Regulators of G Protein Signaling Proteins as Determinants of the Rate of Desensitization of Presynaptic Calcium Channels*

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Norepinephrine inhibits ω-conotoxin GVIA-sensitive presynaptic Ca2+ channels in chick dorsal root ganglion neurons through two pathways, one mediated by Go and the other by Gi. These pathways desensitize at different rates. We have found that recombinant Ga interacting protein (GAIP) and regulators of G protein signaling (RGS)4 selectively accelerate the rate of desensitization of Gi- and Go-mediated pathways, respectively. Blockade of endogenous RGS proteins using antibodies raised against Ga interacting protein and RGS4 slows the rate of desensitization of these pathways in a selective manner. These results demonstrate that different RGS proteins may interact with Gi and Go selectively, giving rise to distinct time courses of transmitter-mediated effects.

Voltage-dependent calcium channels are the primary triggers for electrically evoked release of chemical transmitters. Understanding the mechanisms underlying their regulation is central to the development of a molecular picture of key events in neuronal signaling. Calcium channels are well known targets for inhibition by receptor-G protein pathways, and multiple forms of inhibition have been described (1, 2). The termination of the response or desensitization represents a main mechanism for controlling synaptic strength in an activity-dependent manner. Much of the knowledge about agonist-dependent desensitization comes from studies of ionotropic receptors such as GABAergic1 and glutamatergic receptors. In addition to the ionotropic receptor-mediated fast synaptic transmission, signaling in the nervous system relies heavily on G protein-coupled receptors for more prolonged and sustained synaptic action.

Norepinephrine (NE) inhibits N-type (ω-conotoxin GVIA-sensitive) Ca2+ channels in embryonic dorsal root ganglion (DRG) neurons through two pathways (3, 4), one mediated by Gi, and the other by Go. Low concentrations of NE (<10 μM) produce reductions in N current with no changes in its time course of activation (termed “steady-state inhibition” (SSI)), whereas higher NE concentrations (>10 μM) evoke a slowing of the activation kinetics termed “kinetic slowing” (KS) (3). Use of purified G protein subunits has demonstrated that KS induced by NE requires the activation of Go, and αi is the functional subunit (4). By contrast, SSI is mediated by βγ released from Gi (4) and requires the activation of protein kinase C. Depolarizing prepulses reverse KS, leaving SSI unaffected (5).

This inhibition of the N-type calcium current is a transient phenomenon as the response desensitizes under prolonged exposure to 100 μM NE (6). The onset of desensitization in DRG neurons requires receptor-mediated G protein activation. Although both KS and SSI require the activation of the same G protein-coupled receptor kinase, GRK3 (6), each exhibits different rates of desensitization.

Recently, a new family of proteins, regulators of G protein signaling (RGS), has been found to play a role in desensitization (7–14). RGS proteins block G protein function by accelerating the rate of GTP hydrolysis (15, 16). Experiments presented in this paper show that RGS proteins selectively alter the time course of desensitization of the two signaling pathways. Although recent reports have shown that there is selectivity in the actions of RGS proteins on Gi- and Go-mediated pathways (17, 18), little is known about the ability of these proteins to interact with different members of the Gi subfamily. Here we show that RGS4 and GAIP selectively interact with the Gi- and Go-mediated pathways. RGS4 alters the rate of desensitization of the Gi- and voltage-independent inhibition of calcium current whereas GAIP accelerates the rate of desensitization of the Gi- and voltage-dependent inhibition of calcium current. Furthermore, our data suggest that a domain outside of the RGS domain might be responsible for the G protein subtype selectivity in RGS actions.

EXPERIMENTAL PROCEDURES

Cell Culture—Embryonic chick sensory neurons were grown in culture as described previously (3). Dorsal root ganglia were dissected from 11–12-day-old embryos. Cells were plated at a density of ~50,000 cells per collagen-coated 35-mm tissue culture dish and studied between 1 and 3 days in vitro.

Electrophysiology—Whole-cell recordings were performed as described previously (19). For extracellular application, agents were diluted into standard extracellular saline and applied via a wide-bore (140 μM inner diameter) pipette, which exchanges solutions with sub-second kinetics. For the experiments presented in this report, calcium current has been corrected for rundown by measuring calcium current as a function of time in control cells without transmitter. Cells used for experiments exhibited a rundown of the current of ~2%/min.

Data Analysis—Data were filtered at 3 kHz, acquired at 10–20 kHz,
and analyzed using PulseFit (HEKA) and Igor (WaveMetrics) on a Macintosh computer. Strong depolarizing conditioning pulses (to 80 mV) that precede test pulses (to 0 mV) reverse NE-induced KS without affecting SSI. Such conditioning pulses have no effect on control currents recorded in the absence of NE. During the application of the transmitter, test pulse currents measured before and after the conditioning pulse are subtracted to yield the KS component. Test pulses measured following the conditioning pulse are subtracted from control currents (measured in the absence of NE) to yield the SSI component. Integration of these two currents gives measurements of total charge entry carried by the two modulatory components.

Recombinant Proteins—Rat pGBT9 Ga\(_{\alpha1}\) (originally a gift from E. Neer (9)), human Ga\(_{\alpha1}\), and human Ga\(_{\alpha4}\) (a generous gift from Dr. Druey), and the RGS domain of rat Ga\(_{\alpha1}\)-(80–206) were subcloned in pET28a (Novagen) and expressed in BL21 (DE3) host. Escherichia coli strains were loaded per lane and separated by electrophoresis on denaturing 15% SDS-PAGE gels. Histidine-tagged proteins were eluted with 250 mM imidazole. 4 °C for 15 min at 12,000 g. mix was added. Tubes containing charcoal slurry were centrifuged at 15,000 × g for 40 min in 50 mM HEPES (pH 8.0), 5 mM EDTA, 1 mM dithiothreitol, and 0.05% polyoxyethylene 10 lauryl ether (C12E10) at 30 °C. The temperature was then reduced to 4 °C. All GTP hydrolysis measurements were performed under single turnover conditions at 4 °C (15). Reactions were started by addition of GRS mix consisting of 15 mM MgSO\(_4\), 150 mM GTP, and 2 mM (final concentrations) of each GaIP/RGS4 polypeptide. 50-μl aliquots were removed at the indicated times and added to 750 μl of a 5% (v/v) charcoal slurry in 50 mM NaCl, 250 mM NaCl, 5 mM imidazole, 30 μM AlCl\(_3\), 20 mM NaF, 100 μM GDP, 1% Tween 20, and 200 μg/ml lysozyme for Ga\(_{\alpha1}\). After sonication, 100,000 × g supernatants were applied to Ni\(_2^+\)-nitrilotriacetate resin (Qiagen, Chatsworth, CA), and histidine-tagged proteins were eluted with 250 mM imidazole.

**GTPhase-activating Protein Assays—**250 μM Ga\(_{\alpha1}\) was loaded with [γ-32P]GTP (1 μM) for 40 min in 50 mM HEPES (pH 8.0), 5 mM EDTA, 1 mM dithiothreitol, and 0.05% polyoxyethylene 10 lauryl ether (C12E10) at 30 °C. The blots were washed sequentially with 2x SSC, 0.5% SDS at 25 °C, 1x SSC, 0.25% SDS at 42 °C, and 0.2x SSC, 0.05% SDS at 65 °C, and exposed to film.

**RESULTS**

We generated functional recombinant proteins in *E. coli* tagged on their N termini with a hexahistidine epitope for purification purposes. Ga\(_{\alpha1}\), RGS4, and Ga\(_{\alpha4}\) were able to speed up basal GTPhase activity of Ga\(_{\alpha1}\) triggered with 15 mM MgSO\(_4\) (Fig. 1B). These results are in agreement with previous reports (14, 15).

To test the effects of these RGS proteins, current amplitudes were recorded from the cell bodies of embryonic chick sensory neurons 1–5 days after plating. Application of 100 μM NE induced a 55 ± 7% decrease in calcium current. To separate the voltage-dependent (KS) from the voltage-independent (SSI) component, we used a +80-mV, 15-ms pulse with a 5-ms interval between the conditioning and test pulses (5). This inhibition is transient as the response is maximally desensitized by 2 min. The NE-mediated KS and SSI desensitize at different rates (6) (Fig. 1A).

In yeast two-hybrid experiments GaIP interacts more effectively with Ga\(_{\alpha1}\) and Ga\(_{\alpha4}\) than with Ga\(_{\alpha2}\) (9). This suggested that in chick sensory neurons, GaIP might exhibit differential effects on G\(_{\alpha1}\)-mediated KS and G\(_{\alpha4}\)-mediated SSI. Using tight-seal whole-cell recording, DRG neuron calcium current was measured before and during prolonged exposure to transmitter. Currents were evoked by a 10-ms voltage step from −80 to 0 mV every 10 s. Cells were equilibrated with control or RGS-containing (0.2–200 nM) internal solution for 10 min prior to transmitter application; calcium current as a function of time was measured to check for any agonist-independent effect that the recombinant protein might have. In control cells (n = 16), NE inhibited N-type calcium current by an average of 40% (25% SSI and 15% KS). In GaIP-treated cells, the KS component was eliminated (n = 14), whereas SSI was unaffected (Fig. 2A). Inclusion of GaIP in the recording solution blocked the G\(_{\alpha4}\)-mediated pathway. This result was corroborated in experiments in which GABA was used. GABA via GABA\(_A\) receptors induced kinetic slowing and steady-state inhibition of voltage-dependent calcium channels in chick DRG neurons by pathways mediated through G\(_{\alpha4}\) (4). GaIP blocked the GABA\(_A\)-mediated inhibition of the N-type calcium current (Fig. 2A). Inclusion of GaIP in the recording internal solution did not have an effect on the magnitude of the basal calcium current or the percentage of rundown of calcium current over time.

Experiments performed using more frequent depolarizations (every 2 s) revealed that the apparent block of the response is a result of an alteration in the time course of the NE-mediated
inhibition of calcium. In control cells (Fig. 2B), NE inhibits the calcium current and the effect persists during the experiment for the first 40 s in the presence of agonist. Cells that contained GAIP in the internal recording solution exhibited a maximal recovery in 8 s despite the continued presence of 100 μM NE. This accounts for the lack of response observed when the cells were depolarized every 10 s. Lower concentrations of the protein (2 nM) accelerated the rate of desensitization without altering the magnitude of the response. GAIP failed to alter the time course of the G_i-mediated response or onset of the inhibition.

It has been shown that RGS4 can alter the GTPase activity of both G_i and G_q. Inclusion of 200 nM RGS4 in the internal solution selectively accelerated the time course of desensitization of the NE-SSI without altering the KS component. Lower concentrations of RGS4 were also effective in altering the time course of desensitization (Fig. 3B). Recombinant RGS2, RGS12, and RGS14 (0.1–200 nM) did not have an effect on the magnitude of the response or in the magnitude, extent, or rate of desensitization.

A fusion protein containing the RGS core domain of GAIP-(79–206) was used to test whether this domain of the protein was sufficient to alter the time course of desensitization. The fusion protein was introduced into the intracellular environment through passive diffusion from the recording pipette. The core domain of GAIP altered both NE-mediated KS and SSI (Fig. 2C). The rate of desensitization of both inhibitory components was accelerated under these experimental conditions (data not shown). The selectivity of GAIP was lost when the N terminus and C terminus of the protein were removed.

To test whether RGS4 or GAIP plays a role in the desensi-

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**TABLE I**

Effects of RGS antibodies on the rate of desensitization of NE-mediated steady-state inhibition and kinetic slowing

| Antibody             | Kinetic slowing | Steady-state inhibition |
|----------------------|-----------------|------------------------|
| Control              | 60 ± 8          | 100 ± 6                |
| RGS4 N terminus      | 55 ± 9          | 975 ± 18               |
| RGS4 C terminus      | 61 ± 7          | 1000 ± 12              |
| GAIP full-length     | 650 ± 15        | 102 ± 7                |
| GAIP N terminus      | 640 ± 16        | 98 ± 7                 |
| RGS12 (RGS domain)   | 57 ± 3          | 97 ± 5                 |
| RGS2                 | 56 ± 5          | 101 ± 7                |
| RGS10                | 62 ± 6          | 103 ± 6                |

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**FIG. 2.** Effects of GAIP on the magnitude and time course of desensitization of NE-mediated kinetic slowing. A, mean inhibition of calcium current in control cells (open bars) or cells in the presence of 200 nM GAIP (filled bars). Cells were equilibrated for 10 min with control internal recording solution or internal recording solution containing 200 nM GAIP. After equilibration, cells were exposed to 100 μM NE or NE. B, time course of desensitization of NE-mediated kinetic slowing. Peak calcium current was measured as a function of time, before or during the application of 100 μM NE for control cells (squares, n = 6) and cells equilibrated with 200 (triangles, n = 6) or 2 (circles, n = 7) nM GAIP. C, mean inhibition of calcium current in control cells (open bars) or cells in the presence of a fusion protein containing the RGS domain of GAIP (filled bars). Cells were equilibrated for 10 min with control internal recording solution or internal recording solution containing 200 nM fusion protein. After equilibration, cells were exposed to 100 μM NE. Kinetic slowing and steady-state inhibition were calculated using protocol described under “Experimental Procedures.” The number of cells is indicated in parentheses.

**FIG. 3.** Effects of RGS4 on the magnitude and time course of desensitization of NE-mediated kinetic slowing. A, mean inhibition of calcium current in control cells (open bars) or cells in the presence of 200 nM RGS4 (filled bars). Cells were equilibrated for 10 min with control internal recording solution or internal recording solution containing 200 nM RGS4. After equilibration, cells were exposed to 100 μM NE. Kinetic slowing and steady-state inhibition were calculated using protocol described under “Experimental Procedures.” The number of cells is indicated in parentheses. B, time course of desensitization of NE-mediated kinetic slowing. Peak calcium current was measured as a function of time, before or during the application of 100 μM NE for control cells (squares, n = 4) and cells equilibrated with 200 (triangles, n = 8) or 2 (circles, n = 4) nM RGS4.
tization of NE-mediated KS and SSI under physiological conditions, we employed antibodies raised against these proteins to inhibit the endogenous proteins. The antibodies were introduced into sensory neurons through the recording pipette solution and allowed to diffuse into the cytoplasm. Cells were treated with 100 μM NE after 10–12 min. The magnitude of the N-type inhibition, the extent, and the time course of desensitization were compared with cells equilibrated for an equal amount of time with normal internal recording solution. Antibodies raised against RGS4 N terminus or C terminus (20 ng/ml) slowed the time course of desensitization of NE-mediated SSI (Table 1). The KS component was unaffected. Antibodies raised against full-length GAIP or its N terminus (20 ng/ml) increased the half-time for desensitization of NE-mediated KS by a factor of 10. The antibodies did not alter the extent of desensitization. Whereas blocking RGS proteins had a profound effect on the rate of desensitization, no effect was observed on the onset of desensitization, as the initial time at which desensitization could be detected remained unchanged in control and antibody-treated cells. NE-mediated SSI and KS in cells microinjected with antibodies raised against RGS2, RGS12, and RGS10 were no different from control cells (Table 1).

cDNA fragments encoding RGS domains were generated from poly(A)+ RNA of embryonic chick DRG neurons by RT-PCR using degenerate oligonucleotide primers. Two of the fragments exhibited a high degree of nucleotide and amino acid sequence identity with mammalian GAIP and RGS4, respectively (Fig. 4, A and B). Northern blot analysis of embryonic chick RNA indicated that chick GAIP and RGS4 Fragments hybridized at high stringency to transcripts in DRG neuron RNA that are comparable in size to their mammalian counter-

FIG. 4. Chick RGS genes are expressed in DRG neurons. A and D, alignment of amino acid sequences deduced from chick RT-PCR cDNAs with the proteins that exhibited the highest scores on a BLAST search of the GenBankTM data bases. The sequences are as follows: human RGS4 (accession number U27778), rat RGS8 (AB006013), human RGS3 (1710136), human RGS5 (AB008109), human GAIP (1730186), human GzGAP (AP060877), and bovine retina-specific RGS (U89254). B and E, percentage identity and similarity between chick RGS domains and various mammalian RGS proteins as determined by CLUSTALW pairwise alignment (identity/similarity). C and F, autoradiograms of a Northern blot containing poly(A)+ RNA isolated from chick embryo brain, heart, and DRG neurons. The blot was hybridized with a chick RGS4 probe, stripped, and rehybridized with a radioactively labeled chick GAIP fragment. Numbers on the right of the autoradiograms represent the approximate length in nucleotides of the hybridizing transcripts. ch, chick; h, human; r, rat; GzGAP, regulator of Gz-selective protein signaling; ret, bovine retina specific; St, standards; nt, nucleotides.

DISCUSSION
Recent reports have shed light on the functional roles of RGS proteins in the nervous system. RGS9 colocalizes with the other signaling components of the phototransduction machinery in the rod outer segment, and in vitro assays have shown that this RGS protein accelerates the rate of GTP hydrolysis of transducin (21). RGS8 coexpressed with a G protein-coupled receptor and a G protein-coupled inwardly rectifying K+ channel accelerated the kinetics of onset and desensitization of the response (22). In hippocampal CA1 neurons, RGS4 blocks the glutamate receptor (GluR5)-mediated inhibition of K+ current by blocking Gq-mediated activation of phospholipase C (23). The question remains whether the RGS proteins can selectively alter transmitter responses mediated by a specific type of G protein.

About nine different RGS domains have been sequenced by RT-PCR in embryonic dorsal root ganglion neurons. The questions of why a cell needs multiple RGS proteins and whether there is selectivity in their action arise. Our experiments show that there is selectivity in the effects of RGS proteins and that this could potentially lead to differences in the desensitization of G- and G- mediated pathways. We have shown that the endogenous RGS proteins exhibit selectivity, as blocking RGS4 and GAIP with specific antibodies in sensory neurons altered the rates of desensitization of G-mediated SSI and G-mediated KS, respectively.

3 M. Schiff, J. D. Jordan, and M. A. Diversé-Pierluissi, unpublished observation.
The molecular basis for the selectivity of the RGS actions resides in a domain outside of the RGS box, as the RGS domain of GAIP alone did not discriminate between $G_i$- and $G_o$-mediated pathways. Recent reports have shown that molecular domains outside of the RGS box might be important for the targeting or activity of RGS proteins. The localization of RGS4 to the plasma membrane and GAIP to intracellular membranes seems to be dependent on the N terminus of these molecules (20, 24). Recently, the N-terminal domain of RGS4 (and to a lesser degree, its C-terminal domain) was shown to be important for discrimination between specific receptor signaling complexes (25). The N terminus of RGS12 contains a PDZ domain, which interacts with a chemokine receptor (26), and the C terminus of GAIP interacts with a novel PDZ domain containing protein (27). These interactions might also contribute to their selectivity. Besides its potential role in membrane attachment via its cysteine string motif, the N-terminal domain of GAIP might also play an additional role in the selectivity of this RGS protein.

The RGS box might also contribute to the selectivity. The RGS domains of GAIP and RGS4 are structurally very similar, but the conserved Asn-128 residue in RGS4 that makes closest contact to the $G_{o_1}$ subunit is present as Ser-156 in GAIP (28). Although not yet tested, swapping these residues in both molecules might have effects on their selectivity of interaction with different G protein α subunits.

An interesting observation derived from the experiments is that although RGS proteins play a role in determining the rate of desensitization, blockade by antibodies did not prevent desensitization. A plausible explanation is that another molecule is necessary for the onset of desensitization. We have previously shown that GRK3, a G protein-coupled receptor kinase plays a role in desensitization in embryonic chick sensory neurons (6). Future experiments should address the roles of GRK3 and RGS proteins in the onset and kinetics of desensitization.

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