Multiple G-Protein $\beta\gamma$ Combinations Produce Voltage-Dependent Inhibition of N-Type Calcium Channels in Rat Superior Cervical Ganglion Neurons

Victor Ruiz-Velasco and Stephen R. Ikeda

Laboratory of Molecular Physiology, Guthrie Research Institute, Sayre, Pennsylvania 18840

Activation of several G-protein-coupled receptors leads to voltage-dependent (VD) inhibition of N- and P/Q-type Ca^{2+} channels via G-protein $\beta\gamma$ subunits ($\text{G}\beta\gamma$). The purpose of the present study was to determine the ability of different $\text{G}\beta\gamma$ combinations to produce VD inhibition of N-type Ca^{2+} channels in rat superior cervical ganglion neurons. Various $\text{G}\beta\gamma$ combinations were heterologously overexpressed by intranuclear microinjection of cDNA and tonic VD Ca^{2+} channel inhibition evaluated using the whole-cell voltage-clamp technique. Overexpression of $\text{G}\beta 1$ – $\text{G}\beta 5$, in combination with several different $\text{G}\gamma$ subunits, resulted in tonic VD Ca^{2+} channel inhibition. Robust Ca^{2+} channel modulation required coexpression

of both $\text{G}\beta$ and $\text{G}\gamma$. Expression of either subunit alone produced minimal effects. To substantiate the apparent lack of $\text{G}\beta\gamma$ specificity, we examined whether heterologously expressed $\text{G}\beta\gamma$ displaced native $\text{G}\beta\gamma$ from heterotrimeric complexes. To this end, mutant $\text{G}\beta$ subunits were constructed that differentially modulated N-type Ca^{2+} and G-protein-gated inward rectifier K^+ channels. Results from these studies indicated that significant displacement does not occur, and thus the observed $\text{G}\beta\gamma$ modulation can be attributed directly to the heterologously expressed $\text{G}\beta\gamma$ combinations.

Key words: G-protein; N-type Ca^{2+} channel; GIRK channel; $\text{G}\beta\gamma$; ion channel modulation; SCG neurons

Inhibition of neuronal Ca^{2+} channels by G-protein-coupled receptors (GPCR) represents an important mechanism for modulating release of neurotransmitters from presynaptic nerve endings (Dunlap et al., 1995). Although several discrete signaling pathways leading to N-type Ca^{2+} channel inhibition have been identified (Hille, 1994), the most commonly used and best characterized pathway results from activation of GPCR that couple to pertussis toxin-sensitive G-proteins (Ikeda and Dunlap, 1999). After receptor activation, N-type Ca^{2+} channels are inhibited by a membrane-delimited pathway that results in a shift of the channels from a “willing” to “reluctant” mode in which a more depolarized membrane potential is required for channel opening (Bean, 1989). Consequently, the resulting Ca^{2+} channel inhibition is voltage-dependent (VD), i.e., the magnitude of inhibition is dependent on the membrane potential at which channel opening is measured.

Recently, the molecular mechanism underlying VD inhibition of N- and P/Q-type has begun to emerge (Zamponi and Snutch, 1998; Ikeda and Dunlap, 1999). Experiments in which various G-protein subunits were heterologously expressed in neurons or Ca^{2+} channel-expressing cells demonstrated that the $\text{G}\beta\gamma$, rather than the $\text{G}\alpha$, component of heterotrimeric G-proteins was responsible for VD inhibition (Herlitze et al., 1996; Ikeda, 1996). Subsequent studies demonstrated that $\text{G}\beta\gamma$ interacts with various

regions of Ca^{2+} channel α_1 subunits (De Waard et al., 1997; Qin et al., 1997; Zamponi et al., 1997; Furukawa et al., 1998; Canti et al., 1999). Currently, the consensus view of VD inhibition envisions “release” of $\text{G}\beta\gamma$ from the $\text{G}\alpha\beta\gamma$ heterotrimer after GPCR activation, followed by direct binding of $\text{G}\beta\gamma$ to the Ca^{2+} channel. At depolarized potentials, the $\text{G}\beta\gamma$ subunit is believed to unbind from the Ca^{2+} channel α_1 subunit thereby relieving the inhibition and producing biophysical alterations, i.e., “kinetic slowing” of activation and “prepulse facilitation,” which are the electrophysiological signatures of the VD pathway.

Given this mechanism, the question arises whether distinct combinations of $\text{G}\beta\gamma$ confer specificity in regard to VD N-type Ca^{2+} channel modulation. Currently, five $\text{G}\beta$ subunits ($\beta 1$ – $\beta 5$) and eleven $\text{G}\gamma$ subunits ($\text{G}\gamma 1$ – $\text{G}\gamma 12$; $\text{G}\gamma 6$ was renamed $\text{G}\gamma 2$) have been identified from cloning studies (Watson and Arkinstall, 1994; Clapham and Neer, 1997). Although few combinations of $\text{G}\beta$ and $\text{G}\gamma$ are unlikely to participate in modulation because functional $\text{G}\beta\gamma$ monomers do not form or expression is highly restricted, there appear to be a large number of potential combinations that could participate in Ca^{2+} channel modulation. Previously, Ikeda (1996) and Herlitze et al. (1996) reported that expression of $\text{G}\beta 1\gamma 2$, $\text{G}\beta 1\gamma 3$ or $\text{G}\beta 1\gamma 7$, and $\text{G}\beta 2\gamma 3$, respectively, produce VD inhibition of N-type Ca^{2+} channels. Recently, Garcia et al. (1998) reported that overexpression of some $\text{G}\beta$ subunits ($\text{G}\beta 1$, $\text{G}\beta 2$, or $\text{G}\beta 5$) but not others ($\text{G}\beta 3$ or $\text{G}\beta 4$) resulted in N-type Ca^{2+} channel inhibition. The purpose of the present study was to extend these studies by heterologously overexpressing defined $\text{G}\beta\gamma$ combinations and determining which subunit combination(s) produced tonic (i.e., in the absence of GPCR activation) VD inhibition of N-type Ca^{2+} channels in superior cervical ganglion (SCG) neurons. Unlike Garcia et al. (1998), our results indicate that $\text{G}\beta 1$ – $\text{G}\beta 5$ -containing heterodimers are capable of producing VD modulation.

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Correspondence should be addressed to Dr. Stephen R. Ikeda, Laboratory of Molecular Physiology, Guthrie Research Institute, One Guthrie Square, Sayre, PA 18840. E-mail: siked@inet.guthrie.org.

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MATERIALS AND METHODS

Neuron isolation and cDNA microinjection. Neurons from adult rat SCG were prepared using methods described previously (Ikeda, 1997). Briefly, male Wistar rats (175–225 gm) were killed by decapitation using a laboratory guillotine without previous anesthesia, and the SCG was dissected in chilled HBSS. The ganglia were incubated with 0.6 mg/ml collagenase type D (Boehringer Mannheim, Indianapolis, IN), 0.4 mg/ml trypsin (TRL type; Worthington Biochemical Corp., Lakewood, NJ), and 0.1 mg/ml DNase Type I (Sigma, St. Louis, MO) for 60 min in a water bath shaker at 35°C. After incubation, the dispersed neurons were centrifuged twice for 6 min at $50 \times g$ and then resuspended in Minimal Essential Medium (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal calf serum (Atlanta Biologicals, Atlanta, GA), 1% glutamine, and 1% penicillin–streptomycin solution (both from Mediatech, Inc.). The neurons were then plated into 35 mm tissue culture plates coated with poly-L-lysine and stored in a humidified incubator containing 5% CO_2 in air at 37°C.

Nuclear microinjection of plasmids was performed with an Eppendorf (Madison, WI) 5246 microinjector and 5171 micromanipulator ~3–5 hr after plating as described previously (Ikeda, 1997; Ruiz-Velasco and Ikeda, 1998). Plasmids coding for human $\text{G}\beta 2$ and $\beta 3$, mouse $\text{G}\beta 4$, $\text{G}\beta 5$, and $\text{G}\gamma 4$, and bovine $\text{G}\beta 1$, $\text{G}\gamma 1$, $\text{G}\gamma 2$, and $\text{G}\gamma 3$ (all subcloned into the mammalian expression vector, pCI; Promega, Madison, WI) were prepared using anion exchange columns (Qiagen, Chatsworth, CA) and stored in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). Human G-protein-gated inward rectifier K^+ channel 1 (GIRK1) and GIRK4 (Kir 3.1 and 3.4, respectively) and bovine $\text{G}\alpha_{\text{ir}}$ were supplied in pcDNA3.1 (Invitrogen, Carlsbad, CA) and prepared as above. Site-directed mutagenesis of $\text{G}\beta$ subunits was performed using the GeneEditor *in vitro* site-directed mutagenesis kit (Promega) per the manufacturer's instructions. Mutations were confirmed by automated DNA sequencing (ABI 310; Perkin-Elmer, Foster City, CA). Neurons receiving a successful nuclear injection were identified by fluorescence from coexpressed jellyfish green fluorescent protein (pEGFP-N1, 5 ng/ μL ; Clontech Laboratories, Palo Alto, CA) as described previously (Ruiz-Velasco and Ikeda, 1998).

Electrophysiology and data analysis. Ca^{2+} and GIRK channel currents were recorded using the whole-cell variant of the patch-clamp technique (Hamill et al., 1981). Patch pipettes were pulled from glass capillaries (Corning 7052; Garner Glass Co., Claremont, CA) on a P-97 Flaming-Brown micropipette puller (Sutter Instrument Co., San Rafael, CA), coated with Sylgard (Dow Corning, Midland, MI) and fire polished on a microforge. Whole-cell currents were acquired with a patch-clamp amplifier (Axopatch 200A or Axopatch 1C; Axon Instruments, Foster City, CA), analog filtered at 1–2 kHz (–3 dB; four-pole Bessel), and digitized using custom designed software (S3) on a Macintosh Quadra 700 computer (Apple Computer, Cupertino, CA) equipped with a 12-bit analog-to-digital converter board (MacADIOS II; G. W. Instruments, Bedford, MA). Cell membrane capacitance and series resistance (80–85%) were electronically compensated. All experiments were performed at room temperature (21–24°C). Data analysis were performed with the Igor (Wavemetrics, Lake Oswego, OR) software package. Graphs and current traces were produced with Igor, StatView (SAS Institute, Inc., Cary, NC) and Canvas (Deneba Software, Miami, FL) software packages. Data are presented as means $\pm$ SEM. Statistical analysis were performed with GB-Stat PPC (Dynamic Microsystems, Inc., Silver Spring, MD) software package using the one-way ANOVA, followed by the Newman–Keuls test. $p < 0.05$ was considered statistically significant.

For recording Ca^{2+} currents, the pipette solution contained (in mM): 120 *N*-methyl-D-glucamine, 20 tetraethylammonium hydroxide (TEA-OH), 11 EGTA, 10 HEPES, 10 sucrose, 1 CaCl_2 , 4 Mg-ATP, 0.3 Na_2ATP , and 14 Tris creatine phosphate. The pH was adjusted to 7.2 with methanesulfonic acid and HCl (10 mM), and the osmolality was 299–302 mOsm/kg. The external solution consisted of (in mM): 145 TEA-OH, 10 HEPES, 15 glucose, 10 CaCl_2 , and 0.0003 tetrodotoxin (TTX). The pH was adjusted to 7.4 with methanesulfonic acid, and the osmolality was 319–327 mOsm/kg. For recording GIRK currents, the pipette solution contained (in mM): 135 KCl, 11 EGTA, 1 CaCl_2 , 2 MgCl_2 , 10 HEPES, 4 Mg-ATP, and 0.3 Na_2ATP . The pH was adjusted to 7.2 with KOH, and the osmolality was 305 mOsm/kg. The GIRK external solution consisted of (in mM): 130 NaCl, 5.4 KCl, 10 HEPES, 10 CaCl_2 , 0.8 MgCl_2 , 15 glucose, 15 sucrose, and 0.0003 TTX. The pH was adjusted to 7.4 with NaOH, and the osmolality was 326 mOsm/kg.

Stock solutions (10 mM) of norepinephrine (NE)-bitartrate (Sigma) were prepared in H_2O and diluted in the external solution to 10 μM just

before use. Application of drugs to the neuron under study was performed by positioning a custom-designed gravity-fed microperfusion system ~100 μm from the cell as described previously (Ruiz-Velasco and Ikeda, 1998).

RESULTS

Properties of voltage-dependent Ca^{2+} channel inhibition

Kinetic slowing of activation and prepulse facilitation provide a rapid and reliable means of identifying the VD form of Ca^{2+} channel modulation. Figure 1*A* depicts superimposed Ca^{2+} current traces recorded from a control (uninjected) neuron in the absence (*bottom trace*) or presence (*top trace*) of 10 μM NE. In rat SCG neurons, NE acts via α_2 -adrenergic receptors (Schofield, 1990) to produce a well characterized VD inhibition. Ca^{2+} currents were evoked with a voltage protocol consisting of two identical test pulses (+10 mV) separated by a large depolarizing (+80 mV) conditioning pulse (Fig. 1*A*, *bottom*) (Elmslie et al., 1990). Kinetic slowing is illustrated in the current evoked during the prepulse (i.e., the test pulse preceding the conditioning pulse). Before NE exposure, the Ca^{2+} current activation phase was rapid, reaching a plateau within the initial 5–10 msec after onset of the test pulse (Fig. 1*A*, *bottom trace*). In contrast, after receptor-mediated G-protein activation with NE, the current rising phase was slower and biphasic (Fig. 1*A*, *top trace*).

A second property of VD inhibition, prepulse facilitation, is evident when the prepulse and postpulse (i.e., current evoked after the conditioning pulse) current amplitudes are compared. Figure 1*A* shows that, in the absence of NE (*bottom trace*), the conditioning pulse had a minor, although significant, effect on the postpulse current amplitude (Ikeda, 1991). In the presence of NE, however, the postpulse current was much larger than the prepulse current (relief of NE-mediated inhibition) and displayed normal activation kinetics. The facilitation ratio, a parameter calculated by dividing the postpulse by the prepulse current amplitude, increased dramatically during NE application and thus provided a convenient and reliable measure of VD inhibition. Together, these unique properties (kinetic slowing and increased facilitation ratio) allow VD inhibition to be characterized and measured independently of changes in current amplitude. This strategy was used to determine tonic (i.e., in the absence of agonist) VD inhibition produced after expression of $\text{G}\beta\gamma$ subunits.

Expression of different $\text{G}\beta\gamma$ combinations produces VD inhibition

Figure 1*B–D* illustrates the effects of intranuclear microinjection of $\beta 1$ and $\gamma 2$ cDNA (10 ng/ μL per subunit) alone or together on Ca^{2+} currents. Neurons previously coinjected with $\beta 1\gamma 2$ cDNAs displayed dramatic kinetic slowing and prepulse facilitation indicative of large tonic VD inhibition (Fig. 1*B*) as reported previously (Ikeda, 1996). Consistent with this idea, application of NE failed to produce significant effects, indicating near maximal modulation of the channels by expressed $\text{G}\beta\gamma$. Conversely, previous injection of either $\text{G}\beta 1$ (Fig. 1*C*) or $\text{G}\gamma 2$ (Fig. 1*D*) cDNA alone resulted in small and sometimes inconsistent changes (e.g., slightly increased prepulse facilitation) (Fig. 1*E*) in basal current properties. Moreover, application of NE to $\text{G}\beta 1$ - or $\text{G}\gamma 2$ -expressing neurons resulted in large inhibitions similar to those observed in uninjected neurons. Figure 1*E* summarizes the effect of expressing $\text{G}\beta 1$, $\text{G}\gamma 2$, $\text{G}\gamma 4$, and combinations of these subunits on basal (i.e., in the absence of agonist) facilitation ratio and NE-mediated Ca^{2+} current inhibition. Clearly, coexpression of

Figure 1. Facilitation and NE-mediated inhibition of Ca^{2+} currents in SCG neurons expressing $\beta 1$ or γ alone or combined. Superimposed Ca^{2+} current traces evoke with the “double-pulse” voltage protocol (bottom of *A*) in the absence (bottom traces) and presence (top traces) of $10 \mu\text{M}$ NE for control (*A*), $\text{G}\beta 1\gamma 2$ - (*B*), $\text{G}\beta 1$ - (*C*), and $\text{G}\gamma 2$ -expressing (*D*) neurons. Currents were evoked every 10 sec. *E*, Summary graphs of mean $\pm$ SEM basal facilitation and Ca^{2+} current inhibition for neurons expressing $\text{G}\beta 1$ alone or combined with $\text{G}\gamma 2$ and $\text{G}\gamma 4$ subunits. Final concentration of cDNA injected was $10 \text{ ng}/\mu\text{l}$ per subunit. Facilitation was calculated as the ratio of Ca^{2+} current amplitude determined from the test pulse (+10 mV) occurring after (postpulse) and before (prepulse) the +80 mV conditioning pulse. Ca^{2+} current inhibition was measured isochronally 10 msec after initiation of the test pulse (+10 mV) in the absence or presence of $10 \mu\text{M}$ NE. ** $p < 0.01$ versus control. Numbers in parentheses indicate the number of experiments.

Figure 2. Effect of heterologous overexpression of $\text{G}\beta 2$ and $\beta 5$ alone or with $\text{G}\gamma 2$, $\text{G}\gamma 3$, and $\text{G}\gamma 4$ on facilitation and NE-mediated inhibition of Ca^{2+} currents. *A*, *B*, Summary graphs of mean $\pm$ SEM basal facilitation and Ca^{2+} current inhibition for neurons expressing either $\beta 2$ or $\beta 5$ alone and combined with several γ subunits. Final concentration of cDNA injected was $10 \text{ ng}/\mu\text{l}$ per subunit. Basal facilitation and Ca^{2+} current inhibition were calculated as described in Figure 1*E*. Note that scales for both parameters are the same. Numbers in parentheses indicate the number of experiments. Insets show superimposed current traces evoked with the double-pulse voltage protocol (illustrated in Fig. 1*D*) in the absence or presence of $10 \mu\text{M}$ NE for $\beta 2\gamma 4$ - and $\beta 5\gamma 2$ -expressing neurons. * $p < 0.05$ versus control; ** $p < 0.01$ versus control.

$\text{G}\beta 1$ with different $\text{G}\gamma$ subunits produced significantly greater modulatory effect on Ca^{2+} currents than expression of either subunit alone as indicated by the increased facilitation ratio and attenuation of NE-mediated inhibition ($p < 0.01$). These results are similar to those obtained previously (Ikeda, 1996), although in the present experiments the concentration of cDNA injected was 10-fold lower than those used in the former study.

Using this basic experimental paradigm, we next systematically tested the ability of $\text{G}\beta 2$ – $\text{G}\beta 5$, alone and in combination with different $\text{G}\gamma$ subunits, to produce tonic VD inhibition of N-type Ca^{2+} channels. Unless otherwise noted, cDNA coding for the

various G-protein subunits was injected at a concentration of $10 \text{ ng}/\mu\text{l}$. Figure 2 summarizes basal facilitation and NE-mediated Ca^{2+} current inhibition in SCG neurons previously injected with cDNAs encoding $\text{G}\beta 2$ (Fig. 2*A*) or $\text{G}\beta 5$ (Fig. 2*B*) alone or in combination with cDNAs coding for $\text{G}\gamma 2$ – $\text{G}\gamma 4$. As seen with $\text{G}\beta 1$ -expressing neurons, expression of either $\text{G}\beta 2$ or $\text{G}\beta 5$ in the absence of concurrent $\text{G}\gamma$ expression produced no significant alteration in either basal facilitation ratio or NE-mediated inhibition of Ca^{2+} currents when compared with uninjected neurons (from the same neuronal preparations). Coexpression of $\text{G}\beta 2$ with $\text{G}\gamma$ subunits, however, resulted in significantly enhanced

Figure 3. Effect of heterologous overexpression of $\text{G}\beta 3$ and $\text{G}\beta 4$ alone or with $\text{G}\gamma 2$, $\text{G}\gamma 3$, and $\text{G}\gamma 4$ on facilitation and NE-mediated inhibition of Ca^{2+} currents. *A, B*, Summary graphs of mean $\pm$ SEM basal facilitation and Ca^{2+} current inhibition for neurons expressing either $\beta 3$ or $\beta 4$ alone and combined with several γ subunits. Final concentration of cDNA injected was 10 ng/ μl per subunit. Basal facilitation and Ca^{2+} current inhibition were calculated as described in Figure 1*E*. Note that scales for basal facilitation are different. Numbers in parentheses indicate the number of experiments. Insets show superimposed current traces evoked with the double-pulse voltage protocol (illustrated in Fig. 1*D*) in the absence or presence of 10 μM NE for $\beta 3\gamma 2$ - and $\beta 4\gamma 4$ -expressing neurons. * $p < 0.05$ versus control; ** $p < 0.01$ versus control.

basal facilitation ratio, decreased NE-mediated inhibition, and obvious kinetic slowing in the absence of agonist (Fig. 2*A*, inset). Conversely, coexpression of $\text{G}\beta 5$ with various $\text{G}\gamma$ subunits failed to produce significant increases in basal facilitation, although small decreases in NE-mediated Ca^{2+} current inhibition were observed. Increasing the concentration of injected $\text{G}\beta 5$ and $\text{G}\gamma 2$ cDNA to 100 ng/ μl per subunit, however, resulted in significant modulation, yet not when expressed alone. Under these conditions, basal facilitation ratios for control and $\text{G}\beta 5$ - and $\text{G}\beta 5\gamma 2$ -expressing neurons were 1.23 ± 0.04 ($n = 7$), 1.19 ± 0.04 ($n = 5$), and 1.82 ± 0.12 ($n = 12$; $p < 0.05$), respectively (data not shown) (Ikeda, 1996).

The effects of expressing $\text{G}\beta 3$ or $\text{G}\beta 4$ alone or together with $\text{G}\gamma 2$ – $\text{G}\gamma 4$ are summarized in Figure 3. As with the previously tested $\text{G}\beta$ subunits, expression of $\text{G}\beta 3$ produced significant alterations in basal facilitation ratio and NE-mediated inhibition of Ca^{2+} current only when coexpressed with a $\text{G}\gamma$ subunit (Fig. 3*A*). Conversely, injection of $\text{G}\beta 4$ cDNA resulted in a significant increase in basal facilitation ratio and attenuation of NE-mediated inhibition without concurrent injection of $\text{G}\gamma$ cDNA. Coexpression of $\text{G}\beta 4$ with $\text{G}\gamma$ subunits increased the basal facilitation ratio, an effect especially apparent with $\text{G}\gamma 4$ ($p < 0.01$). In fact, the tonic inhibition produced by $\text{G}\beta 4\gamma 4$ was the most potent observed in this study as indicated by the large basal facilitation ratio (~ 4) and greatly attenuated NE-mediated inhibition ($< 10\%$). Expression of $\text{G}\beta 3$ or $\text{G}\beta 4$ with $\text{G}\gamma$ subunits produced characteristic kinetic slowing of the Ca^{2+} current (Fig. 3*A, B*, insets, respectively).

Together, these results suggest that $\text{G}\beta 1$ – $\text{G}\beta 5$, in combination with various $\text{G}\gamma$ subunits, were capable of producing VD modulation of N-type Ca^{2+} channels. With the exception of $\text{G}\beta 4$, coexpression of a $\text{G}\beta$ together with a $\text{G}\gamma$ subunit was required to produce significant effects. At the usual concentration of injected cDNA (10 ng/ μl) used in this study, expression of $\text{G}\beta 5$, alone or together with $\text{G}\gamma$ subunits, produced minimal effects. These results are in agreement with some previously reported results (Ikeda, 1996; Delmas et al., 1998) but discrepant in regard to

other studies (Herlitze et al., 1996; Garcia et al., 1998). At present, the reason for this discrepancy is unclear. The results are especially puzzling because the preparation used in each of these studies was similar (rat sympathetic neurons).

Does heterologously expressed $\text{G}\beta\gamma$ displace native $\text{G}\beta\gamma$?

Meaningful interpretation of the experimental results presented thus far relies on the tacit assumption that heterologously expressed $\text{G}\beta\gamma$ were directly responsible for the observed changes in Ca^{2+} channel properties. The fact that most of the $\text{G}\beta\gamma$ combinations tested produced VD inhibition prompted us to investigate a possible alternative interpretation of the data. It was hypothesized that heterologously expressed $\text{G}\beta\gamma$ could displace native $\text{G}\beta\gamma$ from the G-protein heterotrimer as a result of basal G-protein activation. Under this scenario, the displaced “free” native $\text{G}\beta\gamma$ would interact with N-type Ca^{2+} channels and produce VD inhibition thus leading to interpretive difficulties.

In the absence of overt GPCR stimulation, there appears to be a low level of baseline G-protein activation in SCG neurons. This assumption is based on two previous experimental findings. First, introduction of nonhydrolyzable GTP analogs in SCG neurons (via the patch pipette) results in spontaneous VD inhibition (Ikeda and Schofield, 1989; Ikeda, 1996; Jeong and Ikeda, 1999). Second, a small amount of tonic VD inhibition, as indicated by basal facilitation ratio > 1 , has been documented in SCG neurons (Ikeda, 1991).

To address the issue of displacement, residues on $\text{G}\beta$ were mutated with the goal of imparting properties that would differentiate the actions of heterologously expressed mutant $\text{G}\beta\gamma$ from natively expressed wild-type $\text{G}\beta\gamma$. Two separate sets of mutations were developed based on the crystal structure of $\text{G}\beta\gamma$ (Wall et al., 1995; Sondek et al., 1996) and previous studies examining the effect of multiple discrete $\text{G}\beta$ mutations on effector interaction (Ford et al., 1998; Li et al., 1998). The goal of the $\text{G}\beta$ mutagenesis was twofold. First, we desired a $\text{G}\beta$ that interacted poorly (as $\text{G}\beta\gamma$) with $\text{G}\alpha$ yet retained the ability to modulate N-type Ca^{2+}

Figure 4. Effect of heterologous overexpression of mutant $\text{G}\beta 1$ and $\text{G}\alpha_{tr}$ on basal facilitation and NE-mediated Ca^{2+} current inhibition. Superimposed Ca^{2+} current traces evoke with the double-pulse voltage protocol (bottom of *E*) in the absence (bottom traces) and presence (top traces) of $10 \mu\text{M}$ NE for wild-type $\beta 1\gamma 2$ - and $\text{G}\alpha_{tr}$ -expressing (A), $\beta 1(80)\gamma 2$ - (B), $\beta 1(80)\gamma 2$ - and $\text{G}\alpha_{tr}$ - (C), $\beta 1(80,88,89)\gamma 2$ - (D), and $\beta 1(80,88,89)\gamma 2$ and $\text{G}\alpha_{tr}$ -expressing (E) neurons. F, Summary graphs of mean $\pm$ SEM basal facilitation and Ca^{2+} current inhibition for neurons expressing wild-type and mutant $\text{G}\beta 1\gamma 2$ alone or combined with $\text{G}\alpha_{tr}$. Final concentration of cDNA injected was $10 \text{ ng}/\mu\text{l}$ per subunit. Basal facilitation and Ca^{2+} current inhibition were calculated as described in Figure 1E. * $p < 0.05$ versus control; ** $p < 0.01$ versus control. Numbers in parentheses indicate the number of experiments.

channels. Second, we desired a $\text{G}\beta$ (when combined with $\text{G}\gamma$) that would differentially modulate two effectors, namely N-type Ca^{2+} channels and GIRK-type K^+ channels, that could be assayed electrophysiologically. The first set of $\text{G}\beta$ mutant constructs consisted of a single residue mutation, I80A, that was introduced into $\text{G}\beta 1$ and $\text{G}\beta 4$. The second set of $\text{G}\beta$ mutant constructs consisted of three separate point mutations in $\text{G}\beta 1$ (I80A,N88A,K89A) or $\text{G}\beta 4$ (L55A,N88A,K89A). These residues (L55, I80, N88, and K89) were chosen because alanine mutations at these sites also seemed to weaken the interaction with $\text{G}\alpha$ based on ADP ribosylation and immunoprecipitation assays (Ford et al., 1998; Li et al., 1998) but preserved interaction with N-type Ca^{2+} channels. In addition, alanine mutations of residues L55 and I80 appeared to impair GIRK activation (Ford et al., 1998). It was anticipated that both sets of mutations would possess one or more of the desired properties such that the mutant $\text{G}\beta$ would modulate N-type Ca^{2+} channels but interact poorly with GIRK channels and $\text{G}\alpha$.

Figure 4 illustrates experiments designed to probe the interaction of heterologously expressed mutant and wild-type $\text{G}\beta(+\text{G}\gamma)$ with a heterologously expressed $\text{G}\alpha$, transducin ($\text{G}\alpha_{tr}$). Transducin was chosen as the $\text{G}\beta\gamma$ “sink” or buffer because heterotrimers containing $\text{G}\alpha_{tr}$ are thought to couple only to rhodopsin. Expression of $\text{G}\alpha_{tr}$ neutralized the actions of expressed $\text{G}\beta 1\gamma 2$ (Fig. 4, compare A, B; Fig. 4F, solid bars), consistent with the known high affinity of GDP-bound $\text{G}\alpha$ for $\text{G}\beta\gamma$ (Slepak et al., 1995). Expression of either $\text{G}\beta 1(80\text{A})\gamma 2$ (Fig. 4B) or $\text{G}\beta 1(80\text{A},\text{N}88\text{A},\text{K}89\text{A})\gamma 2$ (Fig. 4D) resulted in an increased basal facilitation ratio (Fig. 4F, gray bars). Coexpression of $\text{G}\alpha_{tr}$ greatly decreased the basal facilitation resulting from expression of $\text{G}\beta 1(80\text{A})\gamma 2$ (Fig. 4C,F, hatched bars) but had a lesser effect on facilitation arising from $\text{G}\beta 1(80\text{A},\text{N}88\text{A},\text{K}89\text{A})\gamma 2$ expression (Fig. 4E,F, hatched bars). Summary of basal facilitation ratio and NE-mediated Ca^{2+} current inhibition data for each of these conditions is illustrated in Figure 4F. Together, the data suggest that the respective $\text{G}\beta 1$

mutants retained the ability to interact with both N-type Ca^{2+} channels and $\text{G}\alpha$ subunits. In the case of $\text{G}\beta 1(80\text{A},\text{N}88\text{A},\text{K}89\text{A})$, both interactions appeared to be weaker when compared with wild-type $\text{G}\beta 1$. However, basal facilitation resulting from this $\text{G}\beta 1$ mutant was also attenuated.

Figure 5 depicts experiments designed to evaluate whether mutant $\text{G}\beta 1$ subunits modulate GIRK-type K^+ channels. GIRK-type K^+ channels are inwardly rectifying channels that are gated by $\text{G}\beta\gamma$ binding (Logothetis et al., 1987; Wickman et al., 1994). The rat SCG neurons used in this study do not express native GIRK-type channels. However, functional GIRK channels can be heterologously expressed in SCG neurons (Ruiz-Velasco and Ikeda, 1998; Fernandez-Fernandez et al., 1999), thereby providing a second effector to evaluate $\text{G}\beta\gamma$ actions (Wickman and Clapham, 1995; Jan and Jan 1997). Heteromultimeric GIRK1 (Kir 3.1) and GIRK4 (Kir 3.4) channels were expressed in SCG neurons as described previously (Ruiz-Velasco and Ikeda, 1998). GIRK currents were elicited at 0.1 Hz from a holding potential of -60 mV in solutions (see Materials and Methods) designed to support K^+ currents. Current amplitude was determined from the peak inward current occurring during a 200 msec voltage ramp from -140 to -40 mV . Figure 5A shows GIRK current amplitude as a function of time for a $\beta 1\gamma 2$ -expressing neuron. In the absence of NE, there was a standing inwardly rectifying current (Fig. 5A, inset a) of $\sim 0.75 \text{ nA}$. Application of NE ($10 \mu\text{M}$; solid bar) induced an additional 1 nA of inward GIRK current (Fig. 5A, inset b) which reversed after removal of agonist. Application of Ba^{2+} (1 mM ; solid bar), an efficient blocker of GIRK channels, rapidly and reversibly reduced the current to near zero (Fig. 5A, inset c). Similar experiments for $\text{G}\beta 1(80\text{A})\gamma 2$ - and $\text{G}\beta 1(80\text{A},\text{N}88\text{A},\text{K}89\text{A})\gamma 2$ -expressing neurons are shown in Figure 5, B and C, respectively. Neither $\text{G}\beta 1$ mutant was capable of activating significant GIRK current, as indicated by the low current amplitude, lack of inward rectification in the current trace (Fig. 5B,C, inset a), and absence of current inhibition

Figure 5. Effect of heterologous overexpression of wild-type and mutant $\text{G}\beta 1$ on GIRK channel activation. Time course of basal and NE-activated GIRK1 and GIRK4 channel currents in $\beta 1\gamma 2$ - (A), $\beta 1(80)\gamma 2$ - (B), and $\beta 1(80,88,89)\gamma 2$ -expressing neurons. Currents were evoked by 200 msec voltage ramps from -140 to -40 mV from a holding potential of -60 mV applied every 10 sec. Filled bars indicate application of $10\text{ }\mu\text{M}$ NE or 1 mM Ba^{2+} and $10\text{ }\mu\text{M}$ NE. Insets show current traces obtained before (a) and after (b) application of NE or NE plus Ba^{2+} (c). D, Box plot showing the 10th, 25th, 50th (median), 75th, and 90th percentiles of peak GIRK currents before (Basal) and after (NE-activated) external application of $10\text{ }\mu\text{M}$ NE. Both the 10th and 90th percentiles are denoted by shorter lines. Numbers in parentheses indicate the number of experiments.

during Ba^{2+} application. However, GIRK currents were still activated after application of NE. Figure 5D summarizes the basal and NE-mediated GIRK current amplitude for $\text{G}\beta 1\gamma 2$ -, $\text{G}\beta 1(80\text{A})\gamma 2$ -, and $\text{G}\beta 1(80\text{A},\text{N}88\text{A},\text{K}89\text{A})\gamma 2$ -expressing neurons. Because of the large scatter in NE-induced GIRK currents (0.2 to 8.8 nA), box plots depicting the 10th, 25th, 50th (median), 75th, and 90th percentiles of the data are shown. The summary data indicate that expression of either $\text{G}\beta 1$ mutant (with $\text{G}\gamma 2$) did not result in the basal activation of GIRK channels as seen with wild-type $\text{G}\beta 1$ -expressing neurons. However, NE-mediated GIRK current activation, presumably arising from the actions of natively expressed $\text{G}\beta\gamma$, was similar for all three conditions. It has been shown in cardiac myocytes that intracellular Cl^- slows the turn-off reaction of GIRK channels leading to a higher sensitivity of GIRK channels to GTP (Nakajima et al., 1992). Unlike Nakajima et al. (1992), in the present study receptor coupling was bypassed such that overexpression of wild-type $\text{G}\beta\gamma$ subunits led to basal activation of GIRK channels (Fig. 5A,D; see Fig. 7A,D). Thus, it is unlikely that the absence of basal GIRK activity in neurons expressing mutant $\text{G}\beta\gamma$ subunits was a result of a direct influence of this anion on GIRK channels. Together, these data do not support displacement of endogenous $\text{G}\beta\gamma$ by heterologously expressed $\text{G}\beta\gamma$.

Parallel studies on $\text{G}\beta 4$

While this work was in progress, a similar study was published by Garcia et al. (1998) in which expression of $\text{G}\beta 3$ or $\text{G}\beta 4$ (alone or together with $\text{G}\gamma$) was reported to produce negligible effects on N-type Ca^{2+} channels of rat SCG neurons. Because in the current study expression of $\beta 4\gamma 4$ resulted in the greatest modulatory effect on Ca^{2+} currents (Fig. 3B), we undertook additional studies to further validate our results. Figure 6A shows Ca^{2+} current traces from a neuron expressing $\beta 4\gamma 4$ and $\text{G}\alpha_{\text{tr}}$ in the absence and presence of NE. In contrast to analogous experiments performed with $\text{G}\beta 1$, expression of $\text{G}\alpha_{\text{tr}}$ was unable to ablate the $\beta 4\gamma 4$ -mediated effects as evidenced by the significant residual basal facilitation (Fig. 6E, solid bars). Whether this differential effect arises from factors innate to the interaction

between the various subunits or differences in expression levels remains to be determined. Expression of the $\text{G}\beta 4$ mutants $\beta 4(80\text{A})$ and $\beta 4(\text{L}55\text{A},\text{N}88\text{A},\text{K}89\text{A})$, concurrently with $\text{G}\gamma 4$, produced large increases in basal facilitation (Fig. 6B,C, gray bars). Similar to wild-type $\text{G}\beta 4\gamma 4$ -expressing neurons, coexpression of $\text{G}\alpha_{\text{tr}}$ reduced, but did not eliminate, basal facilitation resulting from expression of $\beta 4(\text{L}55\text{A},\text{N}88\text{A},\text{K}89\text{A})\gamma 4$ (Fig. 6D,E, hatched bars).

Figure 7 shows the effects of expressing wild-type and mutant $\text{G}\beta 4$ (along with $\text{G}\gamma 4$) on GIRK channels expressed in SCG neurons. As observed for $\beta 1\gamma 2$, expression of $\beta 4\gamma 4$ resulted in significant basal GIRK channel activation (Fig. 7A,D) as indicated by the large inwardly rectifying current present in the absence of agonist (Fig. 7A, inset a) and the large block of current after Ba^{2+} exposure (Fig. 7A, inset c). Application of NE resulted in the recruitment of additional GIRK current (Fig. 7A, inset b). Conversely, expression of either $\beta 4(80\text{A})\gamma 4$ or $\beta 4(\text{L}55\text{A},\text{N}88\text{A},\text{K}89\text{A})\gamma 4$ failed to activate GIRK channels as exemplified by the lack of significant current in the absence of NE and the minimal effect of Ba^{2+} application. Application of NE, however, produced large increases in GIRK current, verifying the successful expression of the channels. The data also indicate that $\text{G}\beta 4$ containing $\text{G}\beta\gamma$ were capable of activating GIRK-type K^+ channels. Together, these data strengthen the argument that heterologously expressed $\text{G}\beta\gamma$ do not significantly displace native $\text{G}\beta\gamma$. Consequently, the VD Ca^{2+} channel modulation produced by expression of $\text{G}\beta 4$ likely arose from direct actions of the expressed proteins.

DISCUSSION

Three main conclusions can be drawn from the results. First, heterologous expression of $\text{G}\beta$ together with $\text{G}\gamma$ are required for optimal modulation of N-type Ca^{2+} channels. Second, all five known $\text{G}\beta$ subunits, when coexpressed with various $\text{G}\gamma$ subunits, are capable of producing VD inhibition of N-type Ca^{2+} channels. Third, heterologous expression of $\text{G}\beta\gamma$ does not result in significant displacement of native $\text{G}\beta\gamma$ from heterotrimeric complexes.

Figure 6. Effect of heterologous overexpression of mutant $\text{G}\beta 4$ and $\text{G}\alpha_{tr}$ on basal facilitation and NE-mediated Ca^{2+} current inhibition. Superimposed Ca^{2+} current traces evoked with the double-pulse voltage protocol (shown in Fig. 4E) in the absence (bottom traces) and presence (top traces) of $10 \mu\text{M}$ NE for wild-type $\beta 4\gamma 4$ and $\text{G}\alpha_{tr}$ - (A), $\beta 4(80)\gamma 4$ - (B), $\beta 4(55,88,89)\gamma 4$ - (C), and $\beta 4(55,88,89)\gamma 4$ and $\text{G}\alpha_{tr}$ -expressing (D) neurons. E, Summary graphs of mean $\pm$ SEM basal facilitation and Ca^{2+} current inhibition for neurons expressing wild-type and mutant $\text{G}\beta 4\gamma 4$ alone or combined with $\text{G}\alpha_{tr}$. Final concentration of cDNA injected was $10 \text{ ng}/\mu\text{l}$ per subunit. Basal facilitation and Ca^{2+} current inhibition were calculated as described in Figure 1E. ** $p < 0.01$ versus control. Numbers in parentheses indicate the number of experiments.

Figure 7. Effect of heterologous overexpression of wild-type and mutant $\text{G}\beta 4$ on GIRK channel activation. Time course of basal and NE-activated GIRK1 and GIRK4 channel currents in wild-type $\beta 4\gamma 4$ - (A), $\beta 4(80)\gamma 4$ - (B), and $\beta 4(55,88,89)\gamma 4$ -expressing neurons. Currents were evoked by 200 msec voltage ramps from -140 to -40 mV from a holding potential of -60 mV applied every 10 sec. Filled bars indicate application of $10 \mu\text{M}$ NE or 1 mM Ba^{2+} and $10 \mu\text{M}$ NE. Insets show current traces obtained before (a) and after (b) application of NE or NE plus Ba^{2+} (c). D, Box plot showing the 10th, 25th, 50th (median), 75th, and 90th percentiles of peak GIRK currents before (Basal) and after (NE-activated) external application of $10 \mu\text{M}$ NE. Both the 10th and 90th percentiles are denoted by shorter lines. Numbers in parentheses indicate the number of experiments.

Coordinated expression of $\text{G}\beta$ and $\text{G}\gamma$ results in optimal modulation

Four of the five $\text{G}\beta$ subunits tested produced no significant alteration in basal facilitation ratio when expressed alone. In addition, expression of several $\text{G}\gamma$ subunits in isolation produced little effect. The exception to this finding, $\text{G}\beta 4$, significantly enhanced basal facilitation, even in the absence of concurrent $\text{G}\gamma$ expression. In all cases, however, it was clear that coinjection of cDNAs coding for both subunits resulted in a much greater modulation of Ca^{2+} channels when compared with neurons expressing only a single component of $\text{G}\beta\gamma$ dimer. It should be

pointed out that, although $\text{G}\beta$ and $\text{G}\gamma$ are transcribed from separate genes, the expressed proteins likely assemble into a functional monomer. *In vitro* studies have demonstrated that strong denaturants are required for $\text{G}\beta\gamma$ dissociation once assembly has taken place (Schmidt and Neer, 1991). Because $\text{G}\gamma$ subunits appear to be required for proper folding of the $\text{G}\beta$ subunit (Clapham and Neer, 1997), it seems unlikely that “unpartnered” $\text{G}\beta$ would possess significant physiological function. The modest effects produced by expression of either $\text{G}\beta$ or $\text{G}\gamma$ alone can be ascribed to pairing with a natively expressed cognate subunit to form functional $\text{G}\beta\gamma$ dimers.

The apparent pairing of $\text{G}\beta 3$ with several different $\text{G}\gamma$ subunits (Fig. 4) requires comment. Based on a tryptic digestion assay, Ray et al. (1995) inferred that $\text{G}\beta 3$ failed to form dimers with several $\text{G}\gamma$ subunits, including some used in this study. However, a recent report from the same laboratory (Richardson and Robishaw, 1999) demonstrated that $\text{G}\beta 3$ isolated from Sf9 insect cells formed functional dimers with $\text{G}\gamma 4$, $\text{G}\gamma 5$, and $\text{G}\gamma 11$ *in vitro*. Hence, the determination as to whether various $\text{G}\beta\gamma$ combinations form functional dimers relies on the assay used.

Multiple $\text{G}\beta\gamma$ combinations produce VD Ca^{2+} channel inhibition

Our results suggest that $\text{G}\beta 1$ – $\text{G}\beta 5$, when coexpressed with several different $\text{G}\gamma$ subunits, are capable of producing VD inhibition of N-type Ca^{2+} channels. In general, expression of $\text{G}\beta 1$ – $\text{G}\beta 4$ with $\text{G}\gamma$ produced qualitatively similar effects. Basal facilitation ratios increased from ~ 1.3 in uninjected cells to near 2–3 in $\text{G}\beta\gamma$ -expressing neurons. In all cases, NE-mediated Ca^{2+} channel inhibition was occluded, although to varying degrees. Given the high degree of sequence homology shared among $\text{G}\beta 1$ – $\text{G}\beta 4$ ($\sim 80\%$), the results were not surprising. Although minor quantitative differences were noted after expression of $\text{G}\beta 1$ – $\text{G}\beta 4$, the absence of a method for quantifying expressed protein levels precludes interpretation of these differences.

In two cases, however, the magnitude of difference in basal facilitation ratios was deserving of comment. First, expression of $\beta 5$ with $\text{G}\gamma$, at the standard cDNA concentration (10 ng/ μl), clearly produced the weakest effects (Fig. 2B). In fact, the concentration of cDNA injected had to be increased 10-fold to obtain statistically significant results (see Results). Although this difference in apparent “potency” could arise from differences in protein expression levels, it should be noted that $\text{G}\beta 5$ appears to be unique among the $\text{G}\beta$ family in several ways: (1) $\text{G}\beta 5$ shares only 53% homology with $\beta 1$ – $\beta 4$ (Yan et al., 1996; Clapham and Neer, 1997); (2) $\text{G}\beta 5$ -containing $\text{G}\beta\gamma$ subunits form heterotrimers only with members of the $\text{G}_{q/11}$ family of $\text{G}\alpha$ subunits (Fletcher et al., 1998); and (3) $\text{G}\beta 5$ interacts with members of the regulators of G-protein signaling family that contain a GGL domain (Snow et al., 1998; Makino et al., 1999). Given these unique properties, we speculate that the weak effects of $\text{G}\beta 5$ arise from factors inherent to this molecule.

In contrast to the results obtained with $\text{G}\beta 5$, expression of $\text{G}\beta 4\gamma 4$ resulted in an unusually large basal facilitation (Fig. 3B). This observation seemed significant for two reasons. First, of the limited $\text{G}\beta\gamma$ combinations tested in this study, expression of $\text{G}\beta 4\gamma 4$ represented the clearest case in which the contribution of a $\text{G}\gamma$ seemed to make a significant difference in regard to basal facilitation. The increase in basal facilitation produced by pairing $\text{G}\beta 4$ with $\text{G}\gamma 4$ cannot be ascribed solely to differences in expression levels because coexpression of $\text{G}\gamma 4$ did not greatly impact the effects of other $\text{G}\beta$ subunits. Thus, the identity of the $\text{G}\gamma$ component may influence the relative potency of a given $\text{G}\beta\gamma$ subunit. Given this finding, the interpretation of $\text{G}\beta$ potency should probably be framed within the context of the particular $\text{G}\gamma$ paired with the $\text{G}\beta$. Second, although expression of $\text{G}\beta 4\gamma 4$ resulted in the largest basal facilitation ratio observed in this study, another study reported that expression of $\text{G}\beta 4$ did not produce significant effects (Garcia et al., 1998). Some possibilities for this discrepancy are discussed below.

While our work was in progress, the aforementioned group published a similarly designed study that addressed questions identical to those posed here. Although the results of both studies are comparable in several aspects, two observations do not appear

immediately reconcilable. Garcia et al. (1998) found that (1) coexpression of $\text{G}\gamma$ did not enhance $\text{G}\beta$ effects and (2) expression of $\text{G}\beta 3$ or $\text{G}\beta 4$ (with and without $\text{G}\gamma$) did not significantly modulate N-type Ca^{2+} channels. These data meshed well with yeast two-hybrid data (presented in the same manuscript) demonstrating that $\text{G}\beta 3$ and $\text{G}\beta 4$, in contrast to $\text{G}\beta 1$, $\text{G}\beta 2$, and $\text{G}\beta 5$, failed to interact with the domain I-II linker of Ca^{2+} channel $\alpha 1\text{B}$ subunits. It should be pointed out, however, that additional $\text{G}\beta\gamma$ interaction domains on Ca^{2+} channel $\alpha 1$ subunits have been identified, including regions on the N and C termini (Zhang et al., 1996; Qin et al., 1997; Page et al., 1998) (for review, see Dolphin, 1998). Therefore, the absence of protein–protein interaction between $\text{G}\beta 3$ or $\text{G}\beta 4$ and the domain I-II linker region does not preclude the possibility that, under *in situ* conditions, multiple regions combine to form a high-affinity binding “pocket” for $\text{G}\beta\gamma$ (Yamada et al., 1998). Because a nearly identical system was used by Garcia et al. (1998) and the present work, plausible explanations accounting for such large discrepancies are limited. It should be noted that the original $\text{G}\beta 4$ cDNA clone (M. I. Simon, California Institute of Technology, Pasadena, CA) that we obtained lacked a start codon, presumably as a result of a spurious mutation that occurred during propagation of the plasmid. Positive results with $\text{G}\beta 4$ were obtained only after inserting a “new” start codon into the clone using the PCR. This same clone was used by Garcia et al. (1998) (B. Hille, personal communication) and likely accounts for the lack of channel modulation seen in this study. In regard to the $\text{G}\beta 3$ results, the level of protein expression may account for discrepant results.

The effects of heterologously expressed $\text{G}\beta\gamma$ do not arise from displacement

tk;2A potential factor confounding meaningful interpretation of our data was the notion that heterologous $\text{G}\beta\gamma$ might, during basal $\text{G}\alpha$ GDP–GTP exchange, displace native $\text{G}\beta\gamma$ from heterotrimeric complexes. To examine this possibility, two strategies based on $\text{G}\beta$ mutagenesis were pursued. First, we sought to develop a $\text{G}\beta$ that would not complex with $\text{G}\alpha$ –GDP but would retain the ability to modulate N-type Ca^{2+} channels. The lack of $\text{G}\alpha$ interaction would render the “displacement hypothesis” moot, thereby simplifying data interpretation. Unfortunately, none of these mutations appeared to completely eliminate $\text{G}\alpha$ interaction based on the ability of heterologously expressed $\text{G}\alpha_{\text{tr}}$ to reverse the effects of $\text{G}\beta\gamma$ expression on basal facilitation ratio (Figs. 4F, 6F). A second strategy to investigate displacement was based on the idea of distinguishing the effects of heterologously expressed $\text{G}\beta\gamma$ from native $\text{G}\beta\gamma$ by examining differential effector interactions. GIRK-type K^{+} channels have been extensively studied in regard to activation by $\text{G}\beta\gamma$ (Wickman and Clapham, 1995). We and others (Ruiz-Velasco and Ikeda, 1998; Fernandez-Fernandez et al., 1999) have demonstrated that functional GIRK-type K^{+} channels can be heterologously expressed in SCG neurons, thus providing a second $\text{G}\beta\gamma$ “detector” in these neurons. As exemplified by the $\text{G}\beta 1(180\text{A})\gamma 2$ data, this strategy appeared to achieve our goals. Both the single (180A) and triple (180A, N88A, and K89A) mutations ablated tonic GIRK activation (Fig. 5) but retained the ability to induce Ca^{2+} channel facilitation (Fig. 4). Moreover, NE-mediated GIRK activation remained intact in the $\text{G}\beta$ mutant expressing neurons, thus suggesting that (1) native $\text{G}\beta\gamma$ was associated with heterotrimeric complexes, i.e., not displaced, and (2) the mutant $\text{G}\beta\gamma$ did not block GIRK activation. Similar results with $\text{G}\beta 4$ confirmed that these findings were not restricted to a single $\text{G}\beta$ subtype. In this regard, a yeast two-hybrid study, analogous to the one mentioned above per-

formed on Ca^{2+} channel domains, suggested that only $\text{G}\beta 1$ and $\text{G}\beta 2$ interacted with the $\beta 1\text{A}$ domain on GIRK1 (Yan and Gautam, 1996). Hence, protein–protein interactions targeted at single domains may not be predicative of functional channel interactions *in situ*. Together, these data render the notion of $\text{G}\beta\gamma$ displacement untenable and strengthen the argument that heterologously expressed $\text{G}\beta\gamma$ directly influence N-type Ca^{2+} channel function.

REFERENCES

- Bean BP (1989) Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature* 340:153–156.
- Canti C, Page KM, Stephens GJ, Dolphin AC (1999) Identification of residues in the N terminus of $\alpha 1\text{B}$ critical for inhibition of the voltage-dependent calcium channel by $\text{G}\beta\gamma$. *J Neurosci* 19:6855–6864.
- Clapham DE, Neer EJ (1997) G Protein $\beta\gamma$ Subunits. *Annu Rev Pharmacol Toxicol* 37:167–203.
- De Waard M, Liu H, Walker D, Scott VES, Gurnett CA, Campbell KP (1997) Direct binding of G-protein $\beta\gamma$ complex to voltage-dependent calcium channels. *Nature* 385:446–450.
- Delmas P, Brown DA, Dayrell M, Abogadie FC, Caulfield MP, Buckley NJ (1998) On the role of endogenous G-protein $\beta\gamma$ subunits in N-type Ca^{2+} current inhibition by neurotransmitters in rat sympathetic neurones. *J Physiol (Lond)* 506:319–329.
- Dolphin AC (1998) Mechanisms of modulation of voltage-dependent calcium channels by G proteins. *J Physiol (Lond)* 50:3–11.
- Dunlap K, Luebke JI, Turner TJ (1995) Exocytotic Ca^{2+} channels in mammalian central neurons. *Trends Neurosci* 18:89–98.
- Elmslie KS, Zhou W, Jones SW (1990) LHRH and GTP- γ -S modify calcium current activation in bullfrog sympathetic neurons. *Neuron* 5:75–80.
- Fernandez-Fernandez JM, Wanaverbecq N, Halley P, Caulfield MP, Brown DA (1999) Selective activation of heterologously expressed G protein-gated K^{+} channels by M2 muscarinic receptors in rat sympathetic neurones. *J Physiol (Lond)* 515:631–637.
- Fletcher JE, Lindorfer MA, DeFilippo JM, Yasuda H, Guilmar M, Garrison JC (1998) The G protein $\beta 5$ subunit interacts selectively with the $\text{Gq}\alpha$ subunit. *J Biol Chem* 273:636–644.
- Ford CE, Skiba NP, Bae H, Daaka Y, Reuveny E, Shekter LR, Rosal R, Weng G, Yang CS, Iyengar R, Miller RJ, Jan LY, Lefkowitz RJ, Hamm HE (1998) Molecular basis for interactions of G protein $\beta\gamma$ subunits with effectors. *Science* 280:1271–1274.
- Furukawa T, Miura R, Mori Y, Strobeck M, Suzuki K, Ogihara Y, Asano T, Morishita R, Hashii M, Higashida H, Yoshii M, Nukada T (1998) Differential interactions of the C terminus and the cytoplasmic I-II loop of neuronal Ca^{2+} channels with G-protein α and $\beta\gamma$ subunits. II. Evidence for direct binding. *J Biol Chem* 273:17595–17603.
- Garcia DE, Li B, Garcia-Ferreiro RE, Hernandez-Ochoa EO, Yan K, Gautam N, Catterall WA, Mackie K, Hille B (1998) G-protein β -subunit specificity in the fast membrane-delimited inhibition of Ca^{2+} channels. *J Neurosci* 18:9163–9170.
- Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cell and cell-free membrane patches. *Pflügers Arch* 391:85–100.
- Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA (1996) Modulation of Ca^{2+} 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 SR (1991) Double-pulse calcium channel current facilitation in adult rat sympathetic neurones. *J Physiol (Lond)* 439:181–214.
- Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein $\beta\gamma$ subunits. *Nature* 380:255–258.
- Ikeda SR (1997) Heterologous expression of receptors and signaling proteins in adult mammalian sympathetic neurons by microinjection. In: *Methods in Molecular Biology* (Challis RA, ed), pp 191–202. Totowa, NJ: Humana.
- Ikeda SR, Dunlap K (1999) Voltage-dependent modulation of N-type calcium channels: role of G protein subunits. *Adv Second Messenger Phosphoprotein Res* 33:131–151.
- Ikeda SR, Schofield GG (1989) Somatostatin blocks a calcium current in rat sympathetic ganglion neurones. *J Physiol (Lond)* 409:221–240.
- Jan LY, Jan YN (1997) Voltage-gated and inwardly rectifying potassium channels. *J Physiol (Lond)* 505:267–282.
- Jeong SW, Ikeda SR (1999) Sequestration of G-protein $\beta\gamma$ subunits by different G-protein α subunits blocks voltage-dependent modulation of Ca^{2+} channels in rat sympathetic neurons. *J Neurosci* 19:4755–4761.
- Li Y, Sternweis PM, Charnecki S, Smith TF, Gilman AG, Neer EJ, Kozasa T (1998) Sites for $\text{G}\alpha$ binding on the G protein β subunit overlap with sites for regulation of phospholipase $\text{C}\beta$ and adenylyl cyclase. *J Biol Chem* 273:16265–16272.
- Logothetis DE, Kurachi Y, Galper J, Neer EJ, Clapham DE (1987) The $\beta\gamma$ subunits of GTP-binding proteins activate the muscarinic K^{+} channel in heart. *Nature* 325:321–326.
- Makino ER, Handy JW, Li T, Arshavsky VY (1999) The GTPase activating factor for transducin in rod photoreceptors is the complex between RGS9 and type 5 G protein β subunit. *Proc Natl Acad Sci USA* 96:1947–1952.
- Nakajima T, Sugimoto T, Kurachi Y (1992) Effect of anions on the G protein-mediated activation of the muscarinic K^{+} channel in the cardiac atrial cell membrane. Intracellular chloride inhibition of the GTPase activity of GK. *J Gen Physiol* 99:665–682.
- Page KM, Canti C, Stephens GJ, Berrow NS, Dolphin AC (1998) Identification of the amino terminus of neuronal Ca^{2+} channel $\alpha 1$ subunits $\alpha 1\text{B}$ and $\alpha 1\text{E}$ as an essential determinant of G-protein modulation. *J Neurosci* 18:4815–4824.
- Qin N, Platano D, Olcese R, Stefani E, Birnbaumer L (1997) Direct interaction of $\text{G}\beta\gamma$ with a C-terminal $\text{G}\beta\gamma$ -binding domain of the Ca^{2+} channel $\alpha 1$ subunit is responsible for channel inhibition by G protein-coupled receptors. *Proc Natl Acad Sci USA* 94:8866–8871.
- Ray K, Kunsch C, Bonner LM, Robishaw JD (1995) Isolation of cDNA clones encoding eight different human G protein γ subunits including three novel forms designated γ_4 , γ_{10} , and γ_{11} subunits. *J Biol Chem* 270:21765–21771.
- Richardson M, Robishaw JD (1999) The $\alpha 2\text{A}$ -adrenergic receptor discriminates between Gi heterotrimers of different $\beta\gamma$ subunit composition in Sf9 insect cell membranes. *J Biol Chem* 274:13525–13533.
- Ruiz-Velasco V, Ikeda SR (1998) Heterologous expression and coupling of G protein-gated inwardly rectifying K^{+} channels in adult rat sympathetic neurons. *J Physiol (Lond)* 513:761–773.
- Schmidt CJ, Neer EJ (1991) *In vitro* synthesis of G protein $\beta\gamma$ dimers. *J Biol Chem* 266:4538–4544.
- Schofield GG (1990) Norepinephrine blocks a calcium current of adult rat sympathetic neurons via an $\alpha 2$ -adrenoceptor. *Eur J Pharmacol* 180:37–47.
- Slepak VZ, Katz A, Simon MI (1995) Functional analysis of a dominant negative mutant of $\text{G}\alpha_{i2}$. *J Biol Chem* 270:4037–4041.
- Snow BE, Krumins AM, Brothers GM, Lee SF, Wall MA, Chung S, Mangion J, Arya S, Gilman AG, Siderovski DP (1998) A G protein γ subunit-like domain shared between RGS11 and other RGS proteins specifies binding to $\text{G}\beta 5$ subunits. *Proc Natl Acad Sci USA* 95:13307–13312.
- Sondek J, Böhm A, Lambright DG, Hamm HE, Sigler PB (1996) Crystal structure of a G-protein $\beta\gamma$ dimer at 2.1 Å resolution. *Nature* 379:369–374.
- Wall MA, Coleman DE, Lee E, Iniguez-Lluhi JA, Posner BA, Gilman AG, Sprang SR (1995) The structure of the G protein heterotrimer $\text{G}\alpha 1\beta 1\gamma 2$. *Cell* 83:1047–1058.
- Watson S, Arkinstall S (1994) G protein γ subunits. In: *The G-protein linked receptor facts book*, pp 350–355. San Diego: Academic.
- Wickman K, Clapham DE (1995) Ion channel regulation by G proteins. *Physiol Rev* 75:865–885.
- Wickman KD, Iniguez-Lluhi JA, Davenport PA, Taussig R, Krapivinsky GB, Linder ME, Gilman AG, Clapham DE (1994) Recombinant G-protein $\beta\gamma$ -subunits activate the muscarinic-gated atrial potassium channel. *Nature* 368:255–257.
- Yamada M, Inanobe A, Kurachi Y (1998) G protein regulation of potassium ion channels. *Pharmacol Rev* 50:723–760.
- Yan K, Gautam N (1996) A domain on the G protein β subunit interacts with both adenylyl cyclase 2 and the muscarinic atrial potassium channel. *J Biol Chem* 271:17597–17600.
- Yan K, Kalyanaraman V, Gautam N (1996) Differential ability to form the G protein $\beta\gamma$ complex among members of the β and γ subunit families. *J Biol Chem* 271:7141–7146.
- Zamponi GW, Snutch TP (1998) Modulation of voltage-dependent calcium channels by G proteins. *Curr Opin Neurobiol* 8:351–356.
- Zamponi GW, Bourinet E, Nelson D, Nargeot J, Snutch TP (1997) Crosstalk between G proteins and protein kinase C mediated by the calcium channel $\alpha 1$ subunit. *Nature* 385:442–446.
- Zhang JF, Ellinor PT, Aldrich RW, Tsien RW (1996) Multiple structural elements in voltage-dependent Ca^{2+} channels support their inhibition by G proteins. *Neuron* 17:991–1003.