing either $G\alpha_{oA}$(C351G) or $G\alpha_{oA}$(G184S: C351G). Currents were evoked by single 50 msec test pulses to +10 mV from a holding potential of −80 mV. *B*, Average concentration–response curves in neurons expressing either PTX-i $G\alpha_{oA}$ or PTX/RGS-i $G\alpha_{oA}$ mutants. Inhibition (percentage) was normalized to that at the maximal NE concentration (30 μM). Data are presented as mean ± SEM, and *numbers* in parentheses indicate the number of neurons tested. The concentration–response curves were fit to the Hill equation: $I = 1/[1 + (k/[NE])^n]$, where *I*, *k*, [NE], and *n* are normalized inhibition, a constant, NE concentration, and Hill factor, respectively. The *n* values for $G\alpha_{oA}$(C351G), $G\alpha_{oA}$(G184S: C351G), $G\alpha_{oA}$(S207D: C351G), and $G\alpha_{oA}$(G184S: S207D: C351G) were 1.5, 1.8, 1.6, and 1.7, respectively.

the Gα subunit mutations interfered with RGS protein interactions. To test this possibility, we measured Ca^{2+} current inhibition over a wide range of NE concentrations (0.1–30 μM). Figure 3*A* illustrates representative time courses of the current inhibition in neurons expressing $G\alpha_{oA}$(C351G) and $G\alpha_{oA}$(G184S:C351G) after sequential application of increasing concentrations of NE. Figure 3*B* summarized the steady-state concentration–response curves for neurons expressing PTX-i $G\alpha_{oA}$ (control) or PTX/RGS-i $G\alpha_{oA}$. When compared with uninjected neurons (non-PTX-treated), NE-induced Ca^{2+} current inhibition in neurons expressing PTX-i $G\alpha_{oA}$, i.e., $G\alpha_{oA}$(C351G), was approximately twofold less potent but equally efficacious (data not shown). In neurons expressing PTX/RGS-i $G\alpha_{oA}$ subunits, the steady-state concentration–response curves were shifted leftward with a rank order of potency (EC_{50}) as follows: $G\alpha_{oA}$(S207D:C351G) (0.28 μM, $n = 5$) > $G\alpha_{oA}$(G184S:C351G) (0.41 μM, $n = 6$) > $G\alpha_{oA}$(G184S:S207D:C351G) (0.50 μM, $n = 4$) > $G\alpha_{oA}$(C351G) (2.35 μM, $n = 4$). The maximal inhibitions produced by NE (30 μM) in neurons expressing $G\alpha_{oA}$(C351G), $G\alpha_{oA}$(G184S:C351G), $G\alpha_{oA}$(S207D:C351G), and $G\alpha_{oA}$(G184S:S207D:C351G) were 52 ± 6, 67 ± 1, 63 ± 3, and 55 ± 2%, respectively. In addition, the Hill factor for the concentration–response curves ranged between 1.5 and 1.8. Together, these data indicate that endogenous RGS proteins influence the agonist potency, and to a minor extent, efficacy, in sympathetic neurons.

Expression of PTX/RGS-i $G\alpha_{oA}$ subunits alters the kinetics of NE-induced Ca^{2+} current inhibition

It has been well established that RGS proteins, acting as GAPs, accelerate Gα-catalyzed GTP hydrolysis *in vitro* (for review, see Dohlman and Thorner, 1997; Koelle, 1997; Berman and Gilman, 1998). These biochemical results implicate RGS proteins in controlling the kinetics of G-protein signaling. In recent electrophysiological studies, heterologously expressed RGS proteins have been shown to accelerate recovery of Ca^{2+} channels from the inhibition mediated by G_z (Jeong and Ikeda, 1998) and $G_{o/i}$

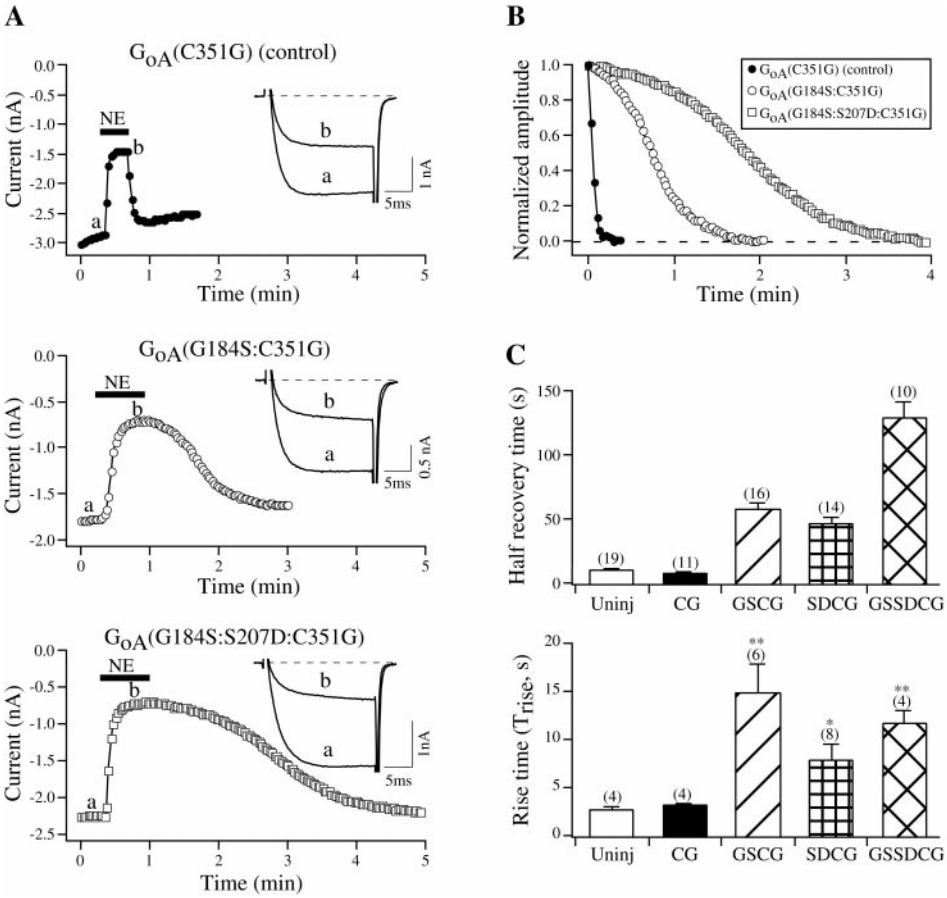

Figure 4. Effects of expression of PTX/RGS-i $G\alpha_{oA}$ mutants on kinetics of NE-mediated current inhibition. *A*, Time courses of NE-induced Ca^{2+} current inhibitions and the current recovery after agonist removal when neurons heterologously expressed $G\alpha_{oA}$(C351G) (*top*), $G\alpha_{oA}$(G184S: C351G) (*middle*), or $G\alpha_{oA}$(G184S: S207D: C351G) (*bottom*), along with $G\beta_1\gamma_2$. The currents were evoked every 2 sec by single 20 msec test pulses to +10 mV from a holding potential of −80 mV. The superimposed current *traces* in the *insets* represent those before (*a*) and during (*b*) agonist applications. *B*, Comparison of the current recovery rates after removal of agonist among $G\alpha_{oA}$(C351G), $G\alpha_{oA}$(G184S: C351G), or $G\alpha_{oA}$(G184S:S207D:C351G). The current amplitudes were normalized, and the *dashed line* indicates complete recovery to the current amplitude before agonist application. *C*, Summary of half recovery time ($t_{0.5}$) and onset time (t_{rise}) for the steady-state current inhibition. The t_{rise} was estimated as a 10–90% rise time. Both parameters were approximated by fitting a polynomial function to the time courses and interpolating the appropriate values (i.e., $t_{0.5}$ and t_{rise}). Curve fitting were performed with the IGOR data analysis package (WaveMetrics). Data are presented as mean ± SEM, and *numbers* in parentheses indicate the number of neurons tested. ANOVA ($p < 0.0001$) followed by Dunnett's *t* test; uninjected control versus *CG*, *GSCG*, *SDCG*, and *GSSDCG*. $^*p < 0.05$; $^{**}p < 0.01$.

(Melliti et al., 1999), and deactivation of GIRK channels (Doupnik et al., 1997; Saitoh et al., 1997; Kovoor et al. 2000). As described in Figure 1, the recovery of Ca^{2+} channels from inhibition was slowed, consistent with a disruption of endogenous RGS actions on expressed PTX/RGS-i $G\alpha_{oA}$ subunits. Therefore, additional experiments were undertaken to quantify these effects. Figure 4*A* illustrates time courses of NE-induced Ca^{2+} current inhibition onset and recovery in neurons expressing PTX-i $G\alpha_{oA}$ (control) or PTX/RGS-i $G\alpha_{oA}$ subunits. For these experiments, the Ca^{2+} currents were evoked every 2 or 5 sec by single 20 msec test pulses to +10 mV from a holding potential −80 mV. In neurons expressing PTX-i $G\alpha_{oA}$, NE (10 μM) produced a rapid onset of inhibition and current recovery after agonist removal similar to that observed in uninjected neurons (Fig. 4*C*) (Zhou et al., 1997). Conversely, the current recovery was dramatically retarded in neurons expressing PTX/RGS-i $G\alpha_{oA}$ (Figs. 4*A*,*B*). To compare the time course of current recovery, the half recovery time ($t_{0.5}$) was determined for the relaxation phase after agonist removal (Fig. 4*B*). A time constant was not calculated because the recovery time course under these circumstances was clearly nonexponential. The mean $t_{0.5}$ for $G\alpha_{oA}$(C351G) (control), $G\alpha_{oA}$(G184S:C351G), $G\alpha_{oA}$(S207D: C351G), and $G\alpha_{oA}$ (G184S:S207D:C351G) -expressing neurons was 9 ± 1 ($n = 19$), 59 ± 5 ($n = 16$), 47 ± 5 ($n = 14$), and 130 ± 13 sec ($n = 10$), respectively (Fig. 4*C*, *top*). Interestingly, decreases in the off rate seemed to be accompanied by decreases in the on rate. Indeed, the onset of the steady-state current inhibition was significantly retarded in neurons expressing PTX/RGS-i $G\alpha_{oA}$ when compared with neurons expressing PTX-i $G\alpha_{oA}$ (Fig. 4*A*). Because the rate of current inhibition onset was relatively rapid in relationship to the frequency of test pulses (therefore limiting temporal resolution), the onset of the NE-induced Ca^{2+} current inhibition was estimated as a 10–90% rise time (t_{rise}) instead of $t_{0.5}$. As summarized in Figure 4*C*, mean t_{rise} for $G\alpha_{oA}$(C351G), $G\alpha_{oA}$(G184S:C351G), $G\alpha_{oA}$(S207D:C351G), and $G\alpha_{oA}$(G184S:S207D:C351G) was 3.4 ± 0.1, 15.0 ± 3.0, 8.0 ± 1.7, and 11.8 ± 1.3 sec, respectively. It should be noted that the slowing of current inhibition onset was not proportional to the slowing of current recovery. Together, these data suggest that endogenous RGS proteins play an important role in controlling the lifetime of Ca^{2+} current inhibition after receptor activation in neurons.

DISCUSSION

Increasing evidence suggests that RGS proteins play an integral role in G-protein signaling. To date, however, information regarding the functional roles of RGS proteins is limited primarily to *in vitro* biochemical assays and heterologous overexpression experiments. Thus, the role played by natively expressed RGS proteins in G-protein signaling remains unclear. A reason for this void in our knowledge is the lack of experimental tools for uncoupling endogenous RGS proteins from G-protein signaling pathways. Thus, the development of the better tools represents a major challenge for understanding the physiological roles subserved by RGS proteins. In this regard, several potential strategies are apparent. First, genetically ablating specific RGS proteins, either acutely (e.g., antisense techniques) or stably (e.g., knock-out mice), might lend insight into endogenous RGS protein functions. However, >20 mammalian RGS proteins have been identified so far, and it is likely that even single cells contain

multiple different RGS protein subtypes (Gold et al., 1997; Kardestuncer et al., 1998). Moreover, of the RGS proteins studied to date, only limited Gα specificity (i.e., $G_{i/o}$ and G_q families) was observed in regard to GAP activity (for review, see Dohlman and Thorner, 1997; Koelle, 1997; Berman and Gilman, 1998). Thus, it seems likely that redundancy of RGS protein actions might confound studies in which single RGS proteins are eliminated. In one study, however, dialysis of neurons with an antibody directed at the RGS4 C terminus appeared to produce a specific result (Diverse-Pierlussi et al., 1999), indicating that this approach may be a fruitful one. A second strategy is to produce a dominant negative mutant RGS protein. Unfortunately, the means of creating such a mutant are not apparent, because mutations that alter GAP activity also disrupt Gα binding (Srinivasa et al., 1998). Finally, it might be possible to construct a mutant Gα subunit that sequesters RGS proteins. It has been shown that RGS4 interacts with a GTPase-deficient mutant of $G\alpha_{i1}$, $G\alpha_{i1}$(R178C) (Berman et al., 1996b). In preliminary experiments, however, expression of $G\alpha_{oA}$(R178C) resulted in apparent sequestration of Gβγ (Ikeda, 1996; Jeong and Ikeda, 1999b), presumably arising from a population of GDP-bound $G\alpha_{oA}$(R178C) (S.-W. Jeong and S. R. Ikeda, unpublished data).

The strategy used in this study originated from the seminal findings of several groups. First, a founding member of the RGS family, SST2, has been shown to desensitize Gβγ-mediated pheromone signaling via direct interaction with GPA1, a Gα subunit in *Saccharomyces cerevisiae* (Dohlman et al., 1996). Biochemical and functional observations demonstrated that a GPA1 mutant ($gpa1^{sst}$) phenotypically mimicked the loss of SST2, suggesting that the mutant was insensitive to RGS GAP activity (DiBello et al., 1998). Subsequent sequencing of the Gα mutant revealed that Gly302, a conserved amino acid in the Gα family, was mutated to Ser. Homologous mutations in mammalian Gα subunits ($G\alpha_{o/i}$ and $G\alpha_q$) resulted in an equivalent phenotype, thus demonstrating the generality of the substitution (DiBello et al., 1998; Lan et al., 1998). Second, mutation of Ser202 to Asp in transducin was shown to abolish interaction with a human retinal RGS isoform, hRGSr, *in vitro* (Natochin and Artemyev, 1998a,b; Posner et al., 1999). Mutation of Ser202 was arrived at by comparing the sequences of RGS-sensitive (i.e., $G\alpha_{i/o}$ and $G\alpha_{q/11}$) and -insensitive Gα subunits (i.e., $G\alpha_s$ and $G\alpha_{12}$) (Berman et al., 1996a; Huang et al., 1997). A problem with using RGS-insensitive Gα mutants for investigating the functional roles of endogenous RGS proteins in neurons is suppressing the activity of endogenous Gα subunits. To this end, we adopted the method used by several laboratories (Taussig et al., 1992; Hunt et al., 1994; Senogles 1994; Kozasa et al., 1996) and subsequently studied in detail by Milligan and colleagues (Wise et al., 1997; Bahia et al., 1998) in which Gα subunits are rendered PTX-insensitive by mutating a Cys residue in the C terminus. We have shown previously that heterologously expressed G-protein heterotrimers (Gαβγs) containing PTX-i Gα subunits functionally replace (as determined from VD Ca^{2+} channel inhibition) natively expressed G-proteins ($G_{o/i}$) after PTX treatment of SCG neurons (Jeong and Ikeda, 2000). Thus, the strategy we used combined these two sets of mutations.

Success in using this method relied on reconstituting VD Ca^{2+} channel inhibition in PTX-treated neurons expressing PTX/RGS-i $G\alpha_{oA}$ subunits. Because the crystal structure of $G\alpha_{i1}$-GDP-AlF_4^- indicates that the mutated Gly and Ser residues are located near Gβγ contact sites within the switch I and II regions, respectively (Lambright et al., 1996), there was concern that heterotrimer formation might be disrupted by the mutations. Preliminary experiments, however, indicated that overexpression of PTX/RGS-i $G\alpha_{oA}$ (without coexpression of Gβγ) attenuated NE-induced Ca^{2+} current inhibition in a manner consistent with the sequestration of Gβγ subunits (data not shown) (Ikeda, 1996; Jeong and Ikeda, 1999b), thus providing evidence for *in situ* heterotrimer formation. Consistent with this observation, successful reconstitution of VD Ca^{2+} channel inhibition was observed for all PTX/RGS-i $G\alpha_{oA}$ subunits (Fig. 1) whenever a functional stoichiometric match (Jeong and Ikeda, 2000) between PTX/RGS-i $G\alpha_{oA}$ and $G\beta_1\gamma_2$ subunits was achieved.

Major features of the NE-induced Ca^{2+} current inhibition reconstituted in neurons expressing PTX/RGS-i $G\alpha_{oA}$ subunits were (1) a leftward shift in the NE concentration–response relationships, (2) a dramatically retarded recovery time course after agonist removal, and (3) an increase in the time to reach steady-state current inhibition after agonist application. Some of these results can be rationalized by the failure of endogenous RGS proteins to interact with PTX/RGS-i $G\alpha_{oA}$ subunits (in the transition conformation) and, consequently, the resulting loss of RGS-mediated GTPase acceleration. In this situation, G-protein heterotrimer reformation is limited by the relatively slow intrinsic GTPase activities of $G\alpha_{oA}$ subunits (Higashijima et al., 1987; Lan et al., 1998). Consequently, the concentration of free Gβγs around the Ca^{2+} channel would remain relatively high for a prolonged time after agonist removal, thus retarding recovery of channels from inhibition. Moreover, because steady-state concentrations of Gα-GTP (and hence free Gβγ) should be determined by the relative rates of Gα-GTP formation and destruction, the increase in agonist potency and modest increase in efficacy can be accommodated by this interpretation as well. These results are consistent with several previous studies in which RGS actions were increased by heterologously overexpressing RGS proteins. For example, expression of RGS4 in COS-7 cells produced a rightward shift in the concentration–response relationship for activation of mitogen-activated protein kinase (Yan et al., 1997). In HEK293 cells, Melliti et al. (1999) showed that expression of RGS3T, RGS3, and RGS8 altered the magnitude and kinetics of N-type Ca^{2+} channel inhibition, as well as produced a rightward shift of the steady-state concentration–response curve. In SCG neurons, Jeong and Ikeda (1998) have shown that overexpression of RGS4 and RGS10 accelerate the recovery of NE-induced inhibition of Ca^{2+} channels reconstituted with $G\alpha_z$. In that study, overexpression RGS10 also attenuated NE-induced Ca^{2+} current inhibition in control SCG neurons, suggesting a rightward shift in the concentration–response curve. Conversely, RGS proteins appear to accelerate deactivation of GIRK currents without an apparent shift in the concentration–response relationship (Doupnik et al., 1997; Saitoh et al., 1997). Although the reason for this difference remains unclear, it possibly suggests that, in addition to GAP functions, RGS proteins subserve other roles, such as increasing Gβγ availability (Bünemann and Hosey, 1998) or effecting receptors/G-proteins coupling (Zeng et al., 1998; Xu et al., 1999).

The slowing of the on rate of steady-state Ca^{2+} current inhibition seen in this study is more difficult to explain within in the framework of established RGS functions. Multiple steps, such as agonist binding to receptor, receptor/G-protein interaction, GDP/GTP exchange rate, and Gβγ interaction with the channel, could contribute to this rate. A possibility that should be considered is that the Gα mutations used in our studies have additional actions, such as altering the receptor-mediated GDP/GTP ex-

change rate. However, *in vitro* experiments indicate that basal GDP/GTP exchange rates are not significantly altered by these $G\alpha$ mutations (Lan et al., 1998). Moreover, the current results are consistent with previous studies in which overexpression of RGS proteins exerted opposite effects, i.e., acceleration of the onset of steady-state Ca^{2+} current inhibition (Jeong and Ikeda, 1998; Melliti et al. 1999) and GIRK activation (Doupnik et al., 1997; Saitoh et al., 1997). Two possibilities might explain this phenomenon. First, RGS proteins may directly or indirectly stimulate GDP/GTP exchange via unknown mechanisms, although at present there is little evidence to support this view (Dohlman and Thorner, 1997; Koelle, 1997; Berman and Gilman, 1998). Second, RGS proteins may affect the receptor/G-protein coupling independently of the GAP activity associated with the RGS core domain (Zeng et al., 1998; Xu et al., 1999), thereby altering the rate of Ca^{2+} channel inhibition. In this regard, it should be mentioned that the term RGS "insensitivity," in the current context, refers solely to the GAP activity of RGS proteins and does not preclude effects that may be conferred by domains lying outside of the RGS core domain. In fact, preliminary data indicate that the kinetics of GIRK channel activation/deactivation after expression of PTX/RGS-i $G\alpha_{oA}$ subunits are altered by noncore RGS domains (Jeong and Ikeda, unpublished data).

Previously, Zhou et al. (1997) have shown that N-type Ca^{2+} channels in SCG neurons recovered from NE-induced inhibition after agonist removal with a rate constant (between 0.09 and 0.18 sec^{-1}) much higher than that reported for the intrinsic GTP hydrolysis rate ($\sim$0.03 sec^{-1}) of $G\alpha_o$ determined *in vitro* (Higashijima et al., 1987). Our results indicate that the actions of endogenous RGS proteins might account for this discrepancy. Thus, in addition to receptors, G-proteins and N-type Ca^{2+} channels, RGS proteins must be added to the membrane-delimited pathway describing VD channel modulation. How RGS proteins contribute to the more integrative functions of N-type Ca^{2+} channels, such as presynaptic inhibition resulting from neurotransmitter release, remains to be determined. It is possible that the strategy used here, i.e., the combined use of PTX- and RGS-insensitive $G\alpha$ subunits, will be useful in defining the functional roles of the endogenous RGS proteins in different systems.

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