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G-Protein-Dependent Facilitation of Neuronal α_{1A}, α_{1B}, and α_{1E} Ca Channels

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Modulation of neuronal voltage-gated Ca channels has important implications for synaptic function. To investigate the mechanisms of Ca channel modulation, we compared the G-protein-dependent facilitation of three neuronal Ca channels. α_{1A}, α_{1B}, or α_{1E} subunits were transiently coexpressed with α_2–δ_b and β_3 subunits in HEK293 cells, and whole-cell currents were recorded. After intracellular dialysis with GTPγS, strongly depolarized conditioning pulses facilitated currents mediated by each Ca channel type. The magnitude of facilitation depended on current density, with low-density currents being most strongly facilitated and high-density currents often lacking facilitation. Facilitating depolarizations speeded channel activation ~1.7-fold for α_{1A} and α_{1B} and increased current amplitudes by the same proportion, demonstrating equivalent facilitation of G-protein-inhibited α_{1A} and α_{1B} channels. Inactivation typically obscured facilitation of α_{1E} current amplitudes, but the activation kinetics of α_{1E} currents showed consistent and pronounced G-protein-dependent facilitation. The onset and decay of facilitation had the same kinetics for α_{1A}, α_{1B}, and α_{1E}, suggesting that Gβγ dimers dissociate from and reassociate with these Ca channels at very similar rates. To investigate the structural basis for N-type Ca channel modulation, we expressed a mutant of α_{1B} missing large segments of the II–III loop and C terminus. This deletion mutant exhibited undiminished G-protein-dependent facilitation, demonstrating that a Gβγ interaction site recently identified within the C terminus of α_{1E} is not required for modulation of α_{1B}.

Key words: Ca channel modulation; neuronal Ca channels; membrane-delimited pathway; G-protein-dependent Ca channel inhibition; presynaptic inhibition; signal transduction; neuronal integration; neuronal plasticity; molecular neuroscience; facilitation; α_{1A}; α_{1B}; α_{1C}; α_{1E}; neurosecretion; electrical excitability

Voltage-gated Ca channels play essential roles in neurosecretion and other neuronal functions (Dunlap et al., 1995). At least six different classes of Ca channel α_1 subunits (α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, and α_{1G}) are expressed in neurons, in which they contribute to the formation of native P/Q-, N-, L-, R-, and T-type Ca channels, respectively (Hofmann et al., 1994; Perez-Reyes et al., 1998). The activities of N-type and P/Q-type channels are known to be modulated by G-protein-dependent pathways (Elmslie et al., 1990; Sah, 1990; Bernheim et al., 1991; Mintz and Bean, 1993), and such modulation is likely to have considerable physiological importance (cf. Kavalali et al., 1997; Koh and Hille, 1997; Wu and Saggau, 1997).

Previous studies in neurons have identified five G-protein-dependent pathways for N-type Ca channel inhibition (Hille, 1994). One pathway is membrane-delimited and may involve only Ca channels, heterotrimeric G-proteins, and neurotransmitter–hormone receptors. Ca channels inhibited via this pathway exhibit positive shifts in the voltage dependence of activation, slowed activation kinetics, and reduced macroscopic current amplitudes; such channels are described as being "reluctant" to open (Bean, 1989). Reluctant channels can be transiently reconverted into "willing" channels by strong or sustained depolarization (Bean, 1989; Elmslie et al., 1990; Ikeda, 1991); this reconversion is known as facilitation.

G-protein-dependent modulation has been extensively studied for native N-type Ca channels (cf. Jones and Elmslie, 1997), and modulation of cloned α_{1A} and α_{1B} Ca channels has been reconstituted in expression systems (Zhou et al., 1995; Zong et al., 1995; Patil et al., 1996; Brody et al., 1997; Herlitze et al., 1997; Page et al., 1997). Interestingly, when neurotransmitter receptors are used to activate G-proteins in a phasic manner, α_{1B} channels are more strongly inhibited and more strongly facilitated than α_{1A} channels (Zhang et al., 1996; Zamponi et al., 1997). To further examine the relative sensitivities of α_{1A} and α_{1B} to G-protein-mediated inhibition, we have compared modulation of these channels by G-proteins tonically activated with GTPγS. Under these conditions, α_{1A} and α_{1B} display very similar magnitudes and kinetics of facilitation, suggesting that other factors in addition to channel primary structure may influence Ca channel–G-protein interactions.

Membrane-delimited Ca channel modulation appears to be effected by Gβγ rather than Gα subunits (Herlitze et al., 1996; Ikeda, 1996; Shekter et al., 1997). It has been proposed that direct interaction with Gβγ occurs at the cytoplasmic I–II loop (De Waard et al., 1997; Zamponi et al., 1997), the C terminus (Qin et al., 1997), or a combination of the first transmembrane domain and the C terminus of the Ca channel α_1 subunit (Zhang et al., 1996; Page et al., 1997). To investigate this issue, we have further studied facilitation of a deletion mutant of α_{1B}. This N-type Ca channel, which lacks large segments of the II–III loop and C terminus, exhibits undiminished G-protein-dependent facilita-

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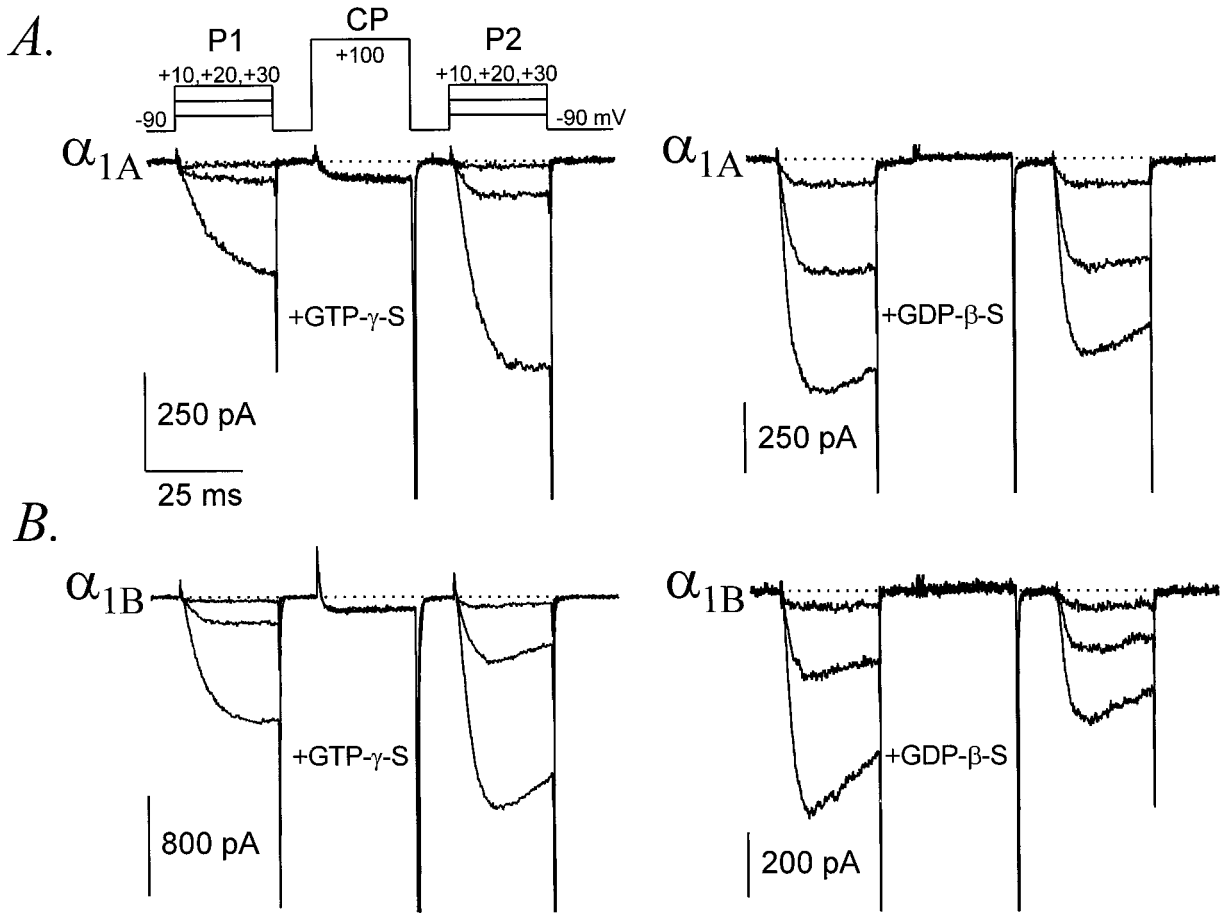

Figure 1. Representative whole-cell Ca currents recorded from HEK293 cells expressing α_{1A} or α_{1B} channels, illustrating G-protein-dependent facilitation. Currents were recorded after ≥5 min of intracellular dialysis with GTPγS or GDPβS, as indicated. The voltage protocol is diagrammed at *top left*. *P1*, *P2*, and *CP* were each 25 msec in duration and were separated by 10 msec intervals. *A*, α_{1A} with GTPγS, data file 97425003; C = 20 pF; R_S = 2.7 MΩ. α_{1A} with GDPβS, data file 97D24008; C = 18 pF; R_S = 3.2 MΩ. *B*, α_{1B} with GTPγS, data file 97403026; C = 16 pF; R_S = 4.8 MΩ. α_{1B} with GDPβS, data file 97D19038; C = 23 pF; R_S = 2.0 MΩ.

tion, demonstrating that a Gβγ interaction site recently identified within the C terminus of α_{1E} (Qin et al., 1997) is not essential for modulation of α_{1B}.

MATERIALS AND METHODS

Cell culture and transfection. Human embryonic kidney (HEK293) cells (CRL 1573) were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium contained 90% DMEM (Life Technologies, Gaithersburg, MD; catalog #11995-065), 10% heat-inactivated horse serum (Life Technologies; catalog #26050-13), and 50 μg/ml gentamicin (Life Technologies; catalog #15710-015). Every 2–3 d, the cells were briefly trypsinized and replated at fourfold lower density. At the time of replating, 35 mm culture dishes (Falcon; catalog #3002) were seeded with ~10^3 cells per dish. Approximately 16 hr later, these cells were transfected using the Ca-PO_4 precipitation technique (Pharmacia, Piscataway, NJ; Cell Phect Kit) with expression plasmids encoding α_{1A} (rabbit brain; Mori et al., 1991), α_{1B} (rabbit brain; Fujita et al., 1993), α_{1C} (rabbit heart; Mikami et al., 1989), or α_{1E} (BII-2, rabbit brain; Niidome et al., 1992) at 1 μg of each cDNA per dish. Cells were simultaneously cotransfected with expression plasmids encoding α_2-δ_b (rat brain; Kim et al., 1992) and β_3 (rabbit brain; Witcher et al., 1993) at 1 μg of each cDNA per dish and also with plasmid EBO-pCD-Leu2 encoding human CD8 protein (ATCC; catalog #59565) at 0.2 μg/dish. Cells expressing CD8 were visually identified by their ability to bind 4.5 μm diameter paramagnetic beads coated with anti-CD8 antibody (Dynal, Great Neck, NY). Decorated cells were selected for electrophysiological analysis (Jurman et al., 1994).

Expression plasmids. The amino acid compositions and construction of expression plasmids encoding α_{1A}, α_{1B}, and α_{1C} have been described previously (Tanabe et al., 1990; Fujita et al., 1993; Adams et al., 1994). cDNAs encoding these α_1 subunits were in the expression vector pKCRH2 (Mishina et al., 1984). The entire coding sequence of α_{1E} was excised from pSPCBII-2 (Wakamori et al., 1994) using *Hin*dIII and *Eco*RI; the resulting ~7.3 kb fragment was ligated into the corresponding sites of pcDNA3.1$^+$ (Invitrogen, San Diego, CA). The construction of pKCRBIII-DD, encoding a double-deletion mutant of α_{1B} ($\alpha_{1B\text{-}DD}$), has been previously described (Zhou et al., 1995). $\alpha_{1B\text{-}DD}$ is missing amino acid residues 829–995 and 1877–2338 from the II–III loop and C terminus, respectively. The cDNA encoding α_2-δ_b (Kim et al., 1992) was in pMT2. The cDNA encoding β_3 was in pcDNA3.

Electrophysiology. Large-bore pipettes were pulled from 100 μl borosilicate micropipettes (VWR Scientific; catalog #53432-921) and filled with a solution containing (in mM:) 155 CsCl, 10 Cs_2 EGTA, 4 Mg ATP, and 10 HEPES, pH 7.4, with CsOH. The pipette solution also contained Li-GTPγS (0.32 mM) or Li-GDPβS (0.30 mM) as noted. Aliquots of pipette solutions were stored at −80°C and kept on ice after thawing. Pipette solutions were filtered at 0.22 μm immediately before use. Pipette tips were coated with paraffin to reduce capacitance and then fire-polished; filled pipettes had DC resistances of 1.0–1.5 MΩ. The bath solution contained (in mM:) 145 NaCl, 40 $CaCl_2$, and 10 HEPES, pH 7.4, with NaOH. Residual pipette capacitance was compensated in the cell-attached configuration using the negative capacitance circuit of the Axopatch 200A amplifier. No corrections were made for liquid junction potentials. Temperature (20–23°C) was continuously monitored using a miniature thermocouple placed in the bath.

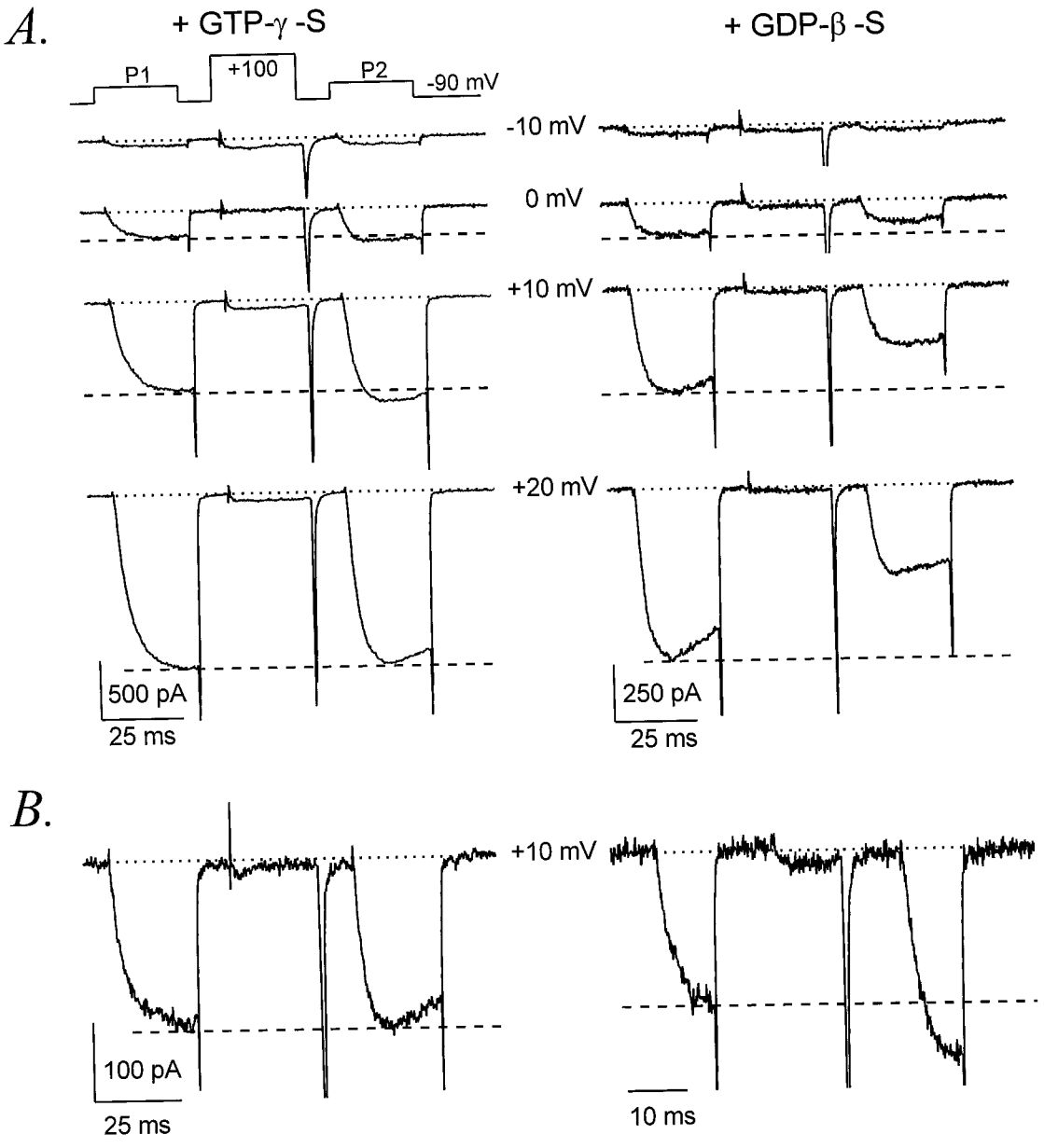

Figure 2. G-protein-dependent facilitation of α_{1E}. *A*, Facilitation of α_{1E} current amplitudes and activation kinetics in a cell dialyzed with GTPγS (*left*) but not in a cell dialyzed with GDPβS (*right*). Voltage protocol as in Figure 1. *Left*, Data file 98403058; C = 32 pF; R_S = 2.8 MΩ. *Right*, Data file 97602030; C = 31 pF; R_S = 2.4 MΩ. *B*, Facilitation of α_{1E} is greatly enhanced by shortening the conditioning and test pulses. *Left*, α_{1E} currents evoked by the standard voltage protocol in which P1, P2, and CP were each 25 msec in duration. Data file 98406202; C = 17 pF; R_S = 3.5 MΩ. *Right*, α_{1E} currents evoked in the same cell by a briefer protocol to the same voltages but with P1 and P2 reduced to 10 msec and CP reduced to 12 msec in duration. Data file 98406204; C = 17 pF; R_S = 3.5 MΩ.

Ca currents were recorded using the whole-cell patch-clamp technique (Hamill et al., 1981). The steady holding potential was normally −90 mV. In all experiments involving GTPγS, cells were dialyzed for ≥5 min before studying G-protein-dependent effects. Currents were filtered at 2–10 kHz using the built-in Bessel filter (four-pole low-pass) of the Axopatch 200A amplifier and sampled at 10–50 kHz using a Digidata 1200 analog-to-digital board installed in a Gateway 486-66V computer. The pCLAMP software programs Clampex and Clampfit (version 6.0.3) were used for data acquisition and analysis, respectively. Figures were made using Origin (version 4.1).

Linear cell capacitance (C) was determined by integrating the area under the whole-cell capacity transient, evoked by clamping from −90 to −80 mV with the whole-cell capacitance compensation circuit of the Axopatch 200A turned off. The average value of C was 22 ± 1 pF (n = 155 cells). Series resistance (R_S) was calculated as $(1/C) \times \tau$, where τ is the time constant for decay of the whole-cell capacity transient. Cells exhibiting more than one τ were rejected. Because pipette resistances and cell capacitances were relatively small, τ was usually <100 μsec, and R_S was <5 MΩ without using the series resistance compensation circuit of the amplifier; when required, this circuit was used to reduce τ and R_S by 30–80%. The average values of τ and R_S in the reported experiments (n = 155) were 71 ± 4 μsec and 3.3 ± 0.1 MΩ, respectively. Because maximal Ca currents were typically <1 nA, voltage errors were usually <5 mV. The DC resistance of the whole-cell configuration was routinely >1 GΩ. All illustrated and analyzed currents have been corrected for linear capacitance and leakage currents using the −P/6 method. Current densities (expressed in picoamperes per picofarad) were calculated as peak Ca current divided by C. Time constants for activation of Ca currents were estimated by fitting the activating phase of currents with a single exponential function.

A standard "facilitation" voltage protocol was used, consisting of two identical test pulses (P1 and P2) separated by a conditioning pulse (CP) to +100 mV (Fig. 1). Unless otherwise noted P1, P2, and CP were each

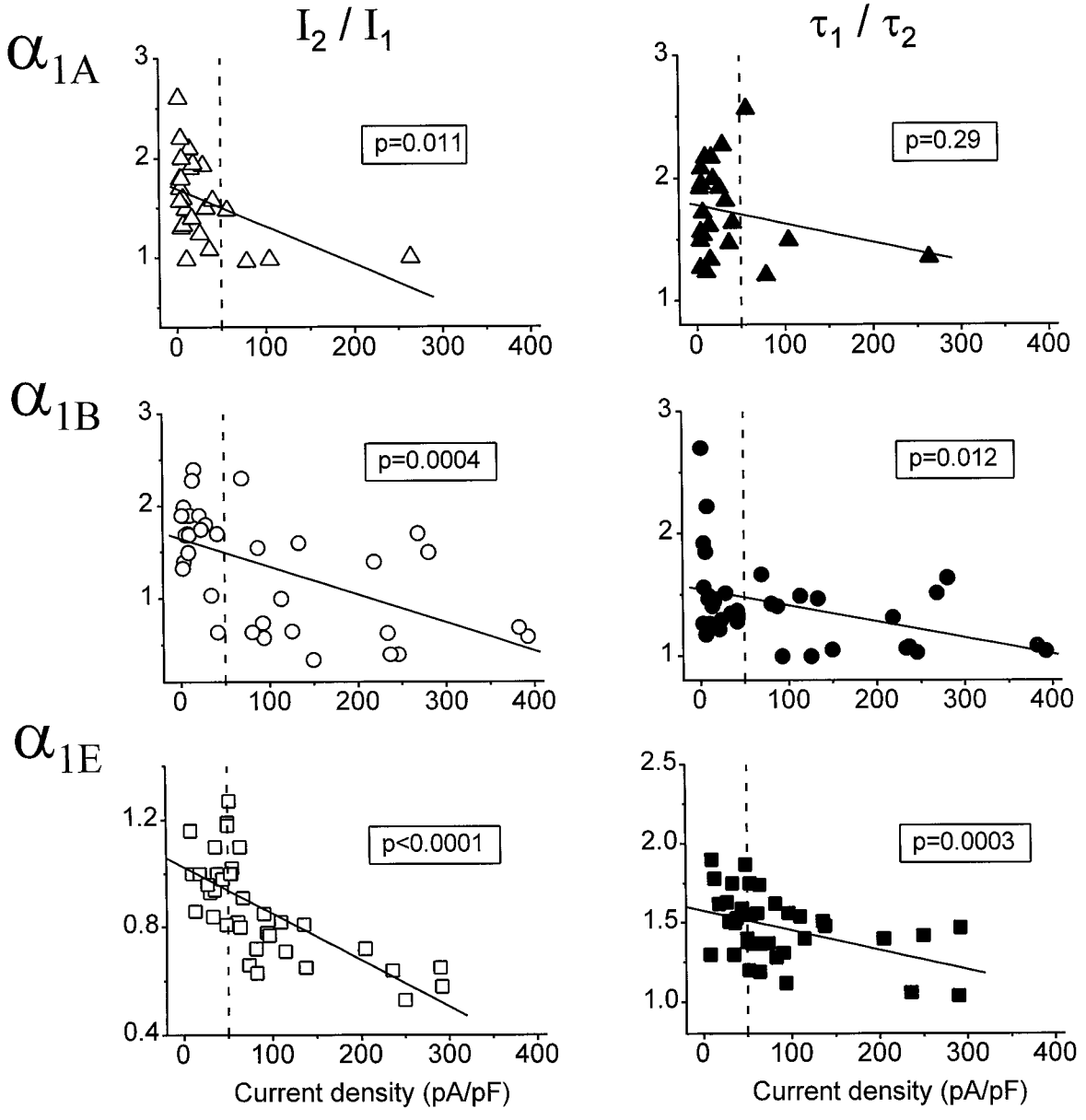

Figure 3. The magnitude of facilitation is negatively correlated with current density. The ratio I_2/I_1 (*left panels*) or τ_1/τ_2 (*right panels*) is plotted as a function of maximal Ca current density for cells expressing α_{1A} (n = 26), α_{1B} (n = 35), or α_{1E} (n = 37). I_1 and I_2 are the amplitudes measured at the time of peak inward Ca current evoked by P1 and P2, respectively, of the standard voltage protocol (Fig. 1). P1 and P2 were to +30 mV (for α_{1A} and α_{1B}) or +10 mV (for α_{1E}); facilitation was maximal at these voltages (Figs. 4–6). The pipette solution contained GTPγS. For the plots shown, current densities were determined within 60 sec of establishing the whole-cell configuration, and facilitation was calculated for currents recorded after ≥5 min of whole-cell dialysis. The *lines* are linear regressions; the p value listed in each plot indicates the statistical significance of the correlation coefficient. When current densities determined after >5 min of whole-cell dialysis were used as the independent variable, the p values were 0.0008, 0.0001, and 0.0003 for I_2/I_1 ratios and 0.27, 0.012, and 0.036 for τ_1/τ_2 ratios of α_{1A}, α_{1B}, and α_{1E}, respectively. All subsequent comparisons of α_{1A}, α_{1B}, and α_{1E} used only currents having initial densities of ≤50 pA/pF (*dashed vertical line*).

25 msec long and separated by 10 msec repolarizations to −90 mV. This voltage protocol induced maximal facilitation (see Fig. 9). Successive episodes of the voltage protocol were separated by 10 sec intervals.

Statistical analysis. Groups of data were compared using one-way ANOVA or a two-tailed, unpaired t test, as appropriate. Averaged data are presented in the text and figures as mean ± SEM.

RESULTS

Facilitation of α_{1A} and α_{1B}

Figure 1 illustrates whole-cell Ca currents mediated by α_{1A} or α_{1B} Ca channels coexpressed with α_2–δ_b and β_3 subunits in HEK 293 cells. After dialyzing cells with GTPγS for several minutes, α_{1A} and α_{1B} currents exhibited slowed activation and reduced amplitudes, reflecting inhibition of the underlying Ca channels through a G-protein-dependent pathway. As expected, the inhibited α_{1A} or α_{1B} channels could be facilitated by a conditioning depolarization. Less pronounced facilitation was also observed with GTP instead of GTPγS in the pipette solution (data not shown). In contrast, facilitation was absent from cells dialyzed with GDPβS.

Ca current amplitudes decreased during dialysis with GTPγS. After ≥ 5 min of whole-cell recording, α_{1A} currents had decreased to 59 ± 7% (n = 26 cells) of the initial amplitude (recorded within 60 sec of establishing the whole-cell configura-

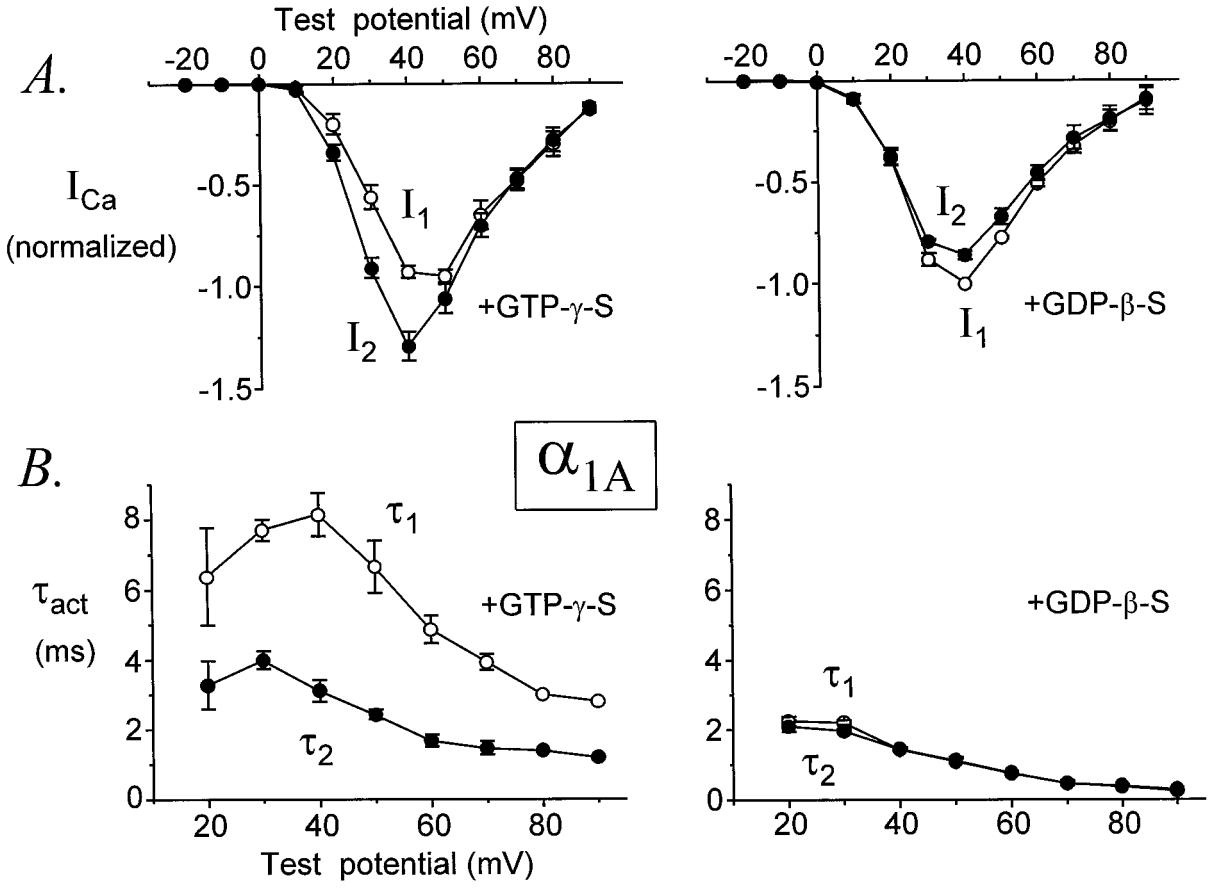

Figure 4. Voltage dependence of facilitation for α_{1A}. *A,* Current–voltage relationships with GTPγS or GDPβS in the pipette solution. I_1 and I_2 were normalized to the maximal I_1 recorded in each cell and then averaged. *B*, Average values of τ_1 and τ_2; the time constants for activation of I_1 and I_2, respectively, are plotted as a function of test potential. Data are from seven (GTPγS) and four (GDPβS) cells. Voltage protocol as in Figure 1.

tion), and α_{1B} currents had decreased to 56 ± 6% (n = 35). These decreases probably reflect the onset of G-protein-dependent inhibition combined with Ca channel run-down. By way of contrast, during ≥5 min dialysis with GDPβS, the amplitudes of α_{1A} currents increased to 122 ± 7% (n = 9), and those of α_{1B} currents increased to 149 ± 7% (n = 10) of initial amplitudes. These increases likely result from Ca current run-up combined with removal of preexisting G-protein-dependent inhibition.

Facilitation of α_{1E}

It is now well established that α_{1A} and α_{1B} are inhibited through G-protein-dependent pathways (Herlitze et al., 1996; Toth et al., 1996; Zhang et al., 1996; Zamponi et al., 1997). In contrast, whether α_{1E} is also modulated by the same pathways has been unclear. Some previous studies have concluded that α_{1E} is inhibited by G-proteins (Yassin et al., 1996; Mehrke et al., 1997; Qin et al., 1997; Shekter et al., 1997), whereas others have concluded that it is insensitive to G-protein inhibition (Bourinet et al., 1996; Toth et al., 1996; Page et al., 1997). To examine this issue further, we studied facilitation of α_{1E} under the same experimental conditions as α_{1A} and α_{1B}.

Dialysis with GTPγS decreased the amplitude of α_{1E} currents to 74 ± 1% (n = 18) of initial levels, suggesting the development of G-protein-mediated inhibition. Consistent with this interpretation, dialysis with GDPβS increased α_{1E} current amplitudes to 151 ± 12% of initial levels (n = 7). α_{1E} currents exhibited significant kinetic slowing (Diverse-Peirluissi et al., 1995), activating at +10 mV with an average time constant (τ_1) of 4.4 ± 0.3 msec in the presence of intracellular GTPγS (n = 18) compared with 2.9 ± 0.5 msec (n = 7) with internal GDPβS. As illustrated in Figure 2, kinetic slowing of α_{1E} currents could be reversed by a conditioning depolarization, consistent with inhibition of α_{1E} channels through a membrane-delimited pathway. Using the standard voltage protocol (as in Fig. 1), we observed a slight facilitation of α_{1E} current amplitudes in some cells; one example is illustrated in Figure 2*A*. However, in most cells α_{1E} current amplitudes were not facilitated. In contrast, nearly all cells dialyzed with GTPγS exhibited significant facilitation of activation kinetics. Facilitation of α_{1E} apparently requires G-protein activation, because it was absent from cells dialyzed with GDPβS.

We also observed that pronounced facilitation of α_{1E} current amplitudes could be produced by shortening the durations of the test and conditioning pulses (Fig. 2*B*). This observation suggests that inactivation of α_{1E} channels in response to the standard voltage protocol usually obscured facilitation of macroscopic current amplitudes.

Facilitation is correlated with current density

Cells transfected with α_{1A}, α_{1B}, or α_{1E} expressed a wide range of Ca current densities (from unmeasurable to ~400 pA/pF). To examine whether the expression level of Ca channels might influence their modulation by G-proteins, we plotted the magnitude of facilitation as a function of the maximal current density in each cell (Fig. 3). Facilitation was quantified as the ratio I_2/I_1, in which

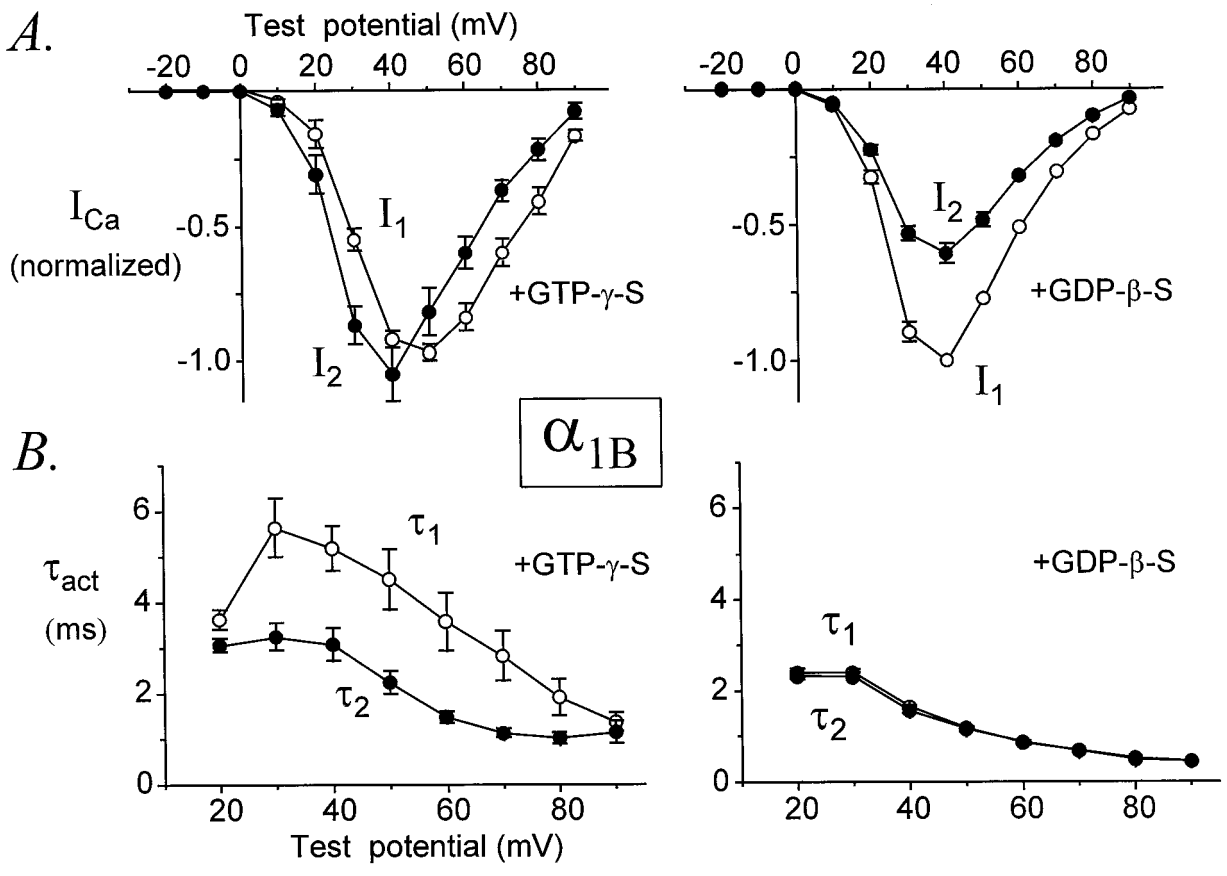

Figure 5. Voltage dependence of facilitation for α_{1B}. Data from six (GTPγS) and four (GDPβS) cells. Legend otherwise as in Figure 4.

I_2 is the peak current evoked by P2, and I_1 is the peak current evoked by P1 of the standard voltage protocol (Fig. 1). As an additional measure of facilitation we used the ratio τ_1/τ_2, where τ_1 is the time constant for activation of I_1, and τ_2 is the time constant for activation of I_2. The plots in Figure 3 reveal that facilitation of α_{1A}, α_{1B}, and α_{1E} is negatively correlated with current density. Thus, low-density currents exhibited the most facilitation and high-density currents exhibited the least facilitation.

Voltage dependence of facilitation

We next compared the voltage dependence of facilitation for α_{1A}, α_{1B}, and α_{1E} Ca channels. To minimize variability attributable to differences in channel density, we restricted our analysis throughout this study to data from cells expressing α_{1A}, α_{1B}, or α_{1E} currents at initial densities of ≤50 pA/pF (Fig. 3, *vertical dashed lines*). As shown in Figure 4*A*, in cells dialyzed with GTPγS, the amplitudes of currents mediated by α_{1A} were significantly facilitated (i.e., I_2 exceeded I_1) at test potentials of +20, +30, +40, and +50 mV, whereas at more positive test potentials I_2 and I_1 were equal. In contrast, the activation rates of α_{1A} currents were facilitated at all test potentials from +20 to +90 mV (Fig. 4*B*). Thus, τ_2 was significantly smaller than τ_1 over the entire range of test potentials at which these time constants could be reliably determined. For comparison, in cells dialyzed with GDPβS no facilitation of current amplitudes or activation kinetics was observed at any test potential (Fig. 4).

Similar results were obtained for α_{1B} (Fig. 5). However, a notable difference was that α_{1B} current amplitudes were facilitated over a smaller range of test potentials (+20, +30, and +40 mV) than were found for α_{1A}. Thus, I_2 was smaller than I_1 at voltages above +40 mV, presumably because of inactivation of α_{1B} channels in response to the standard voltage protocol. In contrast, the activation rates of α_{1B} currents were facilitated at all test potentials from +20 to +80 mV. Thus, for both α_{1A} and α_{1B} the activation rates of currents were facilitated over a much wider range of test potentials than were current amplitudes.

Using the standard voltage protocol, current amplitudes were facilitated in only ~40% (7 of 18) of cells expressing α_{1E} at initial current densities ≤50 pA/pF. Consequently, the average values of I_2 did not exceed those of I_1 (Fig. 6*A*). Nonetheless, the average amplitudes of α_{1E} currents clearly indicated the presence of G-protein-dependent modulation, because there was a much greater difference between I_2 and I_1 in cells dialyzed with GDPβS than in cells dialyzed with GTPγS (Fig. 6*A*). Furthermore, cells dialyzed with GTPγS exhibited kinetic slowing that was almost completely reversed the conditioning pulse (Fig. 6*B*). In contrast, kinetic slowing and facilitation were absent from cells dialyzed with GDPβS. Taken together with results presented in Figure 2, these data demonstrate G-protein-dependent inhibition and facilitation of α_{1E} Ca channels.

No facilitation of α_{1C}

In contrast to α_{1A}, α_{1B}, and α_{1E} subunits, the cardiac α_{1C} subunit was not affected by G-protein activation. In cells dialyzed with GTPγS, I_2 was consistently smaller than I_1, presumably because of Ca-dependent inactivation of α_{1C} (Fig. 7). Also in contrast to α_{1A}, α_{1B}, and α_{1E}, the voltage dependences

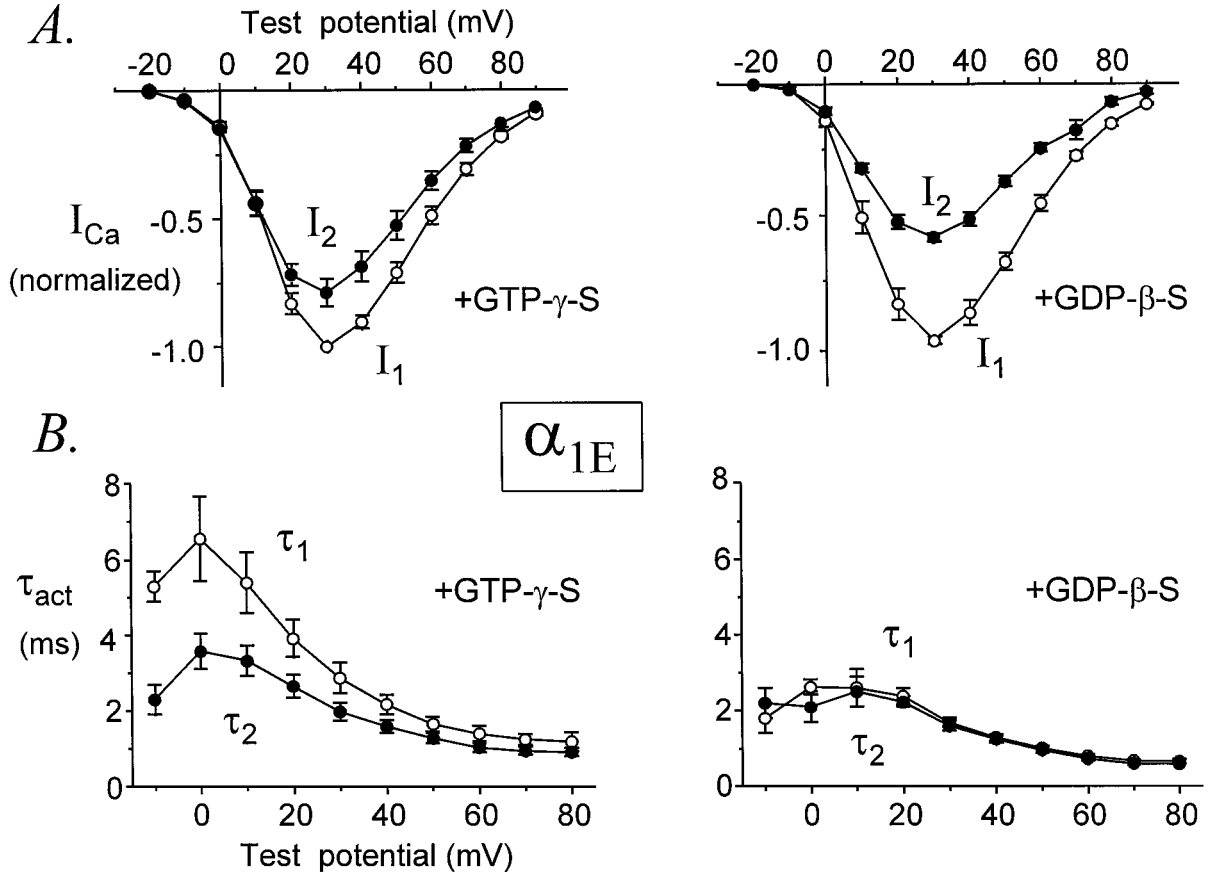

Figure 6. Voltage dependence of facilitation for α_{1E}. Data from eight (GTPγS) and six (GDPβS) cells. Legend otherwise as in Figure 4.

of I_1 and I_2 were not appreciably different for α_{1C} (Fig. 7*B*). Neither was activation of α_{1C} currents speeded by a conditioning depolarization (Fig. 7*C*). Furthermore, the amplitudes of α_{1C} currents decreased less than α_{1A} and α_{1B} currents during dialysis with GTPγS (to 82 ± 8% of initial levels; $n = 11$), and, unlike α_{1A}, α_{1B}, and α_{1E}, the amplitudes of α_{1C} currents did not increase significantly during dialysis with GDPβS (104 ± 5% of control, $n = 4$). In summary, we were unable to detect any significant differences between α_{1C} currents recorded with GTPγS and those recorded with GDPβS in the pipette solution. These results are consistent with previous studies (Bourinet et al., 1996; Toth et al., 1996; Zhang et al., 1996) reporting that α_{1C} is not inhibited by G-proteins.

α_{1A} and α_{1B} are facilitated to similar degrees

Previous studies have concluded that α_{1B} is more strongly inhibited than α_{1A} through G-protein-dependent pathways and also that G-protein-inhibited α_{1B} channels are more strongly facilitated by a conditioning depolarization than α_{1A} channels (Bourinet et al., 1996; Zhang et al., 1996; Zamponi et al., 1997). These studies have used neurotransmitter receptors to induce the phasic activation of G-proteins. To examine whether α_{1B} is also more strongly facilitated in the presence of tonically activated G-proteins, we compared α_{1A} and α_{1B} currents in cells dialyzed with GTPγS. As shown in Figure 8*A*, the average I_2/I_1 ratios of α_{1A} and α_{1B} currents were identical, demonstrating that the amplitudes of α_{1A} and α_{1B} currents were facilitated to the same extent. Control experiments with intracellular GDPβS produced smaller I_2/I_1 ratios for α_{1B} (0.63 ± 0.03; $n = 10$) than for α_{1A} (0.95 ± 0.02; $n = 9$), suggesting that the voltage protocol caused greater inactivation of unmodulated α_{1B} channels than of unmodulated α_{1A} channels.

Figure 8*B* compares the facilitation of α_{1A} and α_{1B} activation kinetics. The average τ_1/τ_2 ratios of α_{1A} and α_{1B} currents were not significantly different, indicating that the conditioning pulse speeded activation of α_{1A} and α_{1B} to the same degree. Thus, once α_{1A} and α_{1B} channels have been inhibited by tonically activated G-proteins, they are equally facilitated by a conditioning depolarization.

Figure 8 also presents data for α_{1E}. With intracellular GTPγS, the average I_2/I_1 ratio for α_{1E} was 1.03 ± 0.03 ($n = 18$), whereas with intracellular GDPβS this ratio was only 0.61 ± 0.06 ($n = 7$), indicating a significant ($p < 0.001$) G-protein-dependent effect. Further evidence of modulation was provided by the substantial facilitation of α_{1E} activation kinetics. In fact, the average τ_1/τ_2 ratios for α_{1E} currents were statistically indistinguishable from those of α_{1A} and α_{1B} currents (Fig. 8*B*). Thus, with intracellular GTPγS these τ_1/τ_2 ratios were 1.79 ± 0.09 ($n = 21$), 1.60 ± 0.09 ($n = 19$), and 1.55 ± 0.05 ($n = 8$) for α_{1A}, α_{1B}, and α_{1E}, respectively ($p = 0.07$). With intracellular GDPβS, these ratios were 1.09 ± 0.03 ($n = 8$), 1.03 ± 0.01 ($n = 9$), and 1.04 ± 0.03 ($n = 7$), respectively ($p = 0.17$). These comparisons further establish the ability of α_{1E} to be modulated through a G-protein-dependent, presumably membrane-delimited pathway.

The kinetics of facilitation are very similar for α_{1A}, α_{1B}, and α_{1E}

Facilitation is thought to reflect dissociation of Gβγ subunits from Ca channels. Facilitation is transient and decays with time after a conditioning depolarization because Gβγ subunits rebind

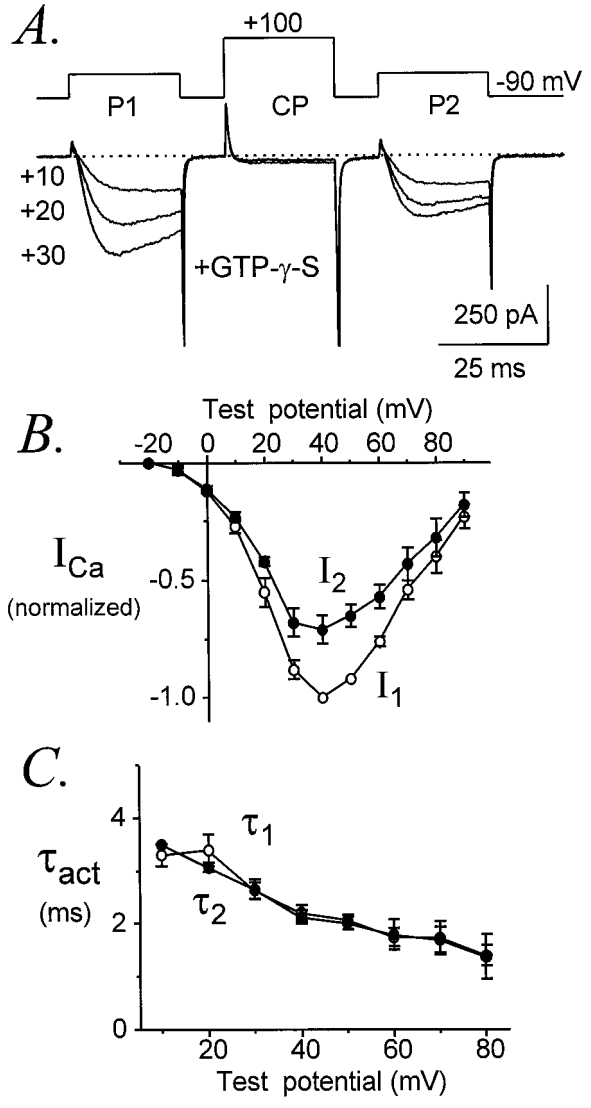

Figure 7. Absence of G-protein-dependent facilitation of α_{1C}. *A*, Representative α_{1C} currents recorded from a cell dialyzed with GTPγS. Data file 97512011; C = 12 pF; R_S = 3.5 MΩ. *B*, Average voltage dependence of I_1 and I_2; data from three cells dialyzed with GTPγS. I_1 and I_2 were normalized by the maximal I_1 in each cell. *C*, Average voltage dependence of τ_1 and τ_2; data from three cells dialyzed with GTPγS. Voltage protocol as in Figure 1.

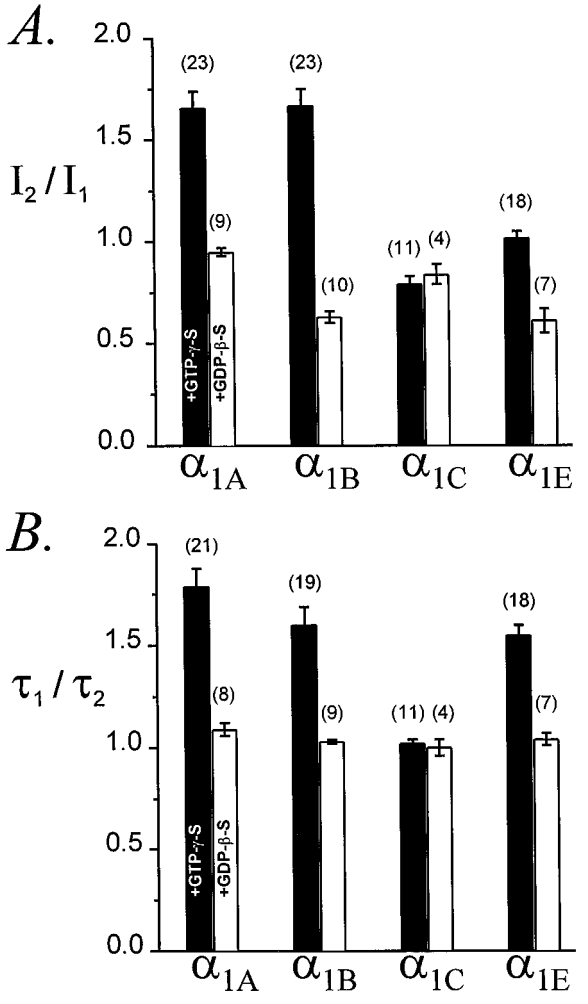

Figure 8. Comparative facilitation of α_{1A}, α_{1B}, α_{1C}, and α_{1E} Ca channels. *A*, Average I_2/I_1 ratios for currents recorded with GTPγS (*filled bars*) or GDPβS (*unfilled bars*) in the pipette. Voltage protocol as in Figure 1. P1 and P2 were to +30 mV (α_{1A}, α_{1B}, and α_{1C}) or +10 mV (α_{1E}). *B*, Average τ_1/τ_2 ratios for the same currents. In cells dialysed with GTPγS, the maximal current densities were 16 ± 3 pA/pF (n = 23) for α_{1A}, 15 ± 3 pA/pF (n = 23) for α_{1B}, 16 ± 5 pA/pF (n = 11) for α_{1C}, and 36 ± 4 pA/pF (n = 18) for α_{1E}. In cells dialysed with GDPβS, the maximal current densities were 20 ± 4 pA/pF (n = 9) for α_{1A}, 12 ± 4 pA/pF (n = 10) for α_{1B}, 6 ± 1 pA/pF (n = 4) for α_{1C}, and 28 ± 7 pA/pF (n = 7) for α_{1E}.

to channels and reestablish inhibition at negative potentials. To further explore the relative modulation of neuronal Ca channels by tonically activated G-proteins, we compared both the onset and the decay of facilitation for α_{1A}, α_{1B}, and α_{1E}.

The onset of facilitation was measured by plotting I_2/I_1 or τ_1/τ_2 ratios of α_{1A} and α_{1B} currents as a function of conditioning pulse duration. For α_{1E} we plotted only τ_1/τ_2 ratios. As shown in Figure 9, the onset of facilitation could be approximated by a single exponential function, producing a time constant (τ_{onset}) to describe this process. As the duration of the conditioning pulse was increased from 0 to 30 msec, the I_2/I_1 ratio increased with a time constant of 4.18 ± 0.18 msec (n = 5) for α_{1A} currents and 5.30 ± 0.20 msec (n = 7) for α_{1B} currents. Although this difference is statistically significant (p = 0.003), it is quite small. Similarly, τ_1/τ_2 ratios increased with time constants of 4.48 ± 0.96 msec (n = 5) for α_{1A} currents, 3.87 ± 0.58 msec (n = 7) for α_{1B} currents, and 3.76 ± 0.42 msec (n = 9) for α_{1E} currents; these time constants are not different (p = 0.72). Thus, facilitation develops with very similar kinetics for α_{1A}, α_{1B}, and α_{1E} Ca channels.

The decay of facilitation was monitored by plotting I_2/I_1 or τ_1/τ_2 ratios as a function of a variable interval (ΔT) between the conditioning pulse and the second test pulse (Fig. 10). Because the decay of facilitation varies with its magnitude (Golard and Siegelbaum, 1993; Elmslie and Jones, 1994), we only compared currents that were facilitated to similar degrees (I_2/I_1 ratios of 1.6 ± 0.1 for α_{1A} and 1.7 ± 0.1 for α_{1B}; and τ_1/τ_2 ratios of 2.1 ± 0.2 for α_{1A}, 1.8 ± 0.2 for α_{1B}, and 1.6 ± 0.1 for α_{1E}). The decays of I_2/I_1 and τ_1/τ_2 were fit by single exponential functions, and time constants for reinhibition ($\tau_{reinhib}$) were obtained. I_2/I_1 ratios decayed with an average time constant of 48 ± 8 msec (n = 7) for α_{1A} currents and 48 ± 3 msec (n = 6) for α_{1B} currents (p = 0.94). τ_1/τ_2 ratios decayed with an average time constant of 51 ± 8 msec

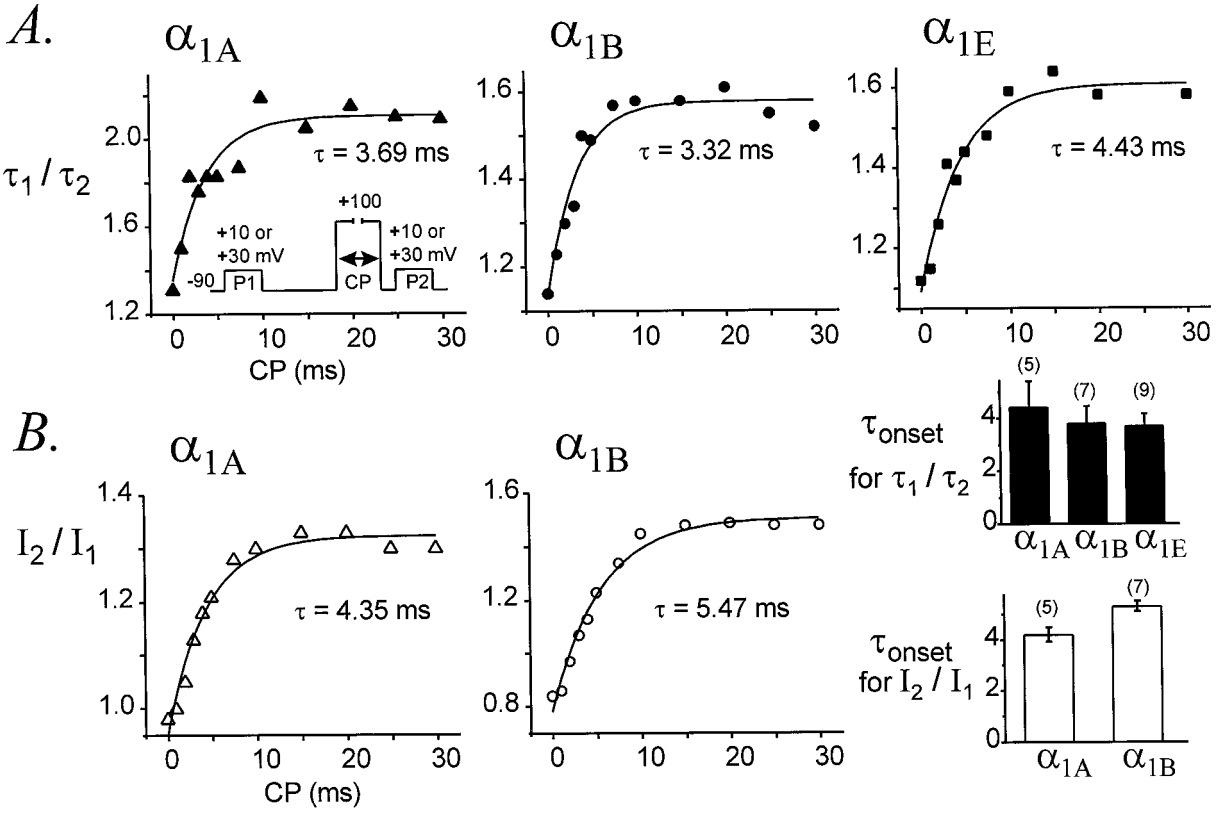

Figure 9. Facilitation develops with similar time course for α_{1A}, α_{1B}, and α_{1E} channels. τ_1/τ_2 ratios (*A*) and I_2/I_1 ratios (*B*) are plotted as a function of the conditioning pulse (*CP*) duration for representative cells. Plots were fit by single exponential functions to yield time constants for the onset of facilitation (τ_{onset}). Average values of τ_{onset} determined using τ_1/τ_2 or I_2/I_1 ratios are summarized graphically in the *bottom right corner*. The pipette solution contained GTPγS. α_{1A}, Data file 98115076; C = 29 pF; R_S = 2.4 MΩ. α_{1B}, Data file 98129067; C = 27 pF; R_S = 3.8 MΩ. α_{1E}, Data file 98205005; C = 19 pF; R_S = 3.2 MΩ. Data from cells expressing maximal current densities of 28 ± 5 pA/pF (α_{1A}, n = 5), 21 ± 6 pA/pF (α_{1B}, n = 7), and 38 ± 4 pA/pF (α_{1E}, n = 9). Voltage protocol as in Figure 1, except that P1 and CP were separated by 50 msec at −90 mV. Each point is the average of two currents.

(n = 7) for α_{1A} currents, 53 ± 10 msec (n = 6) for α_{1B} currents, and 55 ± 7 msec (n = 8) for α_{1E} currents (p = 0.94). These results indicate that facilitation decays from α_{1A}, α_{1B}, and α_{1E} Ca channels at very similar speeds.

The kinetics of modulation can be represented by the scheme (after Currie and Fox, 1997; Zhou et al., 1997):

$$\text{G}\beta\gamma\text{-CaCh} \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} \text{G}\beta\gamma + \text{CaCh}$$

During a facilitating depolarization to +100 mV, Gβγ subunits should dissociate from the channels. If Gβγ subunits do not also rebind channels during the depolarization, then k_{off} can be approximated by $1/\tau_{onset}$. On repolarization to −90 mV, Gβγ subunits should rebind to channels at a rate equal to k_{on} [Gβγ] + k_{off}. If resting inhibition of Ca channels by Gβγ subunits is strong, as suggested by the absence of a separate, rapidly activating component of current (Fig. 1), then k_{off} should be small, and k_{on} [Gβγ] can be approximated by $1/\tau_{reinhib}$. Assuming that all three channel types experience similar concentrations of Gβγ subunits, our estimates of τ_{onset} and $\tau_{reinhib}$ suggest that k_{off} and k_{on} have very similar values for α_{1A}, α_{1B}, and α_{1E}. Although this argument is not rigorous, it is consistent with the idea that Gβγ subunits dissociate from and reassociate with α_{1A}, α_{1B}, and α_{1E} channels at very similar or identical rates.

Large segments of α_{1B} are unnecessary for its modulation by G-protein

It was previously demonstrated by Zhou et al. (1995) that deleting large portions of the cytoplasmic II–III loop and C terminus from α_{1B} (mutant $\alpha_{1B\text{-}DD}$) does not eliminate G-protein-dependent inhibition or facilitation. However, in their experiments $\alpha_{1B\text{-}DD}$ was expressed in dysgenic myotubes, where the magnitude of facilitation was small and where kinetic slowing was not apparent in either wild-type α_{1B} or mutant $\alpha_{1B\text{-}DD}$ currents, raising the possibility that the native behavior of α_{1B} might not be fully reproduced within the cellular environment of skeletal muscle. To further examine the functional importance of the II–III loop and C terminus in Ca channel modulation, we expressed $\alpha_{1B\text{-}DD}$ in HEK293 cells and quantified its G-protein-dependent facilitation.

In $\alpha_{1B\text{-}DD}$, amino acids 829–995 have been deleted from the II–III loop, and residues 1877–2338 have been deleted from the C terminus (Fig. 11*A*). As illustrated in Figure 11*B*, currents mediated by $\alpha_{1B\text{-}DD}$ exhibited strong facilitation of activation rates and current amplitudes. The voltage dependences of inhibited and facilitated $\alpha_{1B\text{-}DD}$ currents (I_1 and I_2, respectively) were very similar (Fig. 11*C*) to currents mediated by the full-length α_{1B} (Fig. 5), confirming that the basic voltage-dependent properties of $\alpha_{1B\text{-}DD}$ were not appreciably changed by its deletions. During ≥5 min of intracellular dialysis with GTPγS, the amplitudes of

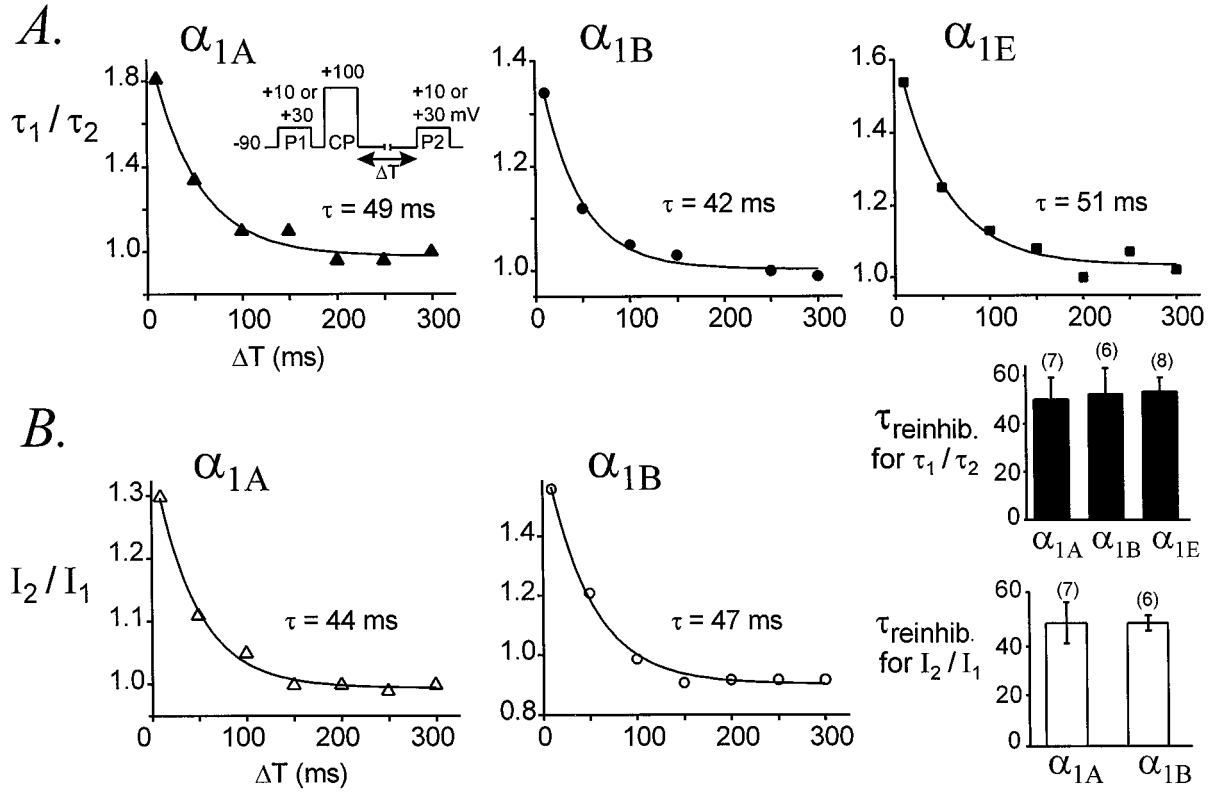

Figure 10. Facilitation decays from α_{1A}, α_{1B}, and α_{1E} Ca channels at the same rate. τ_1/τ_2 ratios (*A*) and I_2/I_1 ratios (*B*) are plotted as a function of ΔT, the variable interval between CP and P2 for representative cells. Each plot was fit by a single exponential function to yield a time constant for reinhibition ($\tau_{reinhib}$). Average values of $\tau_{reinhib}$ are summarized in the bar graphs (*bottom right*). The pipette solution contained GTPγS. α_{1A}, Data file 97731092; C = 24 pF; R_S = 2.9 MΩ. α_{1B}, Data file 97729010; C = 21 pF; R_S = 3.0 MΩ. α_{1E}, Data file 97D24046; C = 16 pF; R_S = 3.7 MΩ. Data from cells expressing maximal current densities of 16 ± 4 pA/pF (α_{1A}, n = 7), 23 ± 6 pA/pF (α_{1B}, n = 6), and 39 ± 6 pA/pF (α_{1E}, n = 8).

$\alpha_{1B\text{-}DD}$ currents decreased to 63 ± 13% (n = 6) of initial levels, comparable to the decrease observed for the full-length α_{1B} (56 ± 6%, n = 35). Interestingly, with intracellular GTPγS the I_2/I_1 ratio for $\alpha_{1B\text{-}DD}$ was significantly larger than for wild-type α_{1B} (2.18 ± 0.15, n = 6; vs 1.67 ± 0.08, n = 23; p = 0.01), suggesting greater facilitation or perhaps less inactivation of the mutant, although the voltage dependence of I_2 suggests that inactivation of $\alpha_{1B\text{-}DD}$ was unaltered. I_2/I_1 ratios (Fig. 11*D*) were also slightly larger for $\alpha_{1B\text{-}DD}$ than for α_{1B} in the presence of intracellular GDPβS (0.86 ± 0.06, n = 5; vs 0.63 ± 0.03, n = 10; p = 0.001). However, the activation kinetics of $\alpha_{1B\text{-}DD}$ and α_{1B} currents were equally facilitated, and τ_1/τ_2 ratios were indistinguishable between wild-type and mutant channels (Fig. 11*E*). Activation rates of $\alpha_{1B\text{-}DD}$ and α_{1B} currents were also identical in the absence of G-protein stimulation. For example, with intracellular GDPβS and at a test potential of +30 mV, τ_1 was 3.1 ± 0.3 msec (n = 5) for $\alpha_{1B\text{-}DD}$ and 3.1 ± 0.2 msec (n = 10) for α_{1B}. These results confirm that amino acids 829–995 and 1877–2338 are not required for modulation of α_{1B} by G-proteins and further demonstrate that these channel regions are not needed for facilitation of current amplitudes or activation kinetics.

DISCUSSION

The magnitude of G-protein-dependent facilitation correlates with Ca current density

Variations in channel density have previously been shown to correlate with significant differences in channel behavior (cf. Adams et al., 1996). In the present study we found that low-density α_{1A}, α_{1B}, and α_{1E} currents exhibited the greatest amount of facilitation, whereas high-density currents often lacked facilitation (Fig. 3). This observation suggests that Ca channel density somehow influences the extent to which G-proteins are able to produce inhibition. A correlation between current density and extent of modulation might result if cells expressing a high density of Ca channel α_1 subunits also expressed an excess of Ca channel β subunits, which are thought to antagonize G-protein-mediated Ca channel inhibition (Campbell et al., 1995) by competing with Gβγ for binding sites on the α_1 subunit (De Waard et al., 1997; Qin et al., 1997). Alternatively, cells expressing high densities of Ca channels might express insufficient G-proteins to inhibit all of the Ca channels, or the endogenous G-proteins of HEK293 cells may not have access to Ca channels expressed at high densities.

α_{1A} and α_{1B} exhibit similar modulation by tonically activated G-proteins

In our experiments, α_{1A} and α_{1B} showed the same amount of G-protein-dependent facilitation. Thus, I_2/I_1 ratios and τ_1/τ_2 ratios were equivalent between α_{1A} and α_{1B} channels (Fig. 8). Furthermore, our measurements of τ_{onset} and $\tau_{reinhib}$ (Fig. 9, 10) strongly suggest that G-proteins dissociate from and reassociate with α_{1A} and α_{1B} Ca channels at the same rates. Our findings that α_{1A} and α_{1B} are equally facilitated and also have the same kinetics of facilitation differ from the results of previous studies (Bourinet et al., 1996; Zhang et al., 1996; Currie and Fox, 1997; Zamponi et al., 1997). Our results may arise from our expression of a particular combination of Ca channel subunits, our consideration of Ca

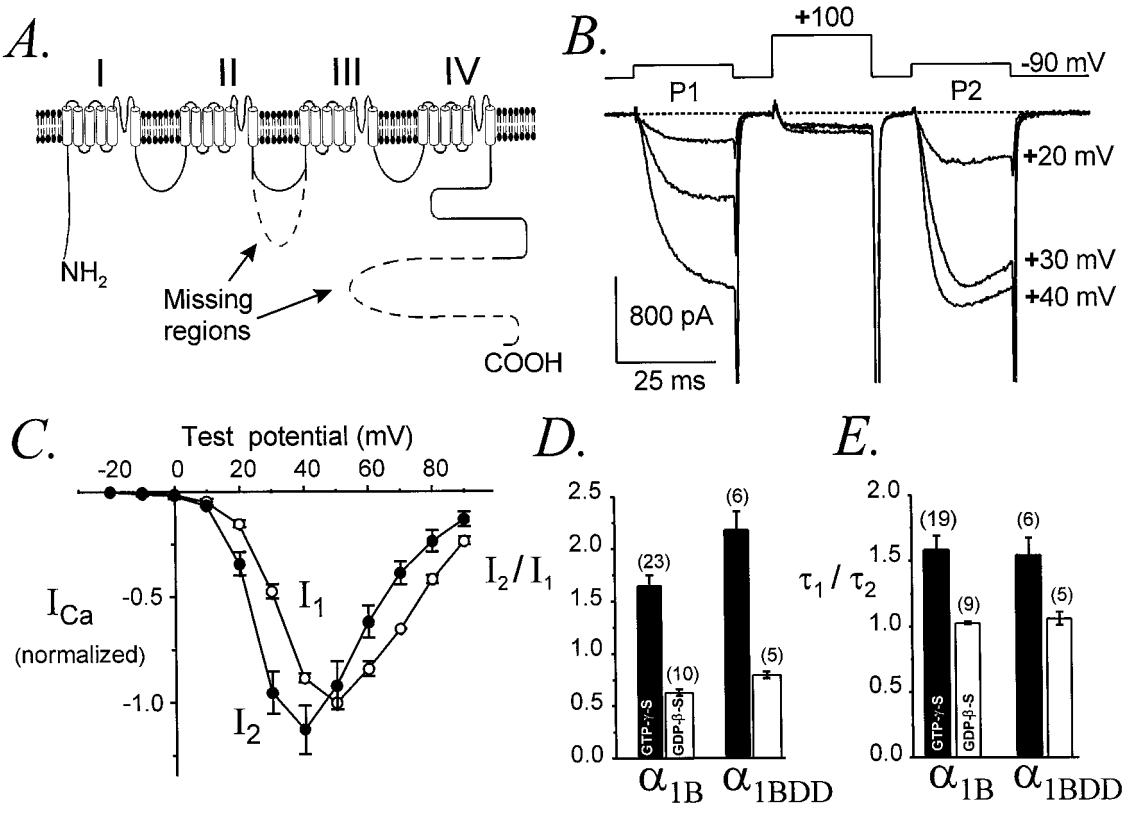

Figure 11. Undiminished G-protein-dependent modulation of $\alpha_{1B\text{-}DD}$. *A*, Diagrammatic representation of the mutant N-type Ca channel $\alpha_{1B\text{-}DD}$, which lacks amino acids 829–995 from the II–III loop and amino acids 1877–2338 from the C terminus. The deleted regions are indicated by *dashed lines*. *B*, Facilitation of $\alpha_{1B\text{-}DD}$ currents, evoked using the standard voltage protocol. Data file 97321108; C = 43 pF; R_S = 3.9 MΩ. *C*, Voltage dependence of inhibited (I_1) and facilitated (I_2) currents mediated by $\alpha_{1B\text{-}DD}$. I_1 and I_2 were normalized to the maximal I_1 in each cell (n = 4). The standard voltage protocol was used. *D*, Facilitation of $\alpha_{1B\text{-}DD}$ current amplitudes is slightly larger than for wild-type α_{1B}. Standard voltage protocol, with P1 and P2 to +30 mV. The pipette contained GTPγS (*filled bars*) or GDPβS (*unfilled bars*). *E*, Facilitation of activation kinetics is identical for $\alpha_{1B\text{-}DD}$ and α_{1B}. With intracellular GTPγS, τ_1/τ_2 ratios were 1.60 ± 0.09 (n = 21) for α_{1B} and 1.56 ± 0.11 (n = 6) for $\alpha_{1B\text{-}DD}$ (p = 0.91). With intracellular GDPβS, τ_1/τ_2 ratios were 1.03 ± 0.01 (n = 5) for α_{1B} and 1.06 ± 0.05 (n = 5) for $\alpha_{1B\text{-}DD}$ (p = 0.41). *D*, *E*, Data from cells with maximal current densities of 15 ± 3 pA/pF (α_{1B}, n = 23) and 18 ± 6 pA/pF ($\alpha_{1B\text{-}DD}$, n = 6) in the GTPγS experiments and 11 ± 5 pA/pF (α_{1B}, n = 5) and 6 ± 1 pA/pF ($\alpha_{1B\text{-}DD}$, n = 5) in the GDPβS experiments.

current density in the data analysis, or our use of GTPγS to produce tonically activated as opposed to phasically activated G-proteins (but see Currie and Fox, 1997 for differential effects of GTPγS on native P/Q- and N-type channels). As one possibility, α_{1B} channels may be localized more closely to neurotransmitter receptors and/or G-proteins than α_{1A} channels, such that α_{1B} would experience a higher concentration of Gβγ subunits after receptor activation. In contrast, during tonic activation of G-proteins with GTPγS, the concentration of Gβγ may be relatively uniform throughout the cell, and differences between the localization of α_{1A} and α_{1B} might not affect their modulation. This possibility is in keeping with the conclusions of Wilding et al. (1995) and Zhou et al. (1997) that activated opioid receptors can only inhibit nearby Ca channels, suggesting that phasically activated G-proteins have a limited range. It is also possible that GTPγS does not activate as many G-proteins, or the same varieties of G-proteins, as activated neurotransmitter receptors.

α_{1E} exhibits G-protein-dependent inhibition and facilitation

In previous studies, Bourinet et al. (1996), Toth et al., (1996), and Page et al. (1997) concluded that α_{1E} was not significantly inhibited by G-proteins, whereas Yassin et al. (1996), Mehrke et al. (1997), Shekter et al. (1997), and Qin et al. (1997) concluded the opposite. Our present results confirm the latter view. Although the amplitudes of α_{1E} currents were not always facilitated using the standard voltage protocol, we observed very consistent facilitation of α_{1E} activation kinetics (Figs. 2, 6, 8). In fact, the average τ_1/τ_2 ratios of α_{1E} currents were indistinguishable from those of α_{1A} and α_{1B} currents (Fig. 8*B*), suggesting that the magnitude of G-protein-dependent facilitation is quite comparable among α_{1A}, α_{1B}, and α_{1E} channels. Our results also indicate that voltage-dependent inactivation can obscure facilitation of α_{1E} current amplitudes. Inactivation produced a similar, although less pronounced, effect on the facilitation of α_{1B} current amplitudes (Fig. 5). Thus, activation kinetics appear to be more sensitive and more reliable than current amplitudes as an index of G-protein-dependent modulation, particularly for channels such as α_{1E} that inactivate at relatively negative potentials.

On the macroscopic level, Ca channel facilitation can be manifested as increased current amplitudes, increased activation rates, or both. Inactivation of some Ca channels can prevent facilitation of macroscopic current amplitudes but should not prevent the facilitated opening (i.e., shorter first latency; Patil et al., 1996) of the remaining noninactivated channels. Thus, facilitation of activation kinetics should occur even if the facilitating depolarizations inactivate some channels. Under physiological

conditions in which Ca channel activation and subsequent Ca influx is triggered by action potentials and other brief depolarizations, a facilitation of Ca channel activation kinetics may be more functionally significant than a facilitation of current amplitudes. Ca channels formed by α_{1E} appear to be localized to neuronal cell bodies and dendrites (Yokoyama et al., 1995). The ability of α_{1E} to undergo G-protein-mediated inhibition and facilitation may therefore be important for the integration and propagation of dendritic electrical signals and possibly also for gene transcription within neuronal cell nuclei.

α_{1C} does not exhibit G-protein-dependent facilitation

The rabbit cardiac α_{1C} subunit did not exhibit kinetic slowing or facilitation in the presence of tonically activated G-proteins. Our results thus agree with previous reports (Bourinet et al., 1994, 1996; Zhou et al., 1995; Zhang et al., 1996) that α_{1C} is not modulated through a membrane-delimited, G-protein-dependent pathway. In our experiments α_{1C} also did not exhibit voltage-dependent facilitation, in contrast to results obtained with the rat neuronal α_{1C} expressed in *Xenopus* oocytes (Bourinet et al., 1994; Cens et al., 1996). Voltage-dependent facilitation may be limited to particular splice variants of α_{1C}, manifested only in certain expression systems, or may require longer conditioning depolarizations than used in the present study.

A G$\beta\gamma$ interaction site identified within the C terminus of α_{1E} is not required for modulation of α_{1B}

We quantified the G-protein-dependent facilitation of a deletion mutant of the N-type Ca channel ($\alpha_{1B\text{-}DD}$) that is missing amino acids 829–995 from the II–III loop and amino acids 1877–2338 from the C terminus (Fig. 11*A*). $\alpha_{1B\text{-}DD}$ displayed the same magnitudes of kinetic slowing and facilitation of activation kinetics as the full-length, wild-type α_{1B} subunit (Fig. 11*D,E*), demonstrating that the missing regions are not required for G-protein-dependent inhibition or facilitation of α_{1B}. This result is particularly significant in view of the recent identification by Qin et al. (1997) of an ~38 amino acid residue sequence within the C terminus of α_{1E} that is required for its inhibition by G$\beta\gamma$. The corresponding region of α_{1B}, which was shown by Qin et al. (1997) to bind G$\beta\gamma$ *in vitro*, is not present within the $\alpha_{1B\text{-}DD}$ mutant. Therefore, this region cannot also be essential for G-protein-dependent modulation of α_{1B}. The results obtained with $\alpha_{1B\text{-}DD}$ thus raise the intriguing possibility that different structural regions of α_{1B} and α_{1E} mediate their interactions with G-proteins.

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