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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

Seong-Woo Jeong and Stephen R. Ikeda

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

The membrane-delimited and voltage-dependent inhibition of N-type Ca^{2+} channels is mediated by $G\beta\gamma$ subunits. Previously, exogenous excess GDP-bound $G\alpha_{oA}$ has been shown to dramatically attenuate the norepinephrine (NE)-mediated Ca^{2+} current inhibition by sequestration of $G\beta\gamma$ subunits in rat superior cervical ganglion (SCG) neurons. In the present study, we determined whether the attenuation of NE-mediated modulation is specific to $G\alpha_{oA}$ or shared by a number of closely related ($G\alpha_{tr}$, $G\alpha_{oB}$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{z}$) or unrelated ($G\alpha_{s}$, $G\alpha_{q}$, $G\alpha_{11}$, $G\alpha_{16}$, $G\alpha_{12}$, $G\alpha_{13}$) $G\alpha$ subunits. Individual $G\alpha$ subunits from different subfamilies were transiently overexpressed in SCG neurons by intranuclear injection of mammalian expression vectors encoding the desired protein. Strikingly, all $G\alpha$ subunits except $G\alpha_{z}$ nearly blocked basal facilitation and NE-mediated modulation. Likewise, VIP-mediated Ca^{2+} current inhibition, which is mediated by cholera toxin-sensitive G-protein, was also completely suppressed by a number of $G\alpha$ subunits overexpressed in neurons. $G\alpha_{s}$ expression produced either enhancement or attenuation of the VIP-mediated modulation—an effect that seemed to depend on the expression level. The onset of the nonhydrolyzable GTP analog, guanylylimidodiphosphate-mediated facilitation was significantly delayed by overexpression of different GDP-bound $G\alpha$ subunits. Taken together, these data suggest that a wide variety of $G\alpha$ subunits are capable of forming heterotrimers with endogenous $G\beta\gamma$ subunits mediating voltage-dependent Ca^{2+} channel inhibition. In conclusion, coupling specificity in signal transduction is unlikely to arise as a result of restricted $G\alpha/G\beta\gamma$ interaction.

Key words: calcium channels; $G\alpha$ subunit; $G\beta\gamma$ subunit; G-proteins; voltage-dependent inhibition; intranuclear injection; sympathetic neuron; coupling specificity

Modulation of N-type Ca^{2+} channels by neurotransmitters occurs via multiple pathways. The most common modulatory pathway involves activation of pertussis toxin (PTX)-sensitive heterotrimeric G-proteins, resulting in a distinct form of membrane-delimited and voltage-dependent inhibition (Hille, 1994). Recently, $G\beta\gamma$ subunits have been shown to mediate the voltage-dependent inhibition of Ca^{2+} currents (Herlitze et al., 1996; Ikeda, 1996). The idea that $G\beta\gamma$ subunits directly interact with Ca^{2+} channels has been supported by the molecular identification of potential $G\beta\gamma$ binding motifs on the intracellular I–II loop (De Waard et al., 1997; Zamponi et al., 1997; Furukawa et al., 1998) and C terminus (Qin et al., 1997). In addition, a recent study suggests that the N terminus of the Ca^{2+} channel $\alpha1$ subunit is also essential for the G-protein-mediated modulation (Page et al., 1998).

With regard to the interaction with Ca^{2+} channels, there seem to be few functional differences among different $G\beta\gamma$ combinations. For example, the Ca^{2+} currents were tonically inhibited by various $G\beta\gamma$ combinations, including $G\beta_1\gamma_2$, $G\beta_1\gamma_3$, $G\beta_1\gamma_7$ (Ikeda, 1996), and $G\beta_2\gamma_3$ (Herlitze et al., 1996) when overexpressed in sympathetic neurons [but see García et al. (1998)]. These observations are similar to the finding that G-protein-activated inwardly rectifying K^+ (GIRK) channels were activated by different $G\beta\gamma$ combinations, with the exception of $\beta_1\gamma_1$ (Wickman et al., 1994). Furthermore, various $G\alpha$ subunits are involved in coupling receptors to ion channels. Activation of different receptors activate GIRK channels via different $G\alpha$ proteins (Lim et al., 1995; Ruiz-Velasco and Ikeda, 1998). Likewise, although $G\alpha_{o}$ appears to be dominantly coupled to receptors mediating the voltage-dependent inhibition of Ca^{2+} channels in neuronal tissues, $G\alpha_{i}$ (Ewald, 1989; Toselli et al., 1989) and $G\alpha_{s}$ (Zhu and Ikeda, 1994) can also participate in this pathway. Overall, these observations argue against the idea that coupling specificity resides at the $G\alpha\beta\gamma$/effector level and thus requires restricted $G\alpha/\beta\gamma$ combinations.

To confirm the modulatory role of $G\beta\gamma$ in the neurotransmitter-mediated inhibition of Ca^{2+} channel currents, the stoichiometry between $G\alpha$ and $G\beta\gamma$ subunits has been disrupted by overexpressing $G\alpha_{oA}$ in sympathetic neurons (Ikeda, 1996). In this experiment, excess GDP-bound $G\alpha_{oA}$ significantly blocked NE-mediated Ca^{2+} current inhibition by creating conditions favoring heterotrimer formation. In the present study, the same strategy was used to test whether the block of norepinephrine (NE)-mediated inhibition is specific to $G\alpha_{oA}$ or shared by members in the same (G_{i}, i.e., $G\alpha_{oB}$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{tr}$, and $G\alpha_{z}$) or different $G\alpha$ subfamilies ($G\alpha_{s}$, $G\alpha_{q/11}$, and $G\alpha_{12}$). In superior cervical ganglion (SCG) neurons, the parallel pathways using PTX-sensitive $G\alpha_{o/i}$ and cholera toxin (CTX)-sensitive $G\alpha_{s}$ converge to the voltage-dependent modulation of Ca^{2+} channels (Zhu and Ikeda, 1994). Thus, a complementary set of experiments was performed to test whether VIP-mediated inhibition is blocked by overexpression of the $G\alpha$ subunits described above.

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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.

Our data showed that a wide variety of Gα subunits can interact with the Gβγ subunits involved in NE-, VIP-, and guanylylimidodiphosphate [Gpp(NH)p]-mediated current inhibition. These results suggest that coupling specificity is unlikely to arise as a result of restricted Gα/βγ interactions.

Some preliminary data have been published previously in abstract form (Jeong and Ikeda, 1997).

MATERIALS AND METHODS

Vectors and chemicals. The clones for G-protein $G\alpha_{oA}$, $G\alpha_{oB}$, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{16}$, $G\alpha_{12}$, and $G\alpha_{13}$ were generously provided by Dr. M. I. Simon (California Institute of Technology). The clones for $G\alpha_s$ and $G\alpha_{i1\text{-}i3}$ were kind gifts from Dr. R. Reed (Johns Hopkins Medical School). The clone for the wild-type $G\alpha_z$ was a generous gift from Dr. H. R. Bourne (University of California San Francisco). The pEGFP-N1 N-terminal fusion vector was purchased from Clontech Laboratories (Palo Alto, CA). The vectors were propagated in either XL-1 Blue or MC1061/p3 *Escherichia coli*, (Stratagene, Cambridge, UK) as appropriate, and purified using Qiagen (Chatsworth, CA) miniprep or maxiprep columns. Chemicals used in experiments were obtained as follows: Gpp(NH)p and NE from Sigma (St. Louis, MO); VIP from Bachem (Torrance, CA).

Dissociation of SCG neurons. SCG neurons were enzymatically dissociated as described previously (Ikeda, 1991; Zhu and Ikeda, 1993). 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 incubated with 1 mg/ml collagenase type D, 0.35 mg/ml trypsin (Boehringer Mannheim Biochemicals, Indianapolis, IN), and 0.1 mg/ml DNase type I (Sigma) in 10 ml of modified Earle's balance salt solution (EBSS, pH 7.4) in a 25 cm^2 tissue culture flask. The EBSS was modified by adding 3.6 gm/l glucose and 10 mM HEPES. The flask was then placed in a shaking water bath at 35°C for 1 hr. After incubation, neurons were dissociated by vigorous shaking of the flask. After centrifugation at 50 × *g* for 5 min, the dispersed neurons were resuspended in MEM containing 10% fetal calf serum, 1% glutamine, and 1% penicillin–streptomycin solution (all from Life Technologies, Grand Island, NY). Neurons were then plated onto polystyrene culture dishes (35 mm) coated with poly-D-lysine and maintained in the humidified atmosphere of a 95% air–5% CO_2 incubator at 37°C. All neurons were used within 24 hr after intranuclear injection of vectors.

Intranuclear injection of vectors. Vectors encoding particular proteins were directly injected into the nucleus of SCG neurons as described previously (Ikeda, 1996, 1997). Briefly, the cDNAs from stock solutions (~1.0 μg/μl) were diluted in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) to a final concentration of 100 ng/μl (per subunit). A reporter vector (pEGFP-N1) encoding a double mutant (F64L:S65T) variant of the green fluorescent protein (GFP) was also included (5 ng/μl) to facilitate later identification of neurons receiving a successful nuclear injection. After centrifugation (16,000 × *g* for 20 min) to remove undissolved particles, the cDNA-containing solution was loaded into a fine borosilicate micropipette and injected into the nucleus of SCG neurons with an Eppendorf 5242 microinjector and 5171 micromanipulator system (Madison, WI) using injection pressure and duration of 120–200 hPa and 0.3 sec, respectively. Neurons successfully expressing particular proteins were easily identified 14–24 hr later by the observation of the fluorescence produced by the GFP under an inverted microscope (Diaphot, Nikon, Japan) equipped with an epifluorescence unit (B-2A filter cube, Nikon).

Electrophysiology. A culture dish containing dissociated SCG neurons was placed on an inverted phase–contrast microscope (Nikon) and superfused at a flow rate of 1–2 ml/min with an external solution as described below. Ionic currents were recorded using a whole-cell variant of the patch-clamp technique (Hamill et al., 1981) as described previously (Ikeda, 1991; Ikeda et al., 1995). Patch electrodes were fabricated from a borosilicate glass capillary (1.65 mm outer diameter, 1.2 mm inner diameter; Corning 7052, Garner Glass Co. Claremont, CA). The patch electrodes were coated with Sylgard 184 (Dow Corning, Midland, MI) and fire-polished on a microforge, and they had resistances of 1.5–2.5 MΩ when filled with an internal solution described below. The bath was grounded by an Ag/AgCl pellet connected via a 0.15 M NaCl/agar bridge. The cell membrane capacitance and series resistance were always compensated (typically >80%) electronically using a patch-clamp amplifier (Axopatch-200; Axon Instruments, Foster City, CA). Voltage protocol generation and data acquisition were performed using custom data acquisition software on a Macintosh Quadra series computer equipped with a MacAdios II data acquisition board (G. W. Instruments, Somerville, MA). Current traces were generally low-pass-filtered at 5 KHz using the four-pole Bessel filter in the clamp amplifier, digitized at 2–5 kHz, and stored on the computer hard drive for later analysis. Ca^{2+} current traces were corrected for linear leakage current as determined from hyperpolarizing pulses. All experiments were performed at room temperature (21–24°C). Drugs were 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. Data were presented as means ± SEM. ANOVA followed by *post hoc* Dunnett's test, as appropriate, were performed to determine statistical significance. $p < 0.05$ was considered significant.

Solutions. For Ca^{2+} current recording, the external solution contained (in mM): 145 tetraethylammonium (TEA)-methanesulfonate (MS), 10 HEPES, 10 $CaCl_2$, 15 glucose, 0.0001 tetrodotoxin (TTX) (pH adjusted to 7.4 with TEA-OH, osmolality 318 mOsm/kg H_2O). Patch pipette contained (in mM) 120 *N*-methyl-D-glucamine (NMG)-MS, 20 TEA-MS, 20 HCl, 11 EGTA, 1 $CaCl_2$, 10 HEPES, 4 MgATP, 0.3 Na_2GTP, 14 creatine phosphate (pH adjusted to 7.2 with TEA-MS, osmolality 297 mOsm/kg H_2O). In experiments designed to activate the overexpressed Gα subunits, 0.5 mM Gpp(NH)p was included in the internal solution. For M-type K^+ channel current (M-current) measurement, the external solution contained (in mM): 150 NaCl, 2.5 KCl, 10 HEPES, 1 $MgCl_2$, 2 $CaCl_2$, and 15 glucose (pH adjusted to 7.4 with NaOH, osmolality 320 mOsm/kg H_2O). The internal solution contained (in mM): 150 KCl, 0.1 K_4BAPTA, 10 HEPES, 4 MgATP, and 0.1 Na_2GTP (pH adjusted to 7.2 with KOH, osmolality 300 mOsm/kg H_2O).

RESULTS

Overexpression of different Gα subunits significantly blocks NE-mediated Ca^{2+} current inhibition

We first determined whether different Gα subunits are able to interact with the Gβγ subunits released on activation of α_2-adrenergic receptors (α_2-ARs). To address this question, Gα subunits from different subfamilies were transiently overexpressed in SCG neurons by intranuclear injection of mammalian expression vectors encoding the desired proteins. Figure 1 illustrates typical whole-cell Ca^{2+} current records obtained in the absence or presence of 10 μM NE from SCG neurons previously injected with selected subunits from four Gα-subfamilies. The Ca^{2+} currents were evoked by 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*). From the current traces, we measured facilitation of Ca^{2+} currents in the absence of agonist (basal facilitation) as well as percent inhibition of Ca^{2+} currents produced by the agonist. Facilitation was defined as the ratio of the postpulse to prepulse current amplitude measured isochronally at 10 msec after the start of the test pulse. In the control neuron, expressing only GFP, Ca^{2+} currents were tonically facilitated (~1.24) by the strong conditioning pulse in the absence of agonist (Fig. 1*A*). Previously, basal facilitation in SCG neurons has been shown to arise from a small degree of tonic G-protein activation (Ikeda, 1991). Application of 10 μM NE produced a typical voltage-dependent inhibition of Ca^{2+} currents characterized by kinetic slowing and an increased prepulse facilitation (from 1.24 to 2.84) (Elmslie et al., 1990). Expression of GFP had little effect on the magnitude of either basal facilitation or NE-mediated Ca^{2+} current inhibition (1.28 ± 0.02, $n = 19$ and 57 ± 2%, $n = 15$, respectively, for uninjected neurons; 1.32 ± 0.02, $n = 39$ and 61 ± 2%, $n = 30$, respectively, for injected neurons) (Fig. 2*A*,*B*). In contrast, basal facilitation was absent after overexpression of Gα subunits. In addition, a slight inacti-

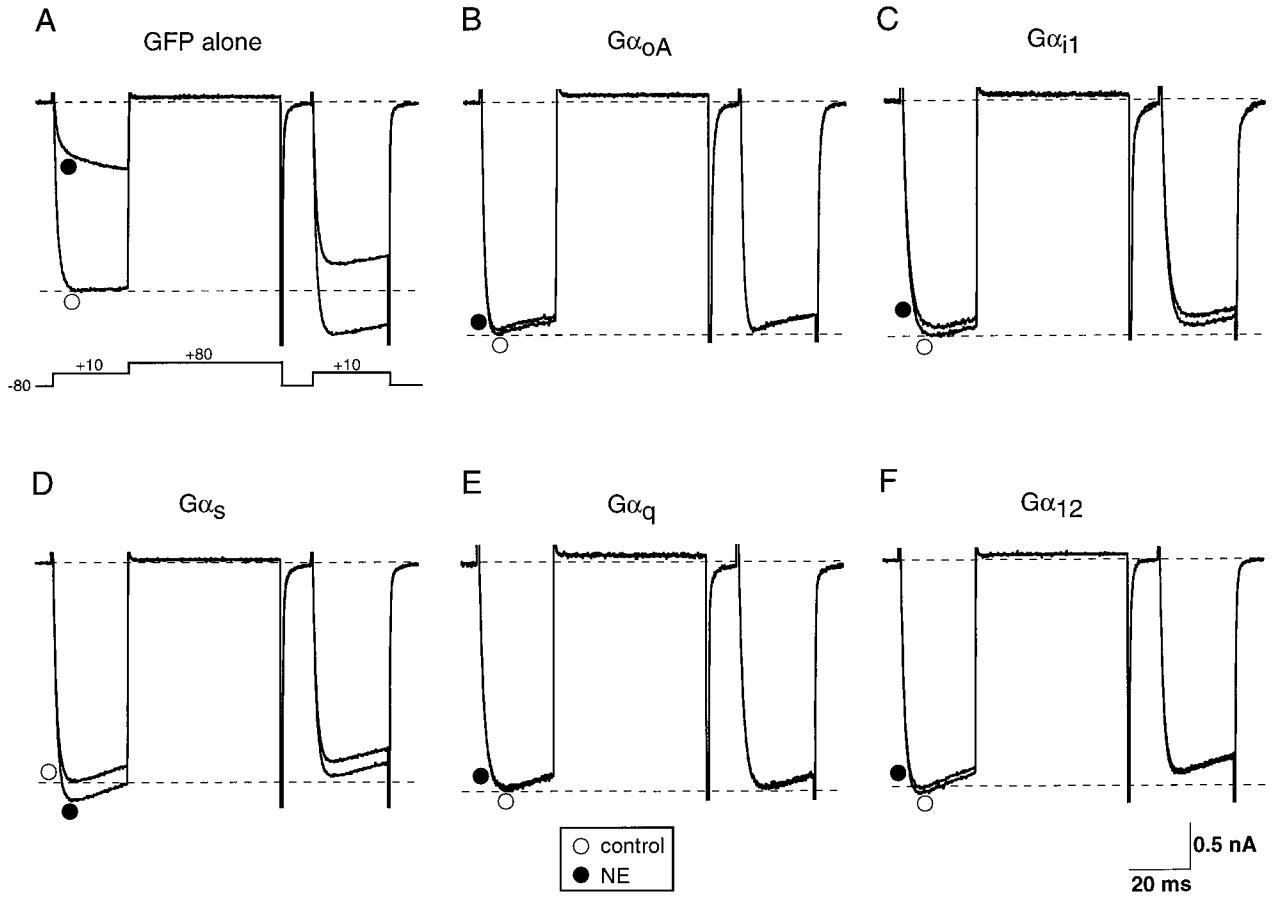

Figure 1. Heterologous overexpression of different Gα subunits abolished NE-mediated Ca^{2+} currents in SCG neurons. Superimposed current traces were recorded in the absence (○) or presence (●) of 10 μM NE from neurons transiently expressing GFP alone (*A*), $G\alpha_{oA}$ (*B*), $G\alpha_{i1}$ (*C*), $G\alpha_s$ (*D*), $G\alpha_q$ (*E*), and $G\alpha_{12}$ (*F*). The Ca^{2+} currents were evoked by a double-pulse protocol consisting of two identical test pulses to +10 mV from a holding potential of −80 mV separated by a large depolarizing conditioning pulse to +80 mV (*inset* in *A*). Note that the basal facilitation in the absence of NE was seen in control neurons (by the *bottom dotted line*) and abolished by the overexpression of Gα subunits.

vation was evident after the large depolarizing conditioning pulse (Figs. 1*B–F*, 2*A*). These results are consistent with the sequestration of endogenous free Gβγ by overexpressed GDP-bound Gα subunits. As shown previously (Ikeda, 1996), the injection of the cDNA encoding for $G\alpha_{oA}$ nearly abolished NE-mediated Ca^{2+} current inhibition (Fig. 1*B*). Likewise, another PTX-sensitive Gα, $G\alpha_{i1}$, was able to disrupt the NE-mediated modulation when overexpressed (Fig. 1*C*). Strikingly, overexpression of other Gα-subfamily members, including $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12}$, produced effects similar to those by $G\alpha_{oA}$ and $G\alpha_{i1}$ (Fig. 1*D–F*). Figure 2*B* summarizes the effects of overexpressing various Gα subunits on NE-mediated Ca^{2+} current inhibition. With the exception of $G\alpha_z$, all tested Gα subunits nearly abolished NE-mediated modulation (Fig. 2*B*). In neurons overexpressing $G\alpha_z$, the NE-mediated modulation was not significantly different from that of control neurons ($p > 0.05$, $n = 8$). Previously, we have shown that $G\alpha_z$ substitutes for $G\alpha_{o/i}$ by coupling to $G\alpha_{o/i}$-coupled receptors, including α_2-ARs (Jeong and Ikeda, 1998). Taken together, these data suggest that various GDP-bound Gα subunits sequester Gβγs released on receptor activation and prevent the NE-mediated Ca^{2+} current inhibition.

Overexpression of different Gα subunits significantly blocks VIP-mediated Ca^{2+} current inhibition

VIP has been shown to produce membrane-delimited and voltage-dependent inhibition of Ca^{2+} currents virtually identical to that seen with α_2-AR stimulation in SCG neurons (Zhu and Ikeda, 1994; Ehrlich and Elmslie, 1995). However, the modulation pathway used by VIP apparently uses a cholera toxin-sensitive G-protein, $G\alpha_s$, instead of $G\alpha_{o/i}$. Thus, we determined the ability of individual Gα subunits to attenuate VIP-mediated Ca^{2+} current inhibition when overexpressed in SCG neurons. Figure 2*C* summarizes the effects of overexpression of various Gα subunits on VIP-mediated Ca^{2+} current inhibition. In uninjected ($n = 8$) and GFP-injected neurons ($n = 15$), the mean percent inhibition produced by 10 μM VIP was 47 ± 3% and 44 ± 2%, respectively. However, in neurons expressing members of subfamilies unrelated to $G\alpha_s$, i.e., $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$, the VIP-mediated Ca^{2+} current inhibition was nearly abolished. $G\alpha_z$ did not reconstitute the VIP response confirming its specificity for G_o/G_i-coupled receptors (Jeong and Ikeda, 1998). When $G\alpha_s$ was overexpressed, the VIP responses were variable and categorized into two groups by correlating NE responses tested in the same neurons. In one group, in which the NE response was minimally affected by overexpression of $G\alpha_s$, the VIP response was significantly enhanced when compared with that in control neurons (44 ± 2%, $n = 15$ for GFP control vs 59 ± 2%, $n = 8$ for $G\alpha_s$, $p < 0.05$). In another group, in which the NE response was completely blocked, however, overexpression of $G\alpha_s$ significantly attenuated the VIP-mediated Ca^{2+} current inhibition (44 ± 2%, $n = 15$ for GFP control vs 29 ± 4%, $n = 4$ for $G\alpha_s$). When two groups of

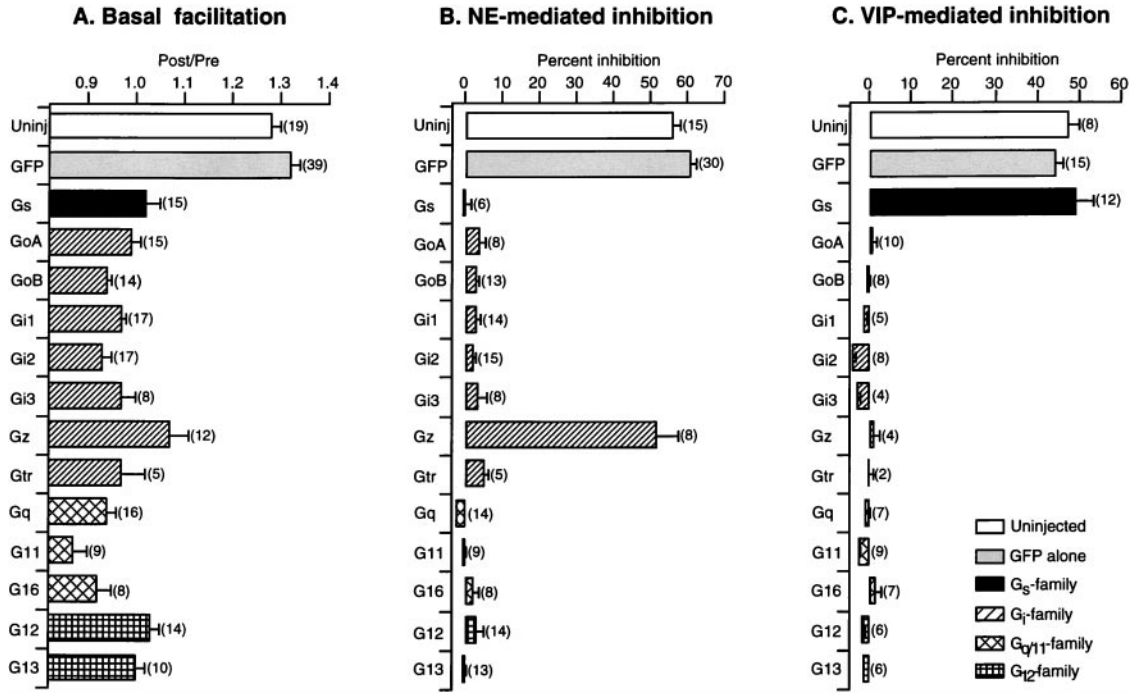

Figure 2. Summary of the effects of different Gα subunits on basal facilitation in the absence of agonist (*A*) and percentage inhibition in the presence of 10 μM NE (*B*) and VIP (*C*). Data are presented as mean ± SEM, and *numbers* in *parentheses* indicate the number of neurons tested.

data were pooled, VIP produced 49 ± 5% ($n = 12$) inhibition, similar to the control value in neurons expressing $G\alpha_s$ (Fig. 2*C*).

Modulation of M-type K^+ channel currents is not blocked by overexpression of Gα subunits

Because all Gα subunits that were tested produced positive effects, we did not confirm the expression of proteins using immunochemical techniques. Instead, we tested whether the heterologously expressed Gα subunits had nonspecific effects on Ca^{2+} current modulation. In sympathetic neurons, inhibition of M-current has been shown to be mediated by the α subunit rather than the βγ subunits of the G_q heterotrimer (Haley et al., 1998; Kammermeier and Ikeda, 1999). Thus, as a negative control for the specific effects of Gα subunits in sequestering Gβγ subunits, the muscarinic modulation of M-current was assessed in SCG neurons expressing different Gα subunits, including $G\alpha_S$, $G\alpha_{oA}$ $G\alpha_{11}$, and $G\alpha_{12}$. The deactivation of M-current was evoked by a test pulse to −60 mV for 0.5 sec from a holding potential of −30 mV (Fig. 3*A*). In a control neuron expressing GFP, 10 μM muscarine produced a significant inhibition of the M-current (Fig. 3*A*). Overexpression of $G\alpha_S$, $G\alpha_{11}$, and $G\alpha_{12}$ subunits did not affect the muscarinic inhibition of the M-currents (Fig. 3*A*). In contrast, M-current inhibition was partially but significantly attenuated in neurons expressing $G\alpha_{oA}$ ($p < 0.05$). As summarized in Figure 3*B*, 10 μM muscarine inhibited the M-current by 83 ± 2% ($n = 8$) in control neurons, and by 80 ± 8% ($n = 5$), 52 ± 11% ($n = 7$), 88 ± 1% ($n = 6$), and 79 ± 10% ($n = 5$), respectively, in neurons expressing $G\alpha_S$, $G\alpha_{oA}$, $G\alpha_{11}$, and $G\alpha_{12}$ subunits. Conversely, overexpression of $G\alpha_q$(Q209L), a GTPase-deficient and constitutively active form of $G\alpha_q$ subunit, virtually eliminated the M-current and thereby the muscarinic modulation ($n = 4$; data not shown), consistent with a previous finding (Haley et al., 1998). Taken together, these data suggest that the overexpressed α subunits interact with Gβγ subunits rather than nonspecific interaction with α_2-ARs, Ca^{2+} channels, or other signaling proteins to block the modulation of Ca^{2+} currents.

Overexpression of Gα subunits significantly delayed Gpp(NH)p-induced Ca^{2+} current inhibition

Overexpression of the $G\alpha_{oA}$ subunit has been shown to significantly delay Gpp(NH)p-mediated facilitation in SCG neurons (Ikeda, 1996). The delay is thought to arise from the ability of Gpp(NH)p, a nonhydrolyzable GTP analog, to irreversibly activate Gα subunits, thereby overcoming the sequestration of Gβγ produced by overexpression of $G\alpha_{oA}$. Thus, we tested whether different Gα subunits could delay the Gpp(NH)p-mediated facilitation when overexpressed in SCG neurons. When uninjected control neurons were dialyzed with 0.5 mM Gpp(NH)p, the mean facilitation was increased from 1.16 ± 0.03 to 2.51 ± 0.11 ($n = 6$) within 4 min (Fig. 4). However, the onset of Gpp(NH)p-mediated facilitation was significantly delayed by overexpression of $G\alpha_{oA}$ ($n = 6$). Likewise, another PTX-sensitive member of the G_i subfamily, $G\alpha_{i2}$, exerted a similar effect on the Gpp(NH)p-mediated facilitation ($n = 2$) (Fig. 4*B*). When PTX-insensitive Gα subunits such as $G\alpha_s$($n = 5$), $G\alpha_z$($n = 4$), $G\alpha_q$ ($n = 5$), and $G\alpha_{13}$ ($n = 5$) were overexpressed, the onset of facilitation by the dialysis of Gpp(NH)p was nearly blocked.

DISCUSSION

One experimental way to affirm Gβγ-mediated signaling is to increase the normal Gα/Gβγ ratio by overexpressing Gα subunits. Excess GDP-bound Gα, with a high affinity for Gβγ subunits, may create conditions favoring heterotrimer formation and thus produce a "Gβγ sink" (Slepak et al., 1995). Suppression of Gβγ-mediated signaling by exogenous GDP-bound $G\alpha_o$ has been demonstrated for different effectors such as adenylyl cyclase (Federman et al., 1992), GIRK channels (Ito et al., 1992; Reuveny et al., 1994; Krapivinsky et al., 1995),), phospholipase Cβ (Katz et al.,

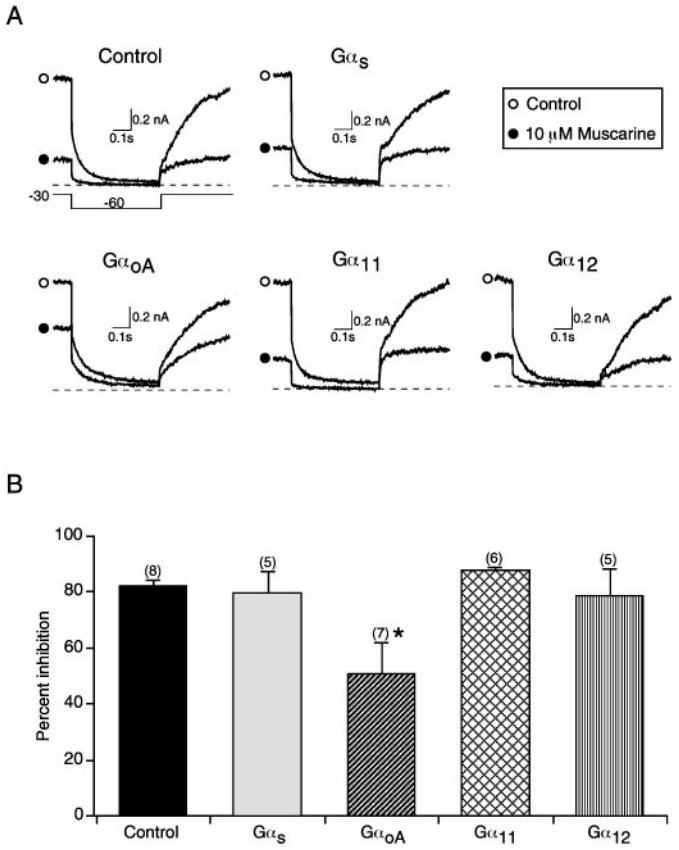

Figure 3. Heterologous overexpression of different Gα subunits failed to abolish the modulation of muscarine-sensitive K^+ current (M-current) in SCG neurons. *A*, Superimposed current traces in the absence (○) or presence (●) of 10 μM muscarine recorded from neurons transiently expressing GFP alone (*Control*), $G\alpha_s$, $G\alpha_{oA}$, $G\alpha_{11}$, and $G\alpha_{12}$. The deactivation of M-current was evoked by a test pulse to −60 mV for 0.5 sec from a holding potential of −30 mV (*inset*). *B*, Summary of effects of different Gα subunits on M-current inhibition. Data are presented as mean ± SEM, and *numbers in parentheses* indicate the number of neurons tested. $^*p < 0.05$ (by *post hoc* Dunnett's test)

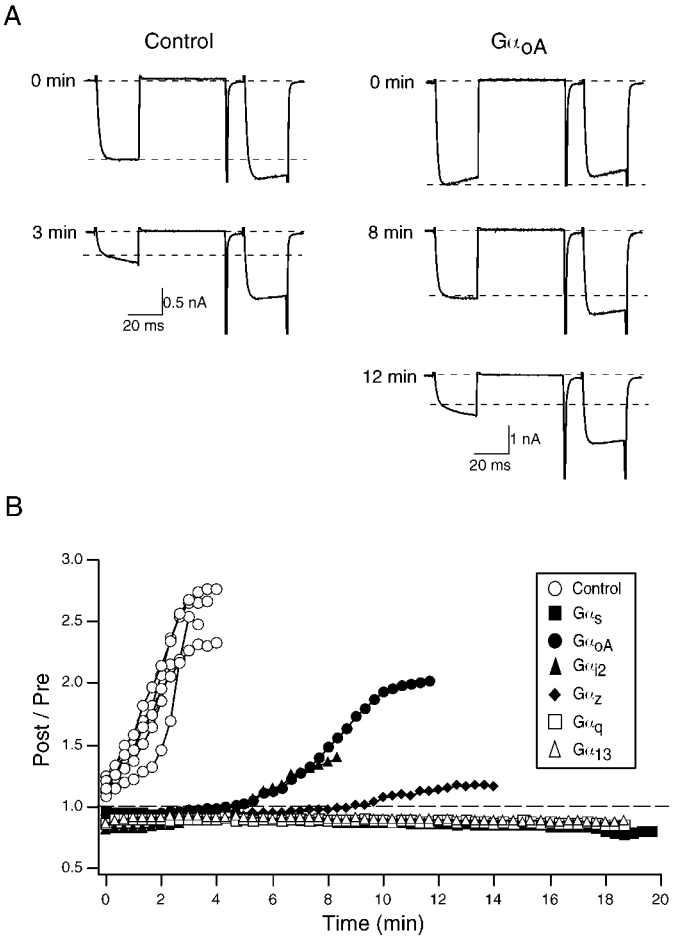

Figure 4. Heterologous overexpression of different Gα subunits significantly delayed Gpp(NH)p-mediated facilitation in SCG neurons. *A*, Representative Ca^{2+} current traces showing time-dependent change of facilitation in control (*left panel*) and $G\alpha_{oA}$-injected (*right panel*) neurons. In both cases, 0.5 mM Gpp(NH)p was present in patch pipettes. *B*, time course of Gpp(NH)p-mediated facilitation in neurons expressing $G\alpha_s$, $G\alpha_{oA}$, $G\alpha_{i2}$, $G\alpha_z$, $G\alpha_q$, and $G\alpha_{13}$. The *dotted line* in *B* indicates the absence of facilitation.

1992), and phosphoinositide 3 kinase (Stephens et al., 1994). Likewise, in SCG neurons, overexpression of GDP-bound $G\alpha_{oA}$ has been shown to eliminate NE-mediated Ca^{2+} current inhibition (Ikeda, 1996). A biochemical study in which ADP-ribosylation by PTX and GTPase activity were measured has demonstrated that recombinant Gα subunits ($G\alpha_s$, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_o$) were able to form heterotrimers with different Gβγ subunits (Ueda et al., 1994). Recently, analysis of immunoprecipitation combined with silver stain and immunoblotting has also demonstrated the random association of Gα subunits with different Gβγ subunits from tissue and cell extracts (Ueda et al., 1998). In addition, another *in vitro* experiment has shown the ability of five Gα subunits ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_o$, $G\alpha_s$, and $G\alpha_q$) to associate with Gβ subunits (β_1 and β_2) (Fletcher et al., 1998). On the basis of these *in vitro* experiments, the interaction between Gα and Gβγ subunits seems to display little specificity. However, an apparent exception to this pattern is the structurally divergent $G\beta_5$ subunit that appears to demonstrate specificity for $G\alpha_q$ (Fletcher et al., 1998). To date, it is unclear whether the nonspecific interaction between Gα and Gβγ subunits occurs in native systems.

In the present study, we showed that the suppression of the NE-mediated Ca^{2+} current inhibition occurred with nearly all Gα subunits, regardless of Gα subfamily ($G\alpha_s$, $G\alpha_{o/i}$, $G\alpha_q$, and $G\alpha_{12}$). Interestingly, overexpression of different Gα subunits also eliminated the current inhibition mediated by cholera toxin-sensitive $G\alpha_s$ activated on VIP receptor activation (Zhu and Ikeda, 1994). In addition, overexpression of GDP-bound Gα subunits significantly delayed the Gpp(NH)p-mediated modulation. Thus, the exogenous GDP-bound Gα seems to interact with Gβγ subunits released from many different endogenous heterotrimers (e.g., $G\alpha_s\beta\gamma$ and $G\alpha_{o/i}\beta\gamma$). This result might not be surprising given the molecular similarity (80–90% identical in sequence) of the known Gβ subunits (except β_5) (Watson et al., 1994) and the results of *in vitro* experiments. It is likely that all effective Gα subunits suppress the current modulation by sequestration of Gβγ subunits because M-current modulation (selected as a negative control), which is mediated by $G\alpha_q$ (Haley et al., 1998), was not abolished by overexpression of Gα subunits. This explanation is also supported by the fact that the NE-mediated Ca^{2+} current inhibition was intact when $G\alpha_o$ was coexpressed with Gβγ subunits (Jeong et al., 1998). Unexpectedly, M-current modulation was partially attenuated when $G\alpha_{oA}$ was overexpressed in SCG neurons. This phenomenon remains to be inves-

tigated. However, it is possible that an extremely high level of GDP-bound $G\alpha_o$ absorbs the $G\beta\gamma$ subunits from G_q heterotrimers coupled to receptors or alters the signaling pathway for M-current modulation by unknown mechanisms.

For the modulation by either NE or VIP there was an exception; that is, NE- and VIP-mediated modulation was not eliminated by overexpression of $G\alpha_z$ and $G\alpha_s$, respectively. Additional experiments showed that $G\alpha_z$ was able to reconstitute the voltage-dependent inhibition of Ca^{2+} currents by different G_o/G_i-coupled receptors when overexpressed in SCG neurons (Jeong and Ikeda, 1998). $G\alpha_z$ did not reconstitute the VIP-mediated current inhibition because of lack of coupling to VIP receptors but was able to interact with the $G\beta\gamma$ responsible for VIP-mediated inhibition. It is unclear how $G\alpha_z$ replaces endogenous $G\alpha_{o/i}$ subunits in neurons. However, the reconstitution of NE-mediated current inhibition by $G\alpha_z$ can be blocked by the extremely high expression of $G\alpha_z$ (data not shown).

When $G\alpha_s$ was overexpressed, we acquired two different groups of results: (1) the enhancement or (2) the attenuation in VIP-mediated modulation in comparison to control. These dual effects can be explained as follows. When the expression level was high (as indirectly judged by block of NE response in the same tested neurons), excess GDP-bound $G\alpha_s$ acted as $G\beta\gamma$ sinks to reduce VIP-mediated inhibition. Conversely, when the expression level was low (as indirectly judged by the unaltered NE response in the same tested neurons), $G\alpha_s$ was capable of forming heterotrimer complexes ($G\alpha_s\beta\gamma$) with endogenous free $G\beta\gamma$ subunits (decreasing basal facilitation), and subsequently coupled to receptors. This is consistent with findings that expression of exogenous $G\alpha_s$ proteins at a low level results in the enhanced modulation by increasing the number of receptor–$G\alpha_s$ complexes (Bertin et al., 1994; Lim et al., 1995; Krumins and Barber, 1997). Studies using non-neuronal heterologous expression systems have generated similar results, i.e., expression of $G\alpha$ subunits decreased the basal facilitation and increased G-protein-mediated modulation (Bourinet et al., 1996; Roche and Treistman, 1998). Thus, whether a certain exogenous $G\alpha$ subunit increases or blocks receptor-mediated current modulation seems to be determined by specificity to a certain receptor and/or expression level of the subunit in the tested cells. Consequently, the question arises as to whether exogenous expression of $G\alpha_o$ might enhance the NE-mediated Ca^{2+} current inhibition in SCG neurons. Unlike the voltage-dependent modulation of Ca^{2+} channels by $G\alpha_s$-coupled receptors (e.g., VIP response), the NE-mediated response appears saturated at maximal concentrations of agonist. This notion is supported by experiments in which the voltage-dependent inhibition of Ca^{2+} currents was unchanged after application of both VIP and α_2-AR agonists together when compared with application of α_2-AR agonist alone (Zhu and Ikeda, 1994; Ehrlich and Elmslie, 1995). Thus, it seems unlikely that exogenous $G\alpha_o$ expressed at low levels would enhance the NE response even after heterotrimer formation with endogenous $G\beta\gamma$ (Herlitz et al., 1996).

The ability of heterologously expressed $G\alpha$ subunits to delay the onset of Ca^{2+} current modulation (Fig. 4) is consistent with the notion of $G\beta\gamma$ buffering. The delay can be rationalized by assuming that basal GDP–GTP exchange results in the binding of Gpp(NH)p to both endogenous and heterologously expressed $G\alpha$ subunits. Because Gpp(NH)p cannot be hydrolyzed, the majority of $G\alpha$ subunits eventually attain the Gpp(NH)p bound state and are thus incapable of binding $G\beta\gamma$ with high affinity. Consequently, the eventual loss of $G\alpha$-GDP results in "release" of free $G\beta\gamma$ and thus voltage-dependent modulation. What is not clear from these data is why $G\alpha_s$ and $G\alpha_z$ produce such a dramatic effect in this assay when compared with their weak ability to attenuate agonist-mediated modulation (Fig. 2). Although we have no definitive explanation for these results, it can be speculated that expression levels, intrinsic GDP–GTP (or Gpp(NH)p) exchange rates (Fields and Casey, 1997), or compartmentalization (Neubig, 1994) may underlie these findings.

When compared with "$G\beta\gamma$ sinks" derived from effector molecules, for example the C terminus of βARK (Koch et al., 1994) or the QEHA peptide (Chen et al., 1995), $G\alpha$ subunits may prove advantageous in regard to probing signaling pathways. First, it is assumed that $G\alpha$–GDP has a greater affinity for $G\beta\gamma$ than effector molecules. Although we are unaware of studies that directly compare these properties, it stands to reason that this is the case because the termination of $G\beta\gamma$ actions is thought to require association with $G\alpha$–GDP. If the affinity of effector molecules for $G\beta\gamma$ exceeded that of $G\alpha$–GDP, termination of signaling could not occur via this mechanism. Recently, a biochemical study has shown that a peptide containing the $G\beta\gamma$-binding motif QXXER failed to inhibit interactions between GDP-bound $G\alpha$ and $G\beta\gamma$ subunits (Chen et al., 1995), thus supporting this idea. Second, effectors such as βARK have been shown to interact with specific $G\beta\gamma$ isoforms (Koch et al., 1994), whereas $G\alpha/G\beta\gamma$ interactions appear relatively nonspecific. Thus, one would predict that overexpression of $G\alpha$–GDP would affect a broader range of $G\beta\gamma$-mediated responses. It should be noted, however, that information concerning heterotrimer formation for the various G-protein subunits is incomplete. Moreover, the ability of βARK isoforms to specifically bind different $G\beta\gamma$ combinations may prove useful for identification purposes once these interactions are better defined. Thus, the information gained by overexpressing $G\alpha$ subunits or effector molecule binding sites may prove complementary in regard to $G\beta\gamma$ signaling.

In summary, the present study showed that a wide variety of GDP-bound $G\alpha$ subunits were able to sequester the $G\beta\gamma$ subunits, resulting in block of NE-, VIP-, and Gpp(NH)p-mediated modulation of N-type Ca^{2+} channels in SCG neurons. These results suggest that coupling specificity in signal transduction is unlikely to arise as a result of restricted $G\alpha/G\beta\gamma$ interaction. Although the interaction between $G\alpha$ and $G\beta\gamma$ subunits is relatively nonspecific, a specific heterotrimer combination may be required for selective coupling of certain receptors to Ca^{2+} channels (for review, see Kalkbrenner et al., 1996). Thus, it is likely that the identity of $G\alpha$ subunits may be one of the major determinants for the selective recognition of a given heterotrimer by a receptor.

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