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G-Protein β-Subunit Specificity in the Fast Membrane-Delimited Inhibition of Ca^{2+} Channels

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We investigated which subtypes of G-protein β subunits participate in voltage-dependent modulation of N-type calcium channels. Calcium currents were recorded from cultured rat superior cervical ganglion neurons injected intranuclearly with DNA encoding five different G-protein β subunits. Gβ_1 and Gβ_2 strongly mimicked the fast voltage-dependent inhibition of calcium channels produced by many G-protein-coupled receptors. The Gβ_5 subunit produced much weaker effects than Gβ_1 and Gβ_2, whereas Gβ_3 and Gβ_4 were nearly inactive in these electrophysiological studies. The specificity implied by these results was confirmed and extended using the yeast two-hybrid system to test for protein–protein interactions. Here, Gβ_1 or Gβ_2 coupled to the GAL4-activation domain interacted strongly with a channel sequence corresponding to the intracellular loop connecting domains I and II of a α_1 subunit of the class B calcium channel fused to the GAL4 DNA-binding domain. In this assay, the Gβ_5 subunit interacted weakly, and Gβ_3 and Gβ_4 failed to interact. Together, these results suggest that Gβ_1 and/or Gβ_2 subunits account for most of the voltage-dependent inhibition of N-type calcium channels and that the linker between domains I and II of the calcium channel α_1 subunit is a principal receptor for this inhibition.

Key words: G-proteins; calcium channel; norepinephrine; yeast 2-hybrid; sympathetic neurons; ion channel modulation

Inhibition of neuronal calcium currents by G-protein-coupled receptors probably occurs in every type of neuron. It is a ubiquitous mode of modulation of electrical activity and synaptic function by neighboring neurons. A common form uses a signaling pathway whose components appear to be restricted to the cell membrane (membrane-delimited), develops in <1 sec, and is voltage-dependent, i.e., the inhibition can be partially relieved by a strong depolarizing pulse (for review, see Hille, 1994). A well studied example is the inhibition of N-type calcium channels by norepinephrine (NE) acting on α_2 adrenergic receptors in superior cervical ganglion (SCG) neurons. This type of inhibition is mediated by $\beta\gamma$-subunits of G-proteins, apparently acting directly on the channel (Herlitze et al., 1996; Ikeda, 1996).

The specificity for Gβ and Gγ subunits has not been investigated, and the target site for the interaction of G-protein $\beta\gamma$ subunits with calcium channels is the subject of some debate. By analogy with other G$\beta\gamma$-effector proteins, several investigators have proposed that the target site on voltage-gated calcium channels includes a QXXER motif in the intracellular loop connecting domains I and II ($L_{I\text{-}II}$) of the α_1 subunits of classes A, B, and E (the P/Q-, N-, and R-type) calcium channels (De Waard et al., 1997; Herlitze et al., 1997; Page et al., 1997; Zamponi et al., 1997). This QXXER sequence overlaps with the region required for interaction with the calcium channel β subunit. In contrast, some investigators argue that an interaction of G$\beta\gamma$ subunits with the C terminus of calcium channel α_1 subunit is the functionally important one (Zhang et al., 1996a; Qin et al., 1997). Here, we inject DNA for various G-protein β subunits into the nucleus of adult rat SCG neurons and find that only certain Gβ subunits will induce inhibition of the endogenous N-type Ca^{2+} currents. Then, we used the yeast two-hybrid assay to look for interactions between Gβ subunits and a domain of the channel. We find that exactly the same Gβ subunits that inhibit current also interact well with the linker between domains I and II of the α_1 subunit of the class B (N-type) calcium channel. Together, these results identify the Gβ subunits that can mediate fast, membrane-delimited, and voltage-dependent inhibition of N-type calcium channels, and they identify a target domain of the channel that has the appropriate binding specificity.

MATERIALS AND METHODS

Cell culture and intranuclear microinjection. Single SCG neurons were enzymatically dissociated from 5-week-old male rats (Sprague Dawley rats were used for the experiments in Figs. 1–3 in Seattle, and Wistar rats were used in the experiments in Figs. 4 and 5 in Mexico) as described previously (Beech et al., 1991; Bernheim et al., 1991). After a 4 hr wait for attachment to the substrate, the neurons were intranuclearly microinjected using an Eppendorf 5242 pressure microinjector and 5171 micromanipulator system (Eppendorf, Madison, WI). The injection solution contained varying amounts of G-protein expression plasmids mixed with two injection markers, 1 mg/ml 10,000 kDa dextran–fluorescein (Molecular Probes, Eugene, OR), and green-fluorescent protein (GFP) plasmid as an expression reporter. Injection at pressures of 10–20 kPa for

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0.5–0.8 sec resulted in no obvious increase in cell volume. After 12–16 hr, successfully injected neurons were identified by their characteristic greenish-blue GFP fluorescence using an inverted microscope equipped with epifluorescence and fluorescein optics.

Electrophysiological recording. Currents were recorded using the whole-cell voltage-clamp technique (Hamill et al., 1981) with a patch-clamp amplifier at room temperature. Pipettes (0.85–2 MΩ) were pulled from microhematocrit glass and fire polished. During recording, neurons were constantly perfused locally (1–2 ml/min) with the appropriate external solution. Solution reservoirs were selected by means of solenoid valves, and solution changes were accomplished in <10 sec. Voltage protocols were generated, and data were digitized, recorded, and analyzed using BASIC-FASTLAB (Indec Systems, Capitola, CA).

To measure calcium currents, we used two sets of solutions. In the experiments of Figures 1–3, the pipette solution contained (in mM): 125 *N*-methyl-D-glucamine, 20 TEA-Cl, 10 HEPES, 0.1 tetracesium-BAPTA, 4 $MgCl_2$, 0.1 leupeptin, 4 Na_2ATP, and 0.3 Na_2GTP, pH adjusted to 7.2 with methanesulfonic acid. The external solution contained (in mM): 140 TEA-OH, 10 HEPES, 2 $CaCl_2$, 15 glucose, 0.0001 TTX, and 0.002 nifedipine, pH adjusted to 7.3 with methanesulfonic acid. In the experiments of Figures 4 and 5, the pipette solution contained (in mM): 125 methanesulfonic acid, 20 TEA-Cl, 10 HEPES, 0.1 tetracesium-BAPTA, 4 $MgCl_2$, 5 MgATP, 0.3 Na_2GTP, and 0.1 leupeptin, pH adjusted to 7.2 with CsOH. The external solution contained (in mM): 162.5 TEA-Cl, 10 HEPES, 8 glucose, 2 $CaCl_2$, 1 $MgCl_2$, 0.0002 TTX, and 0.002 nifedipine, pH adjusted to 7.3 with TEA-OH.

The Ca^{2+} current of rat SCG neurons is carried ~85–90% in N-type channels and the remainder in L-type channels, with no detectable P/Q component (Mintz et al., 1992). Therefore, the N-type Ca^{2+} current could be defined as the component of the current sensitive to 100 μM Cd^{2+} in the presence of 2 μM nifedipine. Currents were sampled at 10 kHz. To emphasize the effects of kinetic slowing of Ca^{2+} current activation, current amplitudes were always taken as the mean value of recorded points between 5 and 6 msec after the start of the depolarizing test pulse. This time is before the current reaches a peak. Because the magnitude of the Ca^{2+} current was dependent on cell size, aggregate current data are presented as current densities normalized to cell capacitance. To avoid one source of systematic bias, experimental and control measurements were alternated whenever possible, and concurrent controls were always performed. Where appropriate, data are expressed as mean ± SE.

Plasmids and materials. The DNAs encoding $G\beta_2$, $G\beta_4$, and $G\beta_5$ were cloned in pcDNA I plasmid, $G\beta_1$ was cloned in pCDM8, $G\beta_3$ was cloned in pCIS, $G\gamma_3$ was cloned in pCI (all from M. Simon, Caltech, Pasadena, CA), and GFP was cloned in pEGFP-N1 (Clontech, Palo Alto, CA). Although not in identical vectors, all G-protein-containing plasmids were driven by the cytomegalovirus promoter. DNA encoding the L_{I-II} loop of rat brain N-type calcium channel (rbB-I) α_{1B} was subcloned into the pAS2–1 vector (Clontech) as an *Eco*RI–*Bam*HI fragment (nt 1069–1449; aa 357–483) to express a GAL4 DNA-binding domain (GBD) hybrid protein. The bovine G-protein β_1 subunit and β_2 subunit genes were subcloned into the pACT vector (Clontech) to express GAL4-activation domain (GAD) hybrid proteins. The plasmids pACT-$G\beta_3$, pACT-$G\beta_4$, and pACT-$G\beta_5$ were described previously (Yan et al., 1996). Plasmids were purified using commercial kits (Qiagen, Valencia, CA). The yeast strain was *PJ69–4A (MAT* **a** *trp1–901 leu2–3,112 ura 3–52 his3–200 gal4*Δ *gal 80*Δ *GAL2-ADE2 LYS2::GAL-HIS3 met2::GAL7-lacZ)* (a gift of Dr. Philip James, University of Wisconsin) (James et al., 1996). This strain contains three reporters (*ADE2*, *HIS3*, and *LacZ*) that can be activated as a result of protein–protein interaction by reconstituted GAL4 transactivator function.

Tetracesium-BAPTA was obtained from Molecular Probes, and all other salts were obtained from Sigma (St. Louis, MO).

Yeast two-hybrid assay. Plasmids were made expressing hybrid proteins consisting either of a specific Gβ subunit coupled to a GAD or an intracellular loop of the α_1 N-type calcium channel fused to a GBD. A plasmid expressing one of the GBD hybrids and the plasmid expressing the GAD hybrid were cotransformed into yeast strain PJ69–4A (usually without introducing any mammalian Gγ subunit). Cells were first plated on a tryptophan-free leucine-free medium to select Trp+ Leu+ transformed cells containing the GBD hybrid- and GAD hybrid-expressing plasmids. To detect protein–protein interactions, these transformants were then transferred onto medium that also lacked adenine and histidine.

Immunoblotting. To check the expression of G-protein subunit hybrids in yeast, transformed yeast cells were grown to saturation phase. Cultures (1.5 ml) were harvested and resuspended in 150 μl of Z buffer (in mM: 30 Na_2HPO_4, 35 $NaHPO_4$, 10 KCl, and 0.4 $MgSO_4$, pH 7.0) with 5% glycerol, 0.5 mM dithiothreitol, 1 μg/ml aprotinin, and 2 μg/ml leupeptin, followed by addition of 0.1 gm of 425–600 μm glass beads (Sigma). The cells were vortexed for 4 min and centrifuged for 10 min at 4°C. The supernatants were saved, and 20 μl cell lysates were fractionated on a SDS gel. The separated proteins were electrophoretically transferred to a nitrocellulose membrane, and the blot was probed with anti-GAD antibody (0.2 μg/ml; Clontech), followed by horseradish peroxidase-conjugated anti-mouse IgG (1:2000; Amersham, Piscataway, NJ). The GAD hybrid proteins were detected by the enhanced chemiluminescence method (Amersham).

RESULTS

We begin with the typical NE-induced modulation of N-type calcium currents. In control SCG neurons, a 20 msec depolarizing test pulse elicited a rapidly activating inward Ca^{2+} current (Fig. 1*A*, *left*), which was primarily carried in N-type channels because the medium contained 2 μM nifedipine to block L-type channels. Perfusion with 10 μM NE reduced the inward current amplitude by ~60% and slowed its rate of activation. To monitor current facilitation, as well as amplitude, we clamped the membrane potential using the voltage protocol in Figure 1*A*. Inward Ca^{2+} currents were evoked every 10 sec with a pair of 20 msec depolarizing test pulses to +10 mV from a holding potential of −80 mV, one before (test pulse 1) and the other after (test pulse 2) a 25 msec prepulse to +125 mV. The depolarizing prepulse transiently relieves much of the NE-induced inhibition, and the resulting facilitation can be seen by comparing currents in test pulse 2 with those in test pulse 1. The "facilitation ratio," defined as the current during test pulse 2 (at 5–6 msec) divided by the current during test pulse 1, is a convenient measure of voltage-dependent membrane-delimited inhibition by certain G-proteins. In standard Ringer's solution, this facilitation ratio is near 1.1, and after treatment with NE, it rises to 1.6.

Dose-dependent suppression of I_{Ca} by overexpression of $G\beta_2$

Previously, we had found that injection of RNA for $G\beta_2$ subunits, with and without RNA for $G\gamma_3$, into the cytoplasm of SCG neurons inhibited Ca^{2+} currents, increased the facilitation ratio, and partially occluded the actions of NE (Herlitze et al., 1996). Ikeda (1996) reported even stronger effects with intranuclear injection of DNA. To optimize conditions for intranuclear injection here, we first determined the dose–response relationship for the action of $G\beta_2$ by varying the concentration of $G\beta_2$ DNA injected, which was always coinjected with 100 ng/μl $G\gamma_3$ and 100 ng/μl GFP DNA. Injection of $G\beta_2$ plasmid at 10, 20, and 100 ng/μl induced progressively larger facilitation and kinetic slowing of activation in standard Ringer's solution (Fig. 1*B–D*). Injection at 600 ng/μl (Fig. 1*E*) gave about the same facilitation as 100 ng/μl. Figure 1*F* shows the dose–response relationship for development of facilitation after these injections of DNA for $G\beta_2$ compared with that after injection of $G\beta_3$. Quite clearly, $G\beta_2$ has a much greater effect than $G\beta_3$. Injection with 100 or 500 ng/μl $G\beta_3$ DNA solution did not induce facilitation (Fig. 1*F*); indeed, injection of $G\beta_3$ slightly reduced facilitation below control levels (facilitation ratio, 1.03 ± 0.02; $n = 10$; $p < 0.05$).

The current traces after injection of $G\beta_2$ show that the progressive increase of facilitation was accompanied by a decreasing effectiveness of NE and a decrease in basal calcium current density. The increasing amounts of expressed $G\beta_2$ subunits presumably occlude the actions of NE and block N-type calcium channels. The facilitation ratio is consistently a more sensitive

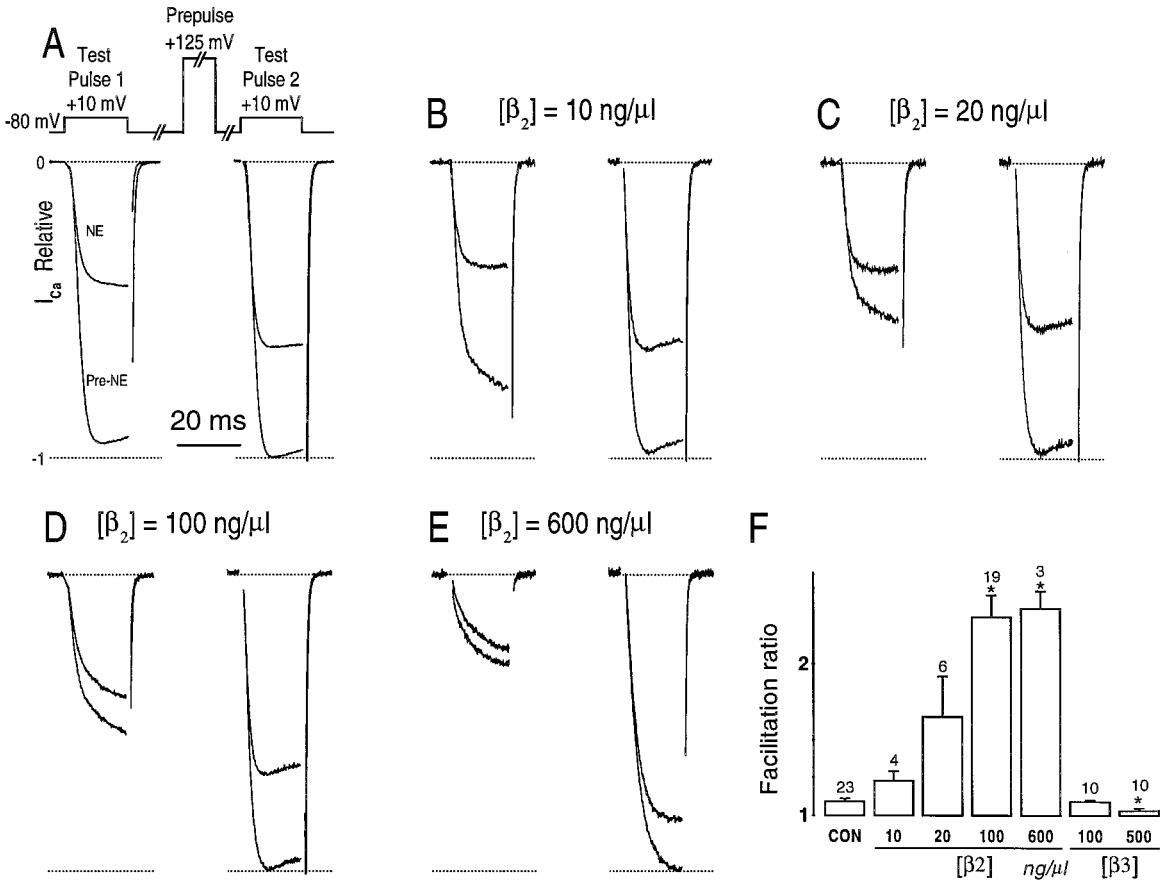

Figure 1. Injecting $G\beta_2$ DNA increases calcium current facilitation. *A–E*, Superimposed calcium current (I_{Ca}) *traces* during a 20 msec depolarization (test pulse) to +10 mV from a holding potential of −80 mV, in the absence (*bottom trace*) or presence (*top trace*) of NE (10 μM), before (*left*) or after (*right*) a 25 msec +125 mV prepulse. Successive *panels* show uninjected cells (*A*) and cells injected with increasing concentrations of $G\beta_2$ DNA (*B–E*). $G\beta_2$ DNA was coinjected with 100 ng/μl $G\gamma_3$ DNA in all cases. *F*, Summary of facilitation ratios for injections of several concentrations of $G\beta_2$ and $G\beta_3$ DNA.

indicator of $G\beta_2$ actions than the occlusion of NE action or the basal current density. From the dose–response curves for these three actions (Fig. 2), we elected to use 100 ng/μl Gβ plasmids in the next experiments.

The *dashed arrow* in Figure 2*A* represents an extrapolated $G\beta_2$ DNA concentration (7 ng/μl) that would be equivalent to the control basal facilitation levels, assuming that the facilitation ratio in the total absence of endogenous Gβ would be the fitted value 0.94.

Identification of the Gβ subtypes that modulate Ca^{2+} currents

The apparent inability of $G\beta_3$ to produce facilitation prompted us to undertake a more systematic study of the effectiveness of the various G-protein β subunits in calcium channel inhibition. As before, we measured facilitation, tonic inhibition, and occlusion of further inhibition by NE. Again in control cells, application of 10 μM NE strongly reduced the Ca^{2+} current magnitude (Fig. 3*A*, *left*). Figure 3, *B* and *D*, illustrates Ca^{2+} currents recorded from neurons that had been injected with 100 ng/μl DNA for $G\beta_1$, $G\beta_2$, or $G\beta_5$, with GFP DNA but without coinjection of a Gγ DNA. In the absence of agonist, the Ca^{2+} currents in neurons injected with DNAs for $G\beta_1$ or $G\beta_2$ displayed kinetic slowing (Fig. 3, *left traces* of each pair) and facilitation (*right traces*) virtually identical to that produced by application of NE in control cells. Coinjection with $G\gamma_3$ DNA did not change the results for $G\beta_1$ (data not shown) or $G\beta_2$ (Fig. 1). Only a small effect was observed in cells injected with $G\beta_5$ DNA, whether alone (Fig. 3*D*) or in combination with $G\gamma_3$ DNA (data not shown). Finally, in neurons injected with DNAs for $G\beta_3\gamma_3$, $G\beta_4\gamma_3$, or CB1λ (a truncated, nonmembrane spanning form of the rat cannabinoid receptor in the pcDNA3 expression vector), the Ca^{2+} current activation was similar to that of uninjected neurons (data not shown).

The quantitative results of these DNA injections for the various Gβ subunits are summarized in Figure 3, *E–G*. The $G\beta_1$ or $G\beta_2$ injections with and without $G\gamma_3$ give indistinguishable, strong, and highly significant effects when compared with cells injected with the control plasmid CB1λ or to uninjected cells. In these $G\beta_1$- or β_2-injected neurons, the facilitation ratio is raised from a mean of 1.1 to 2.3, the inhibition by NE is lowered from 60 to 22%, and the basal calcium current density is lowered from 22 to 9 pA/pF. These actions are significant at the $p < 0.005$ level. Interestingly, both the depression of current and the increase in facilitation ratio are greater than those which occur with 10 μM NE on control cells. By comparison, the effects of injecting DNA for $G\beta_3$, $G\beta_4$, or $G\beta_5$ are weak or negligible, even in the presence of $G\gamma_3$. Of these, $G\beta_5$ appears the most active. Our results suggest that the different Gβ subtypes have different efficacies in voltage-

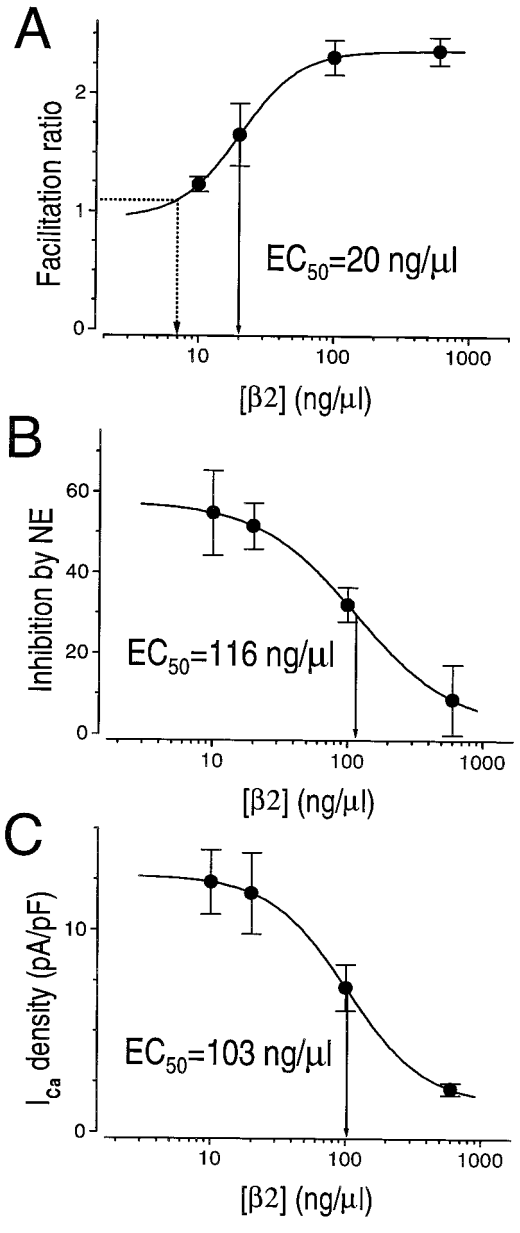

Figure 2. Concentration–response relationships for facilitation ratio, inhibition by NE, and I_{Ca} density. $G\beta_2$ DNA concentrations ([β2]) are plotted on a logarithmic scale. Data were fitted to a Hill function, $y = [(A_1\text{-}A_2)/\{1+([\beta2]/[\beta2]_0)^n\}] + A_2$, with best fitting values of the midpoints, $[\beta2]_0$ = 20.0, 156, and 103 ng/μl (*vertical solid line*); Hill coefficients, n = 2.0, 1.2, and 1.51; low-concentration asymptotes, A_1 = 0.94, 57, and 12.7; and high-concentration asymptotes, A_2 = 2.4, 1.9, and 1.4 for *A*, *B*, and *C*, respectively. The *horizontal dashed line* in *A* indicates the facilitation ratio for control cells (1.1), and the *vertical dashed line* indicates a theoretical injected $G\beta_2$ DNA concentration (7.0 ng/μl) that would be equivalent to this basal facilitation ratio.

dependent inhibition of Ca^{2+} channels, with a rank order of effectiveness: $G\beta_1 = G\beta_2 > G\beta_5 \gg G\beta_4 = G\beta_3$.

Characterization of the weakly active Gβ subtypes

We wanted to explore more fully the reasons for the weak effects of $G\beta_3$, β_4, and β_5 DNA injections. Did the plasmids fail to induce proper synthesis of the corresponding proteins? Were the proteins really inactive on Ca^{2+} channels? Because convenient antibodies for verifying expression of specific Gβ subunits in single injected cells are lacking, we chose less direct approaches. We looked with increased sensitivity for electrophysiological effects on Ca^{2+} channels.

We made two changes in the electrophysiological assay. One was in the concentration of GFP DNA coinjected into the cells. In some systems, it has been found that the overexpression of GFP or other proteins from one plasmid leads to less expression from a second plasmid. Thus, the most brightly fluorescing cells express lower levels of protein from the second plasmid (N. Davidson, personal communication). Therefore, we lowered the concentration of GFP DNA in the injection solution from 100 to 10 ng/μl in the next experiments. A second factor that may have limited our sensitivity to detect occlusion of the NE response is the high concentration of NE we had used (10 μM) in Figures 1, 2, and 3. This concentration is sufficiently above that needed to have a maximal NE action that a small shift of the sensitivity to NE could have been obscured. Therefore, we reduced the NE concentration to 2 μM, which is submaximal.

Figure 4, *A–D*, illustrates Ca^{2+} currents recorded from an uninjected neuron and from neurons injected with $G\beta_3$, $G\beta_4$, and $G\beta_5$ DNAs. In uninjected neurons, 2 μM NE inhibited the Ca^{2+} current by 41 ± 4% (SEM; n = 6), compared with the 55% inhibition obtained with saturating NE (10 μM) (Figs. 1–3). There were statistically significant changes in at least one of the experimental parameters for each injected neuron, suggesting expression of each Gβ subunit. Facilitation was increased in the neurons injected with $G\beta_5$; the inhibition by 2 μM NE was reduced by injection of $G\beta_4$ or $G\beta_5$, and the calcium current density was almost doubled by injection of $G\beta_3$. Further evidence that injection of $G\beta_3$ DNA affects cellular responses is seen in Figure 5, in which neurons were injected with $G\beta_1$ alone, $G\beta_3$ alone, or a mixture of $G\beta_1$ and $G\beta_3$ DNAs. As we have seen before, the facilitation ratio is strongly increased by $G\beta_1$ and not increased by $G\beta_3$; however, coinjection of $G\beta_3$ with the $G\beta_1$ gives significantly less facilitation than with $G\beta_1$ alone, consistent, for example, with competition between the Gβ subunits for formation of active Gβγ complexes. These results show that $G\beta_3$ and $G\beta_4$ DNA injections do have specific electrophysiological effects, even if they do not mimic actions of NE on Ca^{2+} currents. Therefore, these Gβ subunits are expressed at functionally significant levels in our injected neurons.

$G\beta_1$ and $G\beta_2$ interact strongly with the α_1 subunit of N-type calcium channels

It has been demonstrated that $L_{I\text{-}II}$ of the α_1 subunits of classes A, B, and E (P/Q-, N-, and R-type) calcium channels interact directly with the $G\beta_1$ subunit (De Waard et al., 1997; Zamponi et al., 1997) and that this loop may mediate the inhibitory modulation by the activated G-protein (Herlitze et al., 1997; Page et al., 1997). Our results presented above suggest that this interaction is not the same for all of the Gβ subunits. We sought to investigate the mechanism underlying the differential effects of the five different Gβ subunit subtypes by testing the possibility that they interact with the channel with different affinities. We examined the interaction of $L_{I\text{-}II}$ of α_{1B} with the Gβ subunits using the two-hybrid assay, an assay that can detect protein–protein interactions *in vivo* in yeast (Fields and Song, 1989).

Plasmids encoding the GBD-$L_{I\text{-}II}$ hybrid and the various GAD-Gβ hybrids were introduced into a yeast reporter strain in which interactions between two hybrid proteins result in transcriptional activation of two reporter genes, *HIS3* and *ADE2*. These two reporter gene products are involved in histidine and

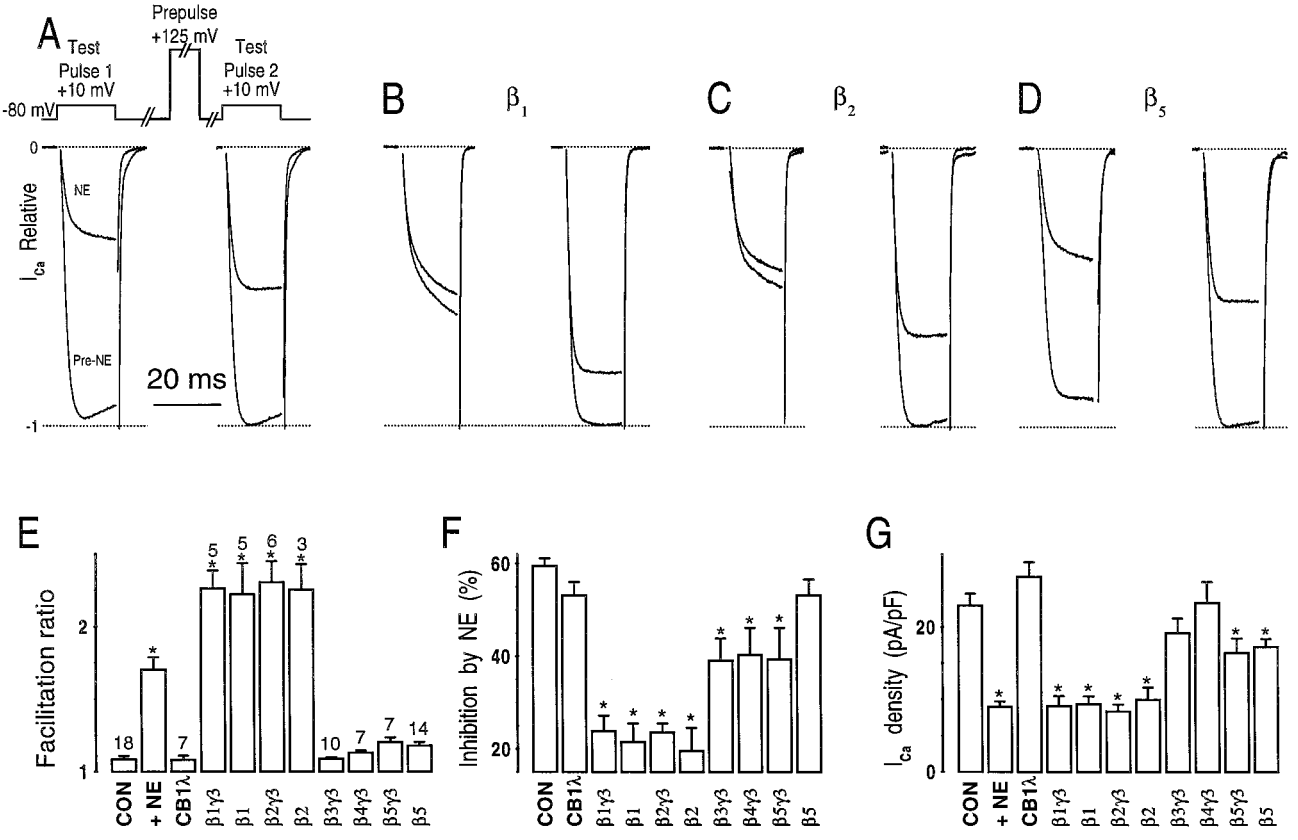

Figure 3. Identification of the Gβ subtypes that cause voltage-dependent modulation of Ca currents. Cells were injected with DNA for G-protein β and γ subunits, as indicated, and currents were recorded by the double-pulse protocol. *A–D*, Current records for uninjected control cell (*A*) and neurons injected with 100 ng/μl $G\beta_1$, $G\beta_2$, or $G\beta_5$ DNA (*B–D*). *E*, Summary data for facilitation ratios; *numbers above* individual *columns* represent the number of tested cells, and the same numbers apply to *F* and *G*. Data plotted as mean ± SEM. $^*p < 0.05$; two-tailed *t* test versus control. *F*, Percent of I_{Ca} inhibited by NE (during test pulse 1). *G*, I_{Ca} density during test pulse 1 in the absence of NE.

adenine synthesis, respectively, and their activation allows the yeast cells to grow on medium lacking histidine and adenine. As shown in Figure 6*A*, coexpression of $L_{I\text{-}II}$ with $G\beta_1$ or $G\beta_2$ results in robust growth of the yeast cells on the selection medium, whereas with $G\beta_5$, the cells grow at a much slower rate. In the case of $G\beta_3$ and $G\beta_4$, no transformed yeast cells were able to grow in the absence of histidine and adenine. The same results were obtained in five experiments. Because transcription signals resulting from the protein–protein interactions in the two-hybrid assay generally correlate with the binding affinity observed from *in vitro* experiments (Li and Fields, 1993; Yan et al., 1996), our data suggest that the order of affinity for $L_{I\text{-}II}$ of α_{1B} is $G\beta_1 = G\beta_2 > G\beta_5 \gg G\beta_3, G\beta_4$.

To exclude the possibility that the tighter binding between $G\beta_1$, $G\beta_2$, and $L_{I\text{-}II}$ is attributable to their more efficient recruitment of endogenous yeast Gγ subunits, we also performed the assay in the presence of overexpressed rat $G\gamma_3$ subunit. The results obtained were the same (data not shown), suggesting that Gγ subunits are not a determinant of the interaction between Gβ and $L_{I\text{-}II}$ in our assay. Hence, activation of the *HIS3* and *ADE2* genes in the assay might be the result of direct interactions between $G\beta_1$, $G\beta_2$, and $G\beta_5$ with the calcium channel $L_{I\text{-}II}$ loop region.

Because an apparent differential affinity of Gβ subtypes for $L_{I\text{-}II}$ of α_{1B} observed in the two-hybrid experiments could also be attributable to differences of their protein level in the yeast cell, we examined their expression by immunoblotting. This method measures the level of expressed protein but does not test correct folding or association with Gγ subunits. Immunoblots of the yeast cell lysates with anti-GAD antibody showed these GAD-hybrid proteins are expressed at similar levels (Fig. 6*B*, *lanes 1–5*). Densitometric scanning and normalization to the amount of total cellular protein in each extract showed that expression of $G\beta_3$, $G\beta_4$, and $G\beta_5$ was higher than expression of $G\beta_1$ and $G\beta_2$. A second experiment gave the same result. These results support our conclusion that, in the yeast two-hybrid assay, $G\beta_1$ and $G\beta_2$ are more effective because they bind with higher affinity to $L_{I\text{-}II}$.

DISCUSSION

Our electrophysiological and protein–protein interaction experiments give concordant results for the β subunits of G-proteins. All Gβ subunits are not equal. Mimicry and occlusion of membrane-delimited voltage-dependent inhibition of N-type Ca^{2+} currents and interaction with the $L_{I\text{-}II}$ loop of α_{1B} (N-type) calcium channels follow the sequence $G\beta_1 = G\beta_2 > G\beta_5 \gg G\beta_3$, $G\beta_4$. The agreement of the two approaches adds weight to the proposal that the functional target of voltage-dependent Gβ interaction includes the $L_{I\text{-}II}$ loop (De Waard et al., 1997; Herlitze et al., 1997; Page et al., 1997; Zamponi et al., 1997). The activity of overexpressed $G\beta_1$ and $G\beta_2$ has been documented before in SCG cells (Herlitze et al., 1996; Ikeda, 1996; Delmas et al., 1998). Unlike our results (Fig. 1), however, Ikeda (1996) and Delmas et al. (1998), who studied only $G\beta_1$, found that a Gγ subunit had to be coexpressed with $G\beta_1$ to have an effect on calcium channels.

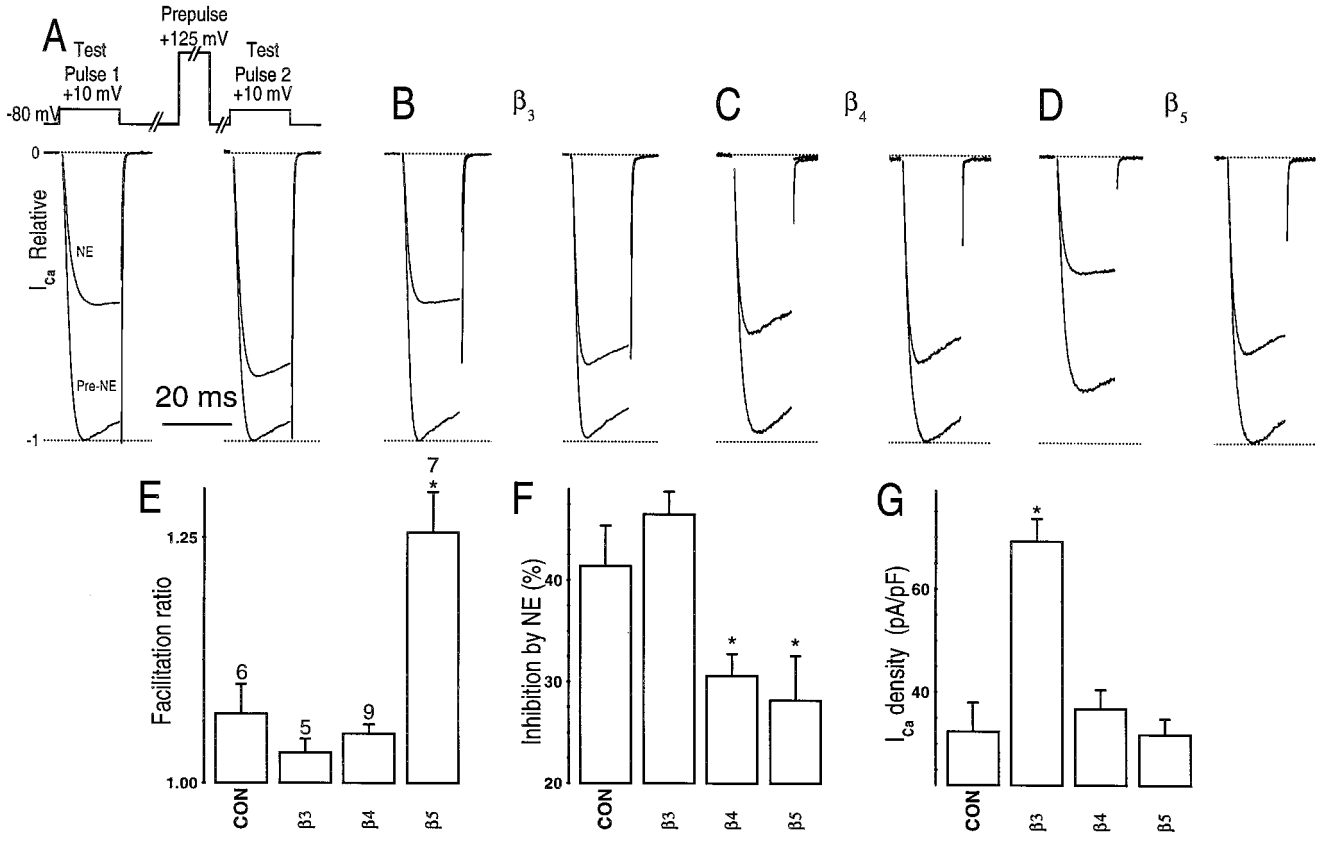

Figure 4. Three weakly active Gβ subtypes. *A–D*, Current records for uninjected control cell (*A*) and neurons injected with 100 ng/μl $G\beta_3$, $G\beta_4$, or $G\beta_5$ DNA (*B–D*). To accentuate possible weak actions by $G\beta_3$, $G\beta_4$, and $G\beta_5$, the concentration of the reporter plasmid (pEGFP-N1) was decreased tenfold to 10 ng/μl, and NE was decreased to 2 μM. *E–G*, Summary data for facilitation ratio, inhibition by NE, and current density in these cells. Note that the axes have been expanded to reveal smaller differences.

We do not know any reason for this clear difference from our work. They tested only $G\gamma_2$, and we tested only $G\gamma_3$. If Gβγ dimers are needed for mimicking action of NE, the Gβ subunits must have combined with endogenous Gγ subunits when we did not inject Gγ DNA. The endogenous complement of Gγ subunits is not known for SCG neurons.

Although the vectors for $G\beta_3$ and $G\beta_4$ did not mimic voltage-dependent Ca^{2+} current inhibition by NE, they did alter electrophysiological properties of the cells. Thus, they were expressed. Unexpectedly, expression of $G\beta_3$ led to a near doubling of the Ca^{2+} current density (Fig. 4). Because the percent of inhibition by NE was unchanged in these cells, the increased current density was primarily caused by an increase in N-type Ca^{2+} current. On the other hand, expression of $G\beta_4$ led to a reduction in modulation by NE, without an increase in facilitation or a decrease in current density. These effects of $G\beta_3$ and $G\beta_4$ might have been caused by interactions with signaling systems in the cell that alter gene expression or act in other ways on the channel. They could also represent direct interactions of $G\beta_3$ and $G\beta_4$ with parts of the channel that do not have the binding specificity we found for the $L_{I\text{-}II}$ loop. It is not likely that $G\beta_3$ or $G\beta_4$ failed to pair with coinjected $G\gamma_3$ subunit, because it has been found previously that this subunit pairs efficiently with the $G\beta_3$ and $G\beta_4$ subunits (Yan et al., 1996). Indeed, in comparison with $G\beta_2\gamma_3$, the reporter activity in a yeast-two-hybrid assay was 55% for $G\beta_3\gamma_3$, 72% for $G\beta_4\gamma_3$, and 83% for $G\beta_5\gamma_3$ (Yan et al., 1996).

The literature does not contain much information on specificity of the actions of Gβ subunits. From our results, the strong membrane-delimited voltage-dependent modulation of N-type calcium channels seen in many neurons is almost certainly mediated by $G\beta_1$ and/or $G\beta_2$ subunits. A similar conclusion may be drawn for the activation of G-protein-coupled inward rectifier K^+ (GIRK) channels. A two-hybrid screen with residues 1–83 of the GIRK1 channel showed interaction with $G\beta_1$ and $G\beta_2$ but not with $G\beta_3$, $G\beta_4$, or $G\beta_5$ (Yan and Gautam, 1996), suggesting that $G\beta_1$ and $G\beta_2$ may be generally involved in membrane-delimited modulation of ion channels. Functional tests of $G\beta_3$, $G\beta_4$, or $G\beta_5$ on GIRK channels have not been published.

Specificity has been investigated in a few other systems. Kleuss et al. (1991, 1992, 1993) used injection of antisense oligonucleotides directed against specific members of the G-protein heterotrimer to study agonist-induced inhibition of L-type calcium channels in GH_3 cells. They established that $G\alpha_{o1}/\beta_1/\gamma_4$ mediates inhibition acting via m_4 muscarinic receptors, whereas $G\alpha_{o2}/\beta_3/\gamma_3$ mediates inhibition by somatostatin receptors. The molecular mechanisms of these signaling pathways have not been worked out, and it is possible that the very high specificity lies at the level of the receptors rather than the (unknown) effector(s). This contrasts with work on adenylyl cyclase II in the two-hybrid system in which all β subunits interact to some degree, and the rank order of interaction was $\beta_1 > \beta_2 > \beta_3 = \beta_5 > \beta_4$ (Yan and Gautam, 1996). Interestingly, β_1 stimulates, whereas β_5 inhibits adenylyl cyclase II (Bayewitch, 1998). Both interactions were strong in the two-hybrid system.

Other systems in which G-protein subtype specificity has been examined include inhibition of adenylyl cyclase I (no difference

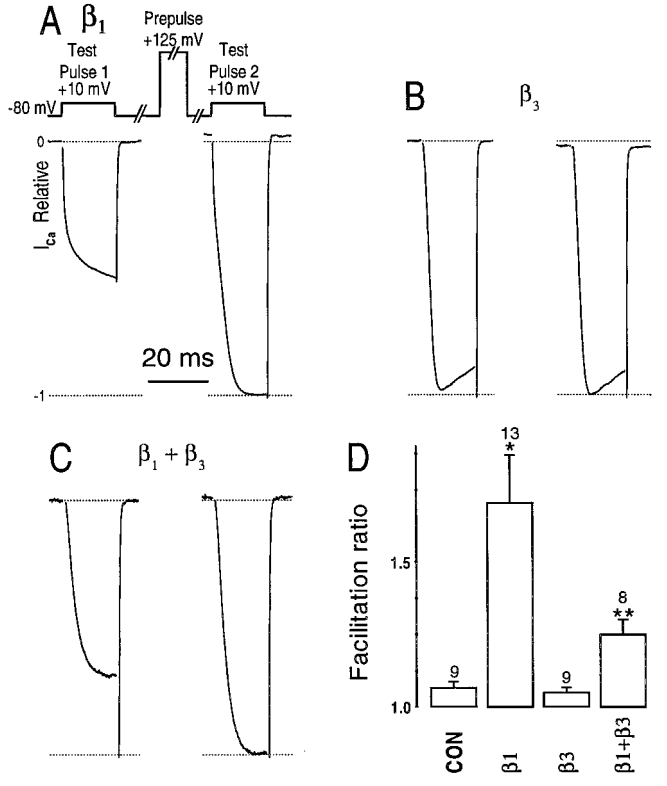

Figure 5. Coinjection of $G\beta_1$ and $G\beta_3$ DNAs. *A–C*, Current *traces* for cells were injected with 100 ng/μl $G\beta_1$ (*A*) or $G\beta_3$ (*B*) or with 100 ng/μl $G\beta_1$ plus 100 ng/μl $G\beta_3$ DNAs (*C*). Same experimental conditions as in Figure 3. *D*, Mean facilitation ratios for these cells. * indicates data significantly different from control. ** indicates data for $G\beta_1$ plus $G\beta_3$ significantly different from control, $G\beta_1$, or $G\beta_3$.

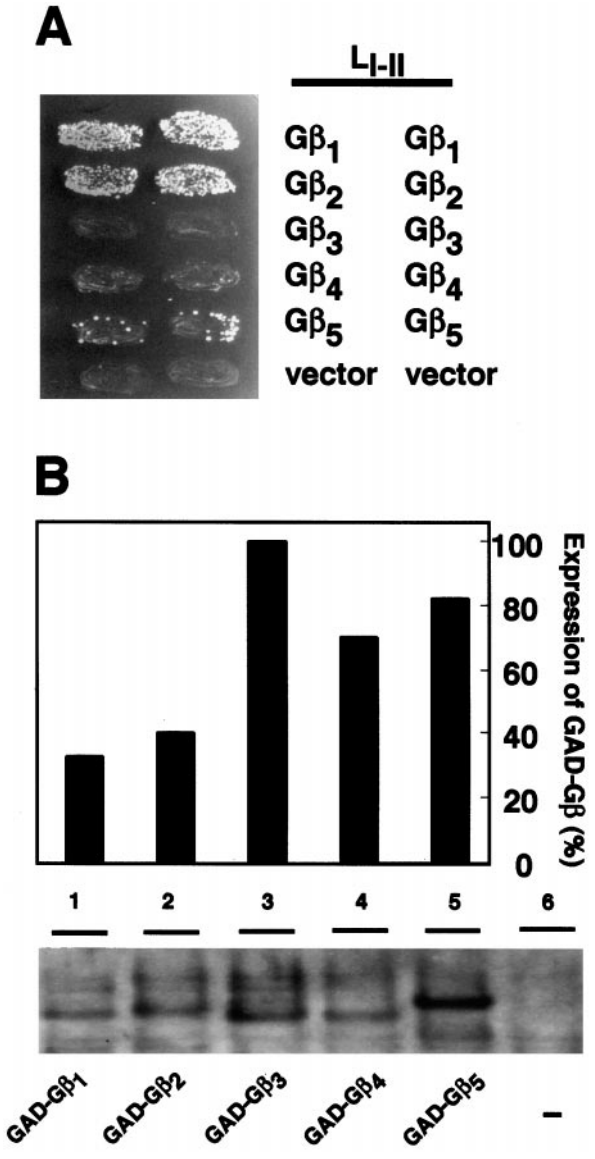

Figure 6. $G\beta_1$ and $G\beta_2$ interact strongly with the α_1 subunit of N-type calcium channels. *A*, Yeast reporter strain PJ69–4A was cotransformed with plasmids encoding GBD-L_{I-II} and the indicated GAD-Gβ subunits. Cells were first plated onto synthetic sucrose medium SS/−Trp/−Leu to select cotransformants. The cotransformants were then replica-plated onto medium SS/−Trp/−Leu/−Ade/−His to select for colonies having successful GBD–GAD interactions. *B*, Expression of GAD-Gβ hybrid proteins in yeast. Yeast lysates were subjected to SDS–PAGE and immunoblotting. The blot was probed with anti-GAD monoclonal antibody and horseradish peroxidase-conjugated to anti-mouse IgG. The proteins were detected by ECL. Yeast lysate without GAD hybrid proteins was loaded in *lane 6* as a negative control. Total protein amounts in the 20 μl lysates quantified by the Bradford assay (Bio-Rad, Hercules, CA) were 1.94, 1.30, 0.72, 0.80, and 1.70 μg in *lanes 1–5*, respectively. Intensity of individual GAD-Gβ bands on the immunoblot was determined by densitometry (Molecular Dynamics, Sunnyvale, CA), normalized to the protein amount in their respective lysates, and plotted as a percent of GAD-Gβ3 to show the relative levels of these hybrid proteins.

between β_1 and β_5; Bayewitch, 1998), activation of MAP kinase (β_1 more efficient than β_5; Zhang et al., 1996b), activation of phospholipase β_2 (no difference between β_1 and β_5; Zhang et al., 1996b), and binding to G-protein receptor kinases (GRKs) (β_1 and β_2, but not β_3, bind to GRK2, and β_1, β_2, and β_3 all bind to GRK3; Daaka, 1997). Thus, in the case of classical G-protein effectors (adenylyl cyclase, phospholipase, and ion conducting channels) specificity of interactions with G-protein β subunit types has previously been noted only in β_5 compared with β_1. However, this is not entirely surprising considering that β_5 is a unique β subunit type that is only 53% identical to the rest and is primarily expressed in brain (Watson et al., 1994). The differential activity of β_1 and β_2 subunits compared with the β_3 and β_4 subunits seen here on Ca^{2+} channels is of interest, because these β subunit types are very similar. In fact, β_1 and β_2 are more similar to β_4 (~90% identity) than β_3 to β_4 (~80% identity) (Yan et al., 1996). The functional differences must, however, be caused by structural difference(s) between the two pairs of proteins. A scan of the sequences to identify residues common to $G\beta_1$ and β_2, but different in β_3 and β_4, yields seven residues: R19, S31, N35, P39, A193, R197, and A305, using $G\beta_1$ numbering. Most of these residues are near regions thought to interact with the Gγ subunit. The molecular basis for the functional difference between β_1/β_2 versus β_3/β_4 may not be restricted to these residues, because β_3 and β_4 diverge individually with respect to the each other, as well as β_1 and β_2 subtypes at many positions in their amino acid sequences. This divergence is also consistent with the differential effects of β_3 compared with β_4 on Ca^{2+} channel properties (Fig. 4). A recent major study by Ford et al. (1998) has looked for residues in $G\beta_1$ that affect interaction with five different effectors, including N-type calcium channels containing α_{1B} subunits. They mutated 15 residues within the interaction domain for Gβ subunits with Gα subunits and found eight that affected the calcium

channel facilitation. However, because all of the 15 residues tested are identical among $G\beta_1$, $G\beta_2$, $G\beta_3$, and $G\beta_4$, these results do not help to explain why two of the subunits are so efficacious and two of them are ineffective on N-type calcium channels.

As mentioned before, the $G\beta_5$ subunit stands out among $G\beta$ subunits. Unlike the others, it is reported to couple almost exclusively to the $G\alpha_q$ subunit in a manner that can prevent the others from binding (Fletcher et al., 1998). In our experiments, injection of the vector encoding $G\beta_5$ produced a small but reliable voltage-dependent inhibition of the Ca^{2+} current and partial occlusion of NE-induced inhibition (Fig. 4). Using a $G\beta_5$ antibody (provided by M. Simon, Caltech) (Watson et al., 1994), we found that SCG neurons express $G\beta_5$ (data not shown), as anticipated from its enrichment in brain, and therefore stimulation of $G\alpha_q$ via m_1 muscarinic or angiotensin II receptors in SCG cells should release $G\beta_5$ subunits. Together, these three results suggest that the small but fast component of calcium channel inhibition seen with receptors linked to $G\alpha_q$ in SCG neurons (such as m_1 muscarinic receptors) (Zhou et al., 1997) could be attributable to $G\beta_5$.

The putative sites of interaction of the G-protein β subunit with the calcium channel have been controversial (e.g., Dolphin, 1998). Typically, experiments examining this interaction have relied on overexpression of appropriately engineered calcium channels in cell lines or *Xenopus* oocytes or on the demonstration of protein–protein interactions with recombinant proteins *in vitro*. We have taken a complementary approach with the two-hybrid system, and our results strongly support the hypothesis that the interaction of Gβ subunits with the linker between homologous domains I and II of the α_1 subunit determines the subunit specificity for voltage-dependent inhibition of N-type calcium channels by G-protein βγ subunits. Although it is likely that G-protein β subunits interact with other regions of the calcium channel, further experimentation will be necessary to determine whether these additional interactions contribute to the voltage-dependent inhibition.

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