0026-895X/00/051064-11$3.00/0
MOLECULAR PHARMACOLOGY

MOL 57:1064–1074, 2000 /12985/822097

Involvement of Regions in Domain I in the Opioid Receptor Sensitivity of α1B Ca^{2+} Channels

ARTHUR A. SIMEN and RICHARD J. MILLER

Department of Neurobiology, Pharmacology, and Physiology, and Committee on Neurobiology, The University of Chicago, Chicago, Illinois

Received September 29, 1999; accepted February 3, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

The structural basis of Ca^{2+} channel inhibition by G proteins has received considerable attention recently, and multiple regions on Ca^{2+} channels that interact with G protein subunits have been identified. We have demonstrated previously that a region extending from the N terminus to the I/II loop of the Ca^{2+} channel is involved in determining the differences between α1B and α1E Ca^{2+} channels with respect to inhibition by G proteins. Here we explore this region of the channel in greater detail in an effort to further define the regions involved in determining inhibition. Chimeric Ca^{2+} channels constructed from α1B and α1E Ca^{2+} channels revealed that the N terminus, the I/II loop, and domain I all play an important role in determining inhibition. We identified a 70-amino acid fragment from domain I that mediates the effects of domain I, and a 50-amino acid fragment from the I/II loop that mediates the effects of the I/II loop. When these regions from α1B were exchanged into α1E, inhibition identical with that of α1B was observed. The differences between α1B and α1E in the identified region of domain I involve residues that are predicted to be almost exclusively extracellular. Mutations to some of the high-affinity G protein binding regions of α1B (α interaction domain, CC14, and a C-terminal Gα binding site) caused relatively little change in inhibition, which suggests that these sites are not necessary individually for G protein-mediated inhibition and may help to explain the small effects of exchanging these regions in isolation.

The inhibition of Ca^{2+} influx through presynaptic Ca^{2+} channels by G proteins is an important mechanism for the modulation of synaptic transmission (Wu and Saggau, 1997; Miller, 1998). The Ca^{2+} channels involved in presynaptic Ca^{2+} entry are usually of the N, P/Q, and R-type (Dunlap et al., 1995; Reid et al., 1998; Wu et al., 1998). Cloned N-type (α1B) Ca^{2+} channels have generally been found to be more sensitive to inhibition by G proteins than are P/Q- (α1A) or R- (α1E) type channels (Bourinet et al., 1996; Toth et al., 1996; Yassin et al., 1996; Zhang et al., 1996; Simen and Miller, 1998; but see Meza and Adams, 1998). These differences in sensitivity to inhibition by G proteins may therefore represent one mechanism mediating the efficacy of synaptic depression by presynaptic receptors.

The structural basis of G protein binding to Ca^{2+} channels has been the subject of a number of studies. G$\beta\gamma$ subunits have been shown to interact with two distinct regions in the I/II loop of nondihydropyridine (DHP)-sensitive Ca^{2+} channels, and one site also interacts with Ca^{2+} β subunits (De Waard et al., 1997; Herlitze et al., 1997; Qin et al., 1997; Zamponi et al., 1997; Hamid et al., 1999). It has also been shown that the C terminus of Ca^{2+} channels can bind G$\beta\gamma$ (Qin et al., 1997) as well as Gα (Furukawa et al., 1998a, 1998b) subunits. Despite a clear role for the I/II loop region in G$\beta\gamma$ binding, this region does not seem to be primarily responsible for the differences in G protein sensitivity between the non-DHP sensitive Ca^{2+} channels (Page et al., 1997; Zhang et al., 1996). Similar to the findings of Zhang et al. (1996) and Stephens et al. (1998), we have previously demonstrated that the insertion of domain I from the human α1B Ca^{2+} channel into the human α1E channel yields a construct with significantly increased sensitivity to G proteins (Simen and Miller, 1998) and that the addition of the I/II loop and the N terminus increased inhibition further. The I/II loop region alone did not affect modulation. The involvement of the N terminus has recently been further defined (Page et al., 1998; Canti et al., 1999).

Although these studies suggest an important role of the N-terminal portion of the channel in determining sensitivity to modulation, it remains unclear what portions of this region are involved and how large their individual contributions are to the overall level of modulation of Ca^{2+} channels. We therefore examined the effects of transfer of regions between α1B (highly modulated) and α1E (minimally modulated) to determine sequences responsible for the differences in G protein sensitivity between the two channels. Our find-

This work was supported by National Institute of Health Grants DA02121, MH40165, NS33826, DK44840, and NS21442. A.A.S. was supported by Grants HD07009 and DA02575.

ABBREVIATIONS: DHP, dihydropyridine; norBNI, nor-binaltorphimine; AID, α interaction domain; ND1, N-terminal domain I region; GID, G protein interaction domain; κOR, κ-opioid receptor.

ings suggest that the N terminus alone contributes substantially to inhibition, a region extending from the S1/S2 loop to the S3/S4 loop of domain I is involved, and that a fragment of the I/II loop spanning the AID region and a downstream G$\beta\gamma$ binding region is also involved in mediating inhibition. The identification of the region of domain I involved is of particular interest because the residues involved are almost entirely extracellular and are therefore unlikely to directly interact with G proteins.

Materials and Methods

Chimeric Ca^{2+} channel α1 subunit constructs were created using methods described previously (Simen and Miller, 1998). Final constructs were confirmed by a combination of restriction analysis and DNA sequencing. The native human α1B construct consisted of residues 1 to 2340 of GenBank 2284339 (gift from Dr. R. Harpold, SIBIA Neurosciences, San Diego, CA). The native human α1E construct consisted of residues 1 to 2271 of GenBank 21082919 (gift from Dr. R. Harpold, SIBIA Neurosciences). The negative chimeras were named by appending the amino acids from α1E that were transferred into α1B to the letter "E," using the numbering system shown in Fig. 5. For example, the construct E120–132 consisted of α1B with amino acids 120 to 132 replaced with the corresponding amino acids from α1E. The positive chimeras were named in a similar fashion, by appending the residues from α1B that were transferred into α1E to the letter "B." For example, the construct B1–93 consisted of residues 1 to 93 from α1B in the α1E background. The construct Δ2037–2087 was created by replacing the cDNA coding for residues 2037 to 2087 of α1B with a *Hin*dIII restriction site, coding for the amino acids RL. The construct Δ1875–2339 was created by deleting the nucleotides coding for amino acids 1875 to 2339 of α1B. The construct BQ1 was created from α1B by site-directed mutagenesis to make the mutations Q383A, E386A, and R387A. The construct BQ2 was created from α1B by site-directed mutagenesis to make the mutations Q383A, Q384A, I385A, E386A, and R387A. tsA-201 cell culture, transfections, solutions for electrophysiological recording, and drug solutions were made as described previously (Simen and Miller, 1998). Cells were transfected with Ca^{2+} α2/δ, Ca^{2+} β1b, and wild-type or recombinant Ca^{2+} α1 subunits, along with the mouse κ-opioid receptor and CD8α. The mouse κ-opioid receptor was a gift from Dr. Graeme Bell (Howard Hughes Medical Institute, University of Chicago, Chicago, IL). Currents were elicited by a dual-pulse protocol consisting of two 50-ms depolarizations (pulse 1 and pulse 2) to test potentials varying from −40 to +40 mV from a holding potential of −90 mV, separated by 800 ms at the holding potential, with a 30-ms, 90-mV depolarization ("prepulse") ending 5 ms before the second pulse (Fig. 1A), at an acquisition rate of 10 kHz and filtered with a four-pole Bessel filter at 2 kHz.

To facilitate comparison of the different recombinant channel constructs, cumulative integrals (CI) were computed on the current voltage data from each construct, and were defined as

$$CI = \int_{-40}^{+40} \mathrm{I(v)} \qquad (1)$$

where I(v) is the observed current amplitude at a particular test potential v. Current amplitudes were estimated from the observed maximum inward current during each 50-ms depolarization, and were scaled to values between 0 and −1 by dividing all four data vectors from each cell [+U69593, −prepulse; +U69593, +prepulse; +nor-binaltorphimine (norBNI), −prepulse; +norBNI, +prepulse] by the absolute value of the largest observed current. Points between the observed test potentials were interpolated by cubic spline interpolation, and the cumulative integrals (CI) were estimated by integration of the resulting cubic spline functions between −40 and +40 mV. Inhibition was then calculated as [CI(pulse 1, norBNI) − CI(pulse 1, U69593)] / CI(pulse 1, norBNI). Because no outward current was observed between these test potentials, changes in the resulting statistic sensitively reflect changes in inward current induced by G protein activation across the entire current voltage curve. Facilitation (corrected for inactivation) was calculated as [CI(pulse 2, U69593) / CI(pulse 1, U69593)] × [CI(pulse 1, norBNI) / CI(pulse 2, norBNI)] − 1. Inactivation by the prepulse was calculated as 1 − CI(pulse 2, norBNI)/CI(pulse 1, norBNI).

Facilitation and inhibition were also measured at individual test potentials by calculating an index of inhibition in terms of the simple current amplitudes (I) at each test potential. This index was computed as [I(pulse 1, norBNI) − I(pulse 1, U69593)]/I(pulse 1, norBNI) and an index of facilitation corrected for inactivation was calculated as [I(pulse 2, U69593)/I(pulse 1, U69593)] × [I(pulse 1, norBNI)/I(pulse 2, norBNI)] as described previously (Simen and Miller, 1998). Activation midpoints ($V_{1/2}$) for selected constructs were calculated by fitting the function $I(v) / I_{max} = [G(v - V_{rev})] / (1 + \exp[(V_{1/2} - v)/K)]$, where I(v) is the observed inward current maximum at potential v and I_{max} is the absolute value of the largest observed inward current.

Statistical analyses were computed on inhibition and facilitation across the entire voltage range using one-way ANOVA followed by the Tukey multiple comparison procedure. These analyses were conducted separately for the three sets of constructs discussed (i.e., positive chimeras, negative chimeras, and chimeras involving G protein binding regions). Each ANOVA also included data from α1B and α1E for comparison.

Results

Quantification of κOR Effects on Ca^{2+} Currents. We have shown previously (Simen and Miller, 1998) that the κ-opioid receptor has activity in the absence of agonist (constitutive activity), that the drug norBNI can suppress this activity (i.e., is an inverse agonist), and that norBNI and U69593 can be used to drive the receptor toward minimal activity and maximal activity, respectively, allowing examination of the full range of G protein effects on Ca^{2+} channels. We have also demonstrated that κOR effects on α1B currents are completely sensitive to pertussis toxin and insensitive to protein kinase C blockers (Simen and Miller, 1998), are completely *N*-ethylmaleimide-sensitive, almost totally voltage dependent, and are not mediated by alterations in intracellular Ca^{2+} (A.A. Simen, R.J. Miller, unpublished observations).

Our laboratory has observed previously that α1B Ca^{2+} channels are much more sensitive to G proteins than are α1E Ca^{2+} channels (Toth et al., 1996; Simen and Miller, 1998), which is similar to the findings of other groups (e.g., Bourinet et al., 1996). To study G protein modulation of these native Ca^{2+} channels as well as mutant and chimeric Ca^{2+} channels, we expressed various Ca^{2+} channel α1 subunits along with α2/δ and β1b Ca^{2+} channel subunits, the κ-opioid receptor, and CD8α in tsA-201 cells. Cells expressing CD8α (as indicated by decoration with anti-CD8 coated beads) were chosen for patch clamping. When barium currents were evoked by a dual pulse protocol (Fig. 1A) with two identical test pulses (pulse 1 and pulse 2) and an intervening strongly depolarizing pulse ("prepulse"), α1B currents evoked from pulse 1 were seen to decrease in amplitude in the presence of U69593. A prepulse (Fig. 1B, top) largely relieved this current inhibition. Currents in the presence of U69593 were compared with currents obtained from the same cell in the

presence of the κ-opioid receptor inverse agonist/antagonist norBNI rather than baseline currents because we have found previously that the κ-opioid receptor is active to some extent in the absence of agonist (Simen and Miller, 1998) and that norBNI can relieve this receptor activity. To summarize the extent of inhibition obtained across the entire range of test potentials, the current amplitudes from pulse 1 were normalized and differences in the area under the current-voltage curves were calculated for currents in the presence of norBNI and U69593 (Fig. 1B, middle). The extent of relief of inhibition by a prepulse was calculated in a similar way by comparing pulse 1 current-voltage curves to pulse 2 current-voltage curves in the presence of U69593 (Fig. 1B, bottom). However, many of the constructs differed in the degree of inactivation caused by the prepulse (discussed below), and an effort was therefore made to correct these data for inactivation essentially as described previously (Simen and Miller, 1998; see *Materials and Methods*).

Inhibition and facilitation were also calculated in this manner for α1E (Fig. 1C). By this method, α1B channels were inhibited by 51 ± 3% and showed 85 ± 9% facilitation (Table 1; Fig. 2, Fig. 3, and Fig. 4). In contrast, α1E channels showed 12 ± 2% inhibition and 4 ± 2% facilitation ($P < .05$ with respect to α1B). These values closely parallel inhibition and facilitation values obtained at a single test potential of +10 mV (45 ± 5% inhibition and 87 ± 12% facilitation for α1B and 7 ± 5% inhibition and 0 ± 3% facilitation for α1E). These indices of inhibition and facilitation obtained by integration across the entire voltage range were highly correlated with inhibition and facilitation calculated by more conventional methods at single test potentials. For example, at a test potential of +10 mV inhibition ($r = 0.78$, $P < .05$; $n = 154$) as well as facilitation ($r = 0.81$, $P < .05$; $n = 154$) were highly correlated with the same indices calculated across the entire voltage range for all the constructs tested in this study. There were, however, important differences in the voltage depen-

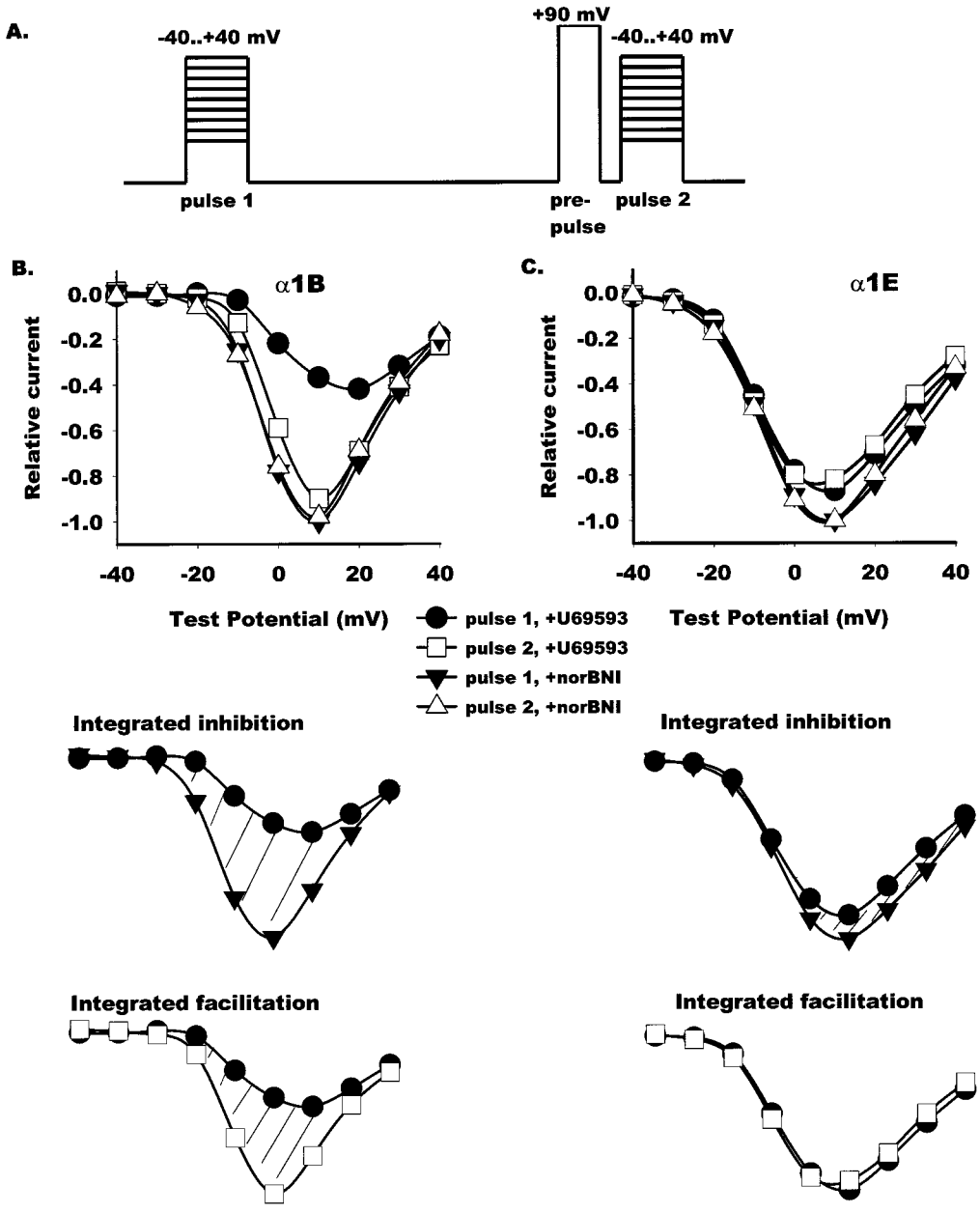


Fig. 1. Examples of κ-opioid receptor effects on α1B and α1E Ca^{2+} currents. Native α1B and α1E Ca^{2+} channels were expressed in tsA-201 cells along with the β1b and α2/δ Ca^{2+} subunits, the κ-opioid receptor, and CD8α. A, currents were evoked with a dual-pulse protocol with two variable 50-ms test-pulses ranging from −40 to +40 mV separated by a 30-ms pulse to +90 mV ("prepulse"). B, top, normalized current amplitudes from a representative α1B expressing cell are shown. Currents in the presence of U69593 (a κOR agonist) were compared with currents in the presence of norBNI (a κOR inverse agonist/antagonist) to minimize constitutive receptor activity, as described previously (Simen and Miller, 1998). Currents were normalized to the largest observed current. The area under the current-voltage curves from the first pulse in the presence of norBNI (see legend) and U69593 (see legend), and the second pulse in the presence of norBNI (see legend) and U69593 (see legend) were calculated. Middle, Inhibition was defined in terms of the fractional difference between the normalized areas of the pulse 1 current in the presence of U69593 compared with norBNI. Bottom, facilitation was defined as the ratio of the areas of the pulse 2 currents in the presence of U69593 to the pulse 1 currents in the presence of U69593. Facilitation was adjusted for inactivation as described in *Materials and Methods.* C, top, representative current-voltage curves from an α1E expressing cell. Middle, integration of inhibition from the current-voltage curves shown in Fig. 1C, top (α1E expressing cell). Bottom, integration of facilitation from the current-voltage curves shown in shown in Fig. 1C, top (α1E expressing cell).

dence of inhibition for certain constructs, as discussed in detail below. A summary of inhibition and facilitation calculated in this manner for α1B, α1E, and a variety of recombinant Ca^{2+} channels is shown in Fig. 2 and Table 1. Current-voltage curves and representative traces from selected constructs are shown in Figs. 3 and 4, respectively.

Negative Chimeras. We have previously determined that a region of α1B extending from the N terminus to the I/II loop could confer high sensitivity to G protein-mediated inhibition when transferred into the α1E background (Simen and Miller, 1998), similar to the results of Stephens et al. (1998). Page et al. (1998) subsequently determined that the N terminus alone was partially responsible for these effects but could not itself bring inhibition up to the level seen in α1B, suggesting an important role for regions within domain I and/or the I/II loop. These findings with regard to the N terminus are consistent with our previous observation that an α1B/α1E chimera bBbEeEeEe was modulated to a greater extent than a chimera eBbEeEeEe (Simen and Miller, 1998) lacking the α1B N terminus.

An examination of sequence alignments between α1E and α1B in domain I (Fig. 5) revealed that the differences between the two channels in this region are largely restricted to the (mainly) extracellular loops (i.e., S1/S2, S3/S4, and S5/S6 loops; Fig. 5). We therefore attempted to identify the regions in domain I contributing to the differences in G protein sensitivity between α1B and α1E by altering these loops in the α1B channel individually and in conjunction with larger regions of domain I to the corresponding sequence in the α1E channel (negative chimeras). Alteration of α1B to correspond

TABLE 1

Inhibition and facilitation calculated across the entire voltage range

Inhibition, facilitation, and statistical comparisons were calculated as described under *Materials and Methods*. Statistical significance with respect to α1B is denoted as ^{B} and statistical significance with respect to α1E is denoted as ^{E}.

Construct	N	Inhibition		Facilitation	
		Mean	S.E.M.	Mean	S.E.M.
α1B	12	0.51	0.03^{E}	0.85	0.09^{E}
α1E	8	0.12	0.02^{B}	0.04	0.02^{B}
E120-351	8	0.28	0.05^{B}	0.22	0.04^{B}
E120-215	8	0.31	0.03^{B}	0.21	0.04^{B}
E120-132	7	0.43	0.04^{E}	0.76	0.11^{E}
E120-132;Δ129-130	8	0.46	0.04^{E}	0.59	0.12^{E}
E185-190	9	0.38	0.05^{E}	0.44	0.05^{B,E}
E250-377	7	0.43	0.05^{E}	0.36	0.09^{B}
E382-432	7	0.50	0.03^{E}	0.77	0.08^{E}
E185-190;382-432	7	0.28	0.06^{B}	0.42	0.04^{B,E}
E120-215;382-432	11	0.28	0.04^{B}	0.32	0.04^{B}
E250-377;382-432	7	0.38	0.07^{E}	0.42	0.08^{B,E}
B1-93	7	0.27	0.06^{B,E}	0.10	0.04^{B}
B1-93;382-432	7	0.37	0.04^{B,E}	0.07	0.01^{B}
B1-93;185-190;382-432	7	0.34	0.02^{B,E}	0.27	0.05^{B}
B1-93;120-190;382-432	8	0.48	0.03^{E}	0.29	0.04^{B}
BQ1	5	0.55	0.09^{E}	0.88	0.21^{E}
BQ2	7	0.47	0.06^{E}	0.76	0.14^{E}
Δ2037-2087	7	0.46	0.06^{E}	0.66	0.10^{E}
Δ1875-2339	7	0.46	0.06^{E}	1.02	0.12^{E}

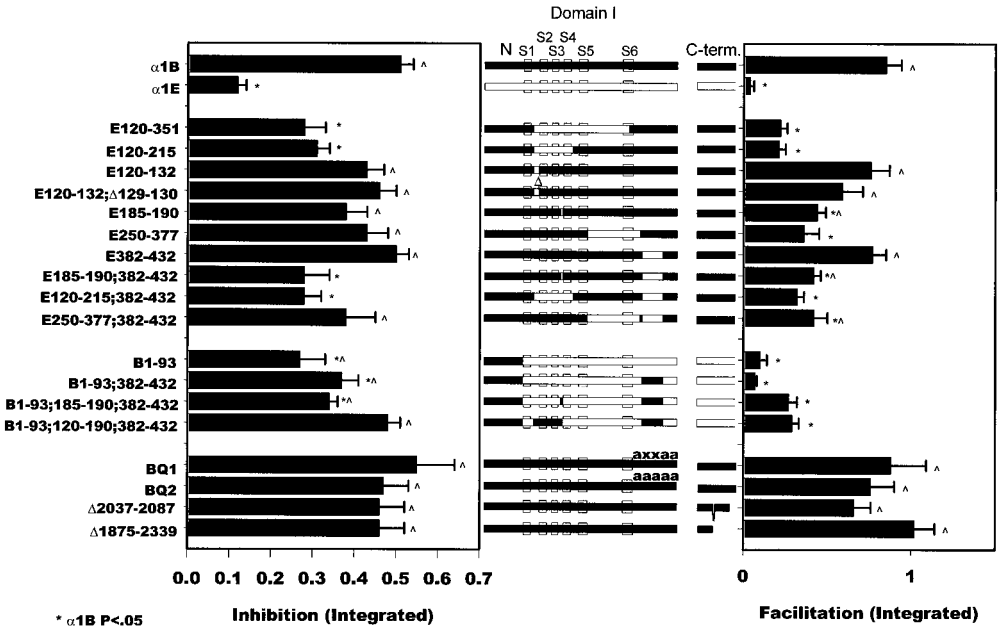


Fig. 2. Summary of inhibition (left) and facilitation (right), quantified across the entire current-voltage curve (Fig. 1; see *Materials and Methods*). Bars represent means, and error bars represent the standard error of the mean. Cartoon depictions of each construct (center) show only the N terminus to I/II loop and C-terminal region of the channel. Values significantly different from α1B are marked with *, and values significantly different from α1E are marked with ^ (4). See Table 1 for numerical values and statistical analyses.

to the sequence of α1E in the region of the S1/S2 loop (construct E120–132), S3/S4 loop (construct E185–190), or S5/S6 loop and proximal I/II loop region (construct E250–377) yielded constructs showing significantly more inhibition than α1E that did not differ significantly from α1B (Fig. 2; Table 1). Larger changes in domain I did, however, reduce inhibition. Exchange of the entire domain I region reduced inhibition significantly (construct E120–351; Fig. 2; Table 1). Exchange of 95 amino acids spanning the S1/S2 to S4/S5 region (construct E120–215) also reduced inhibition significantly (Figs. 2 and 3; Table 1).

We were concerned that alterations in domain I might impair channel function and interfere nonspecifically with G protein inhibition. This was suggested by the fact that some of the changes in domain I (e.g., construct E120–215) caused a rightward shift of the current-voltage relation with respect to α1B and α1E (Fig. 3). We fortuitously identified a clone during the creation of the E120–132 construct with a 2-amino acid deletion at positions 129 and 130 from the C-terminal end of the I S1/S2 region that showed a significant rightward shift of current-voltage relation ($V_{1/2}$ = 14.84 ± 1.45 mV; n = 6) compared with α1B ($V_{1/2}$ = −9.15 ± 1.99 mV; n = 12), α1E ($V_{1/2}$ = −13.62 ± 2.59 mV; n = 11), and E120–132 ($V_{1/2}$ = −7.50 ± 4.69 mV; n = 5; Fig. 3). This construct (construct E120–132; Δ129–130) showed inhibition that did not differ from the E120–132 construct or α1B ($p > .05$ in both cases; Fig. 2; Table 1).

The results from these negative chimeric constructs suggest, therefore, that exchange of the region from S1/S2 to S4/S5 can decrease inhibition as much as exchanging all of

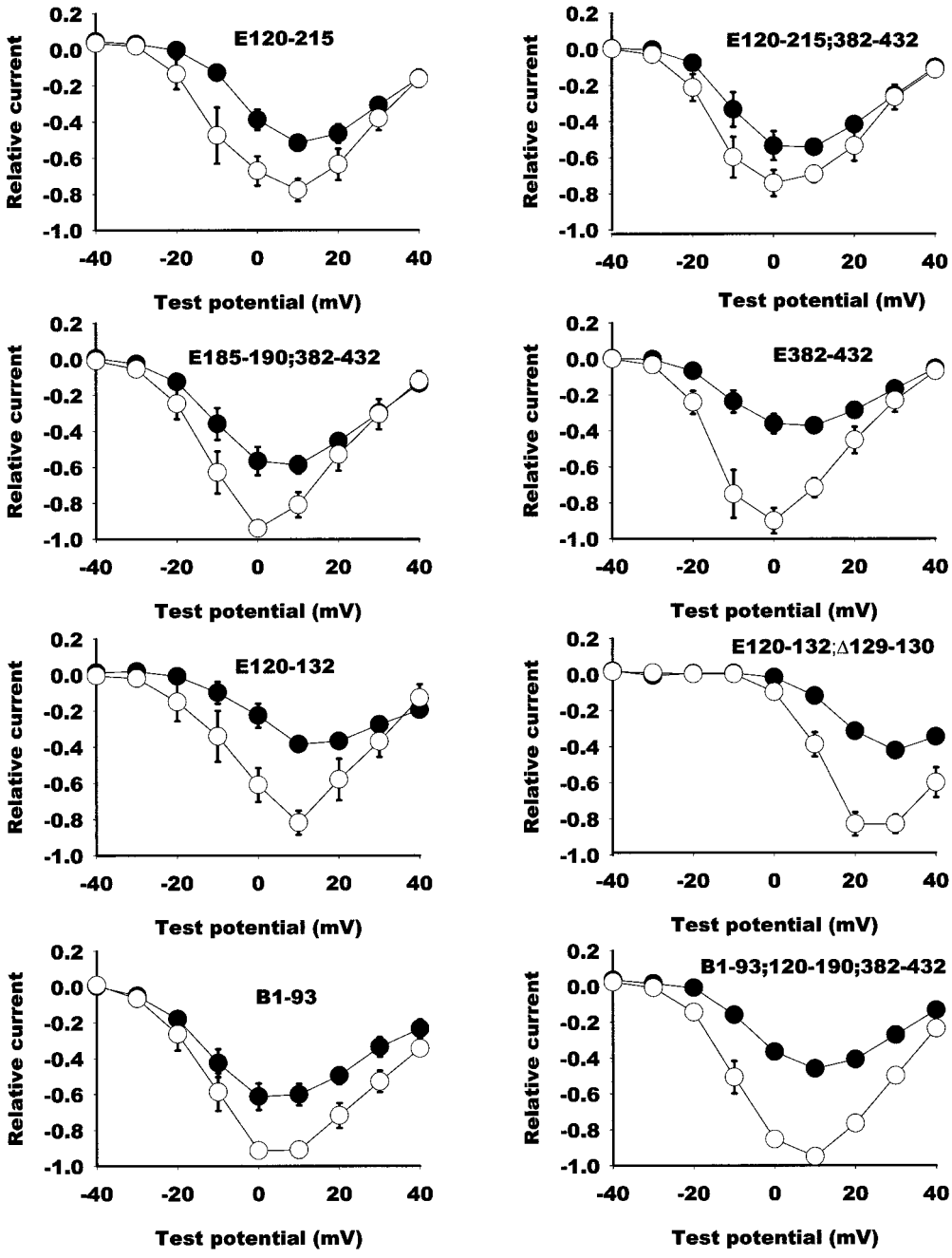


Fig. 3. Current-voltage curves of selected constructs. Error bars represent the standard error of the mean. Sample sizes are shown in Table 1. See Fig. 1 for examples of α1B and α1E current-voltage curves for comparison.

domain I. Because the region from S4 to S4/S5 is identical in the two channels except for one conservative substitution (Val/Ile at position 215 in the S4/S5 loop; Fig. 5) the region from S1/S2 to S3/S4 (positions 120–190) is strongly implicated by these findings. We will refer to this portion of domain I as N-terminal domain I region (ND1). Therefore, this ND1 region consisting of 70 amino acids is likely to be the portion of domain I involved in determining the differences in inhibition between α1B and α1E. More direct evidence for involvement of this region and the lack of involvement of position 215 based on positive chimeras is discussed below.

We had shown previously that the α1B I/II loop, when exchanged along with domain I into α1E, increased inhibition to some extent (Simen and Miller, 1998). However, we saw no effect of exchange of the I/II loop alone, consistent with the results of other groups (Zhang et al., 1996; Page et al., 1997). This suggests that alterations in the I/II loop and domain I may interact in some manner to affect inhibition. Therefore, by creating double negative chimeras, we attempted to identify such interactions by exchanging regions in domain I along with regions in the I/II loop from α1E into α1B. The I/II loop is known to mediate G$\beta\gamma$ binding by virtue of the AID region as well as a downstream G$\beta\gamma$ binding region (De Waard et al., 1997; Zamponi et al., 1997). The I/II loop also mediates Ca^{2+} channel β subunit binding by way of the AID region (Pragnell et al., 1994). We will refer to the portion of the downstream G$\beta\gamma$ site identified by Zamponi et al. (1997) as the G protein interaction domain (GID). To determine whether these regions of the I/II loop implicated in G$\beta\gamma$ binding are sufficient to account for the effects of the I/II loop, we created a series of chimeras with a stretch of 50 amino acids from the I/II loop spanning the region from AID to the GID region. This alteration to α1B had no significant effect on inhibition (construct E250–377; Fig. 2; Table 1) and only small changes were seen in combination with alterations of the S5/S6 loop and the proximal I/II loop region (construct E250–377;382–432; Fig. 2; Table 1). However, in combination with a 95-amino acid stretch from the S1/S2 to S4/S5 regions (construct E120–215;382–432) or the S3/S4 loop alone (construct E185–190;382–432), inhibition was significantly reduced with respect to α1B (Figs. 2, 3, and 4; Table 1). Therefore, these double chimeras suggest that the AID-GID segment of the I/II loop is capable of affecting inhibition in a manner that parallels the effects that we observed previously of exchange of the entire I/II loop in positive chimeras (Simen and Miller, 1998). Facilitation generally paralleled inhibition (Fig. 2; Table 1) and is discussed in detail below.

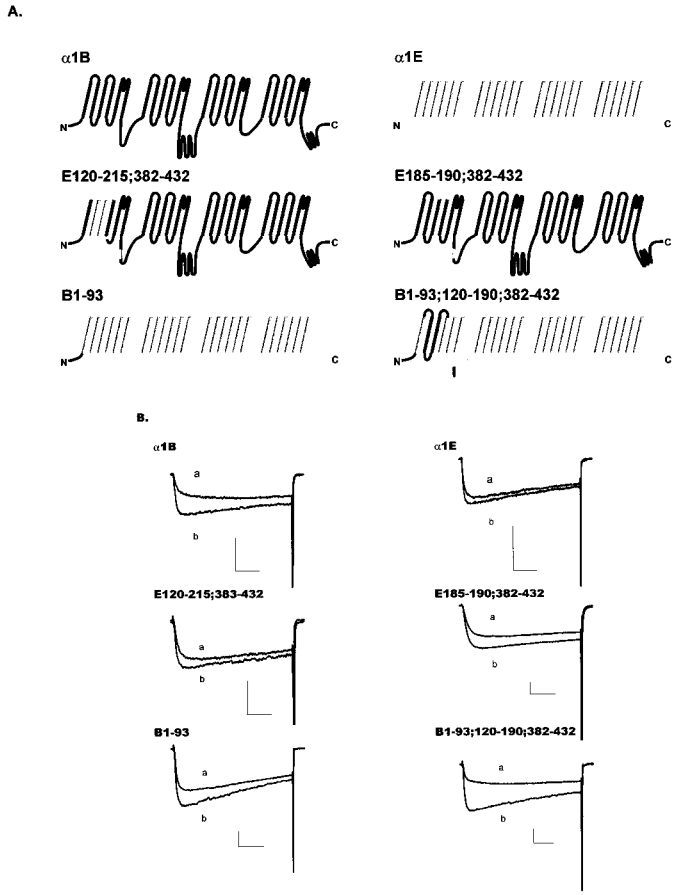
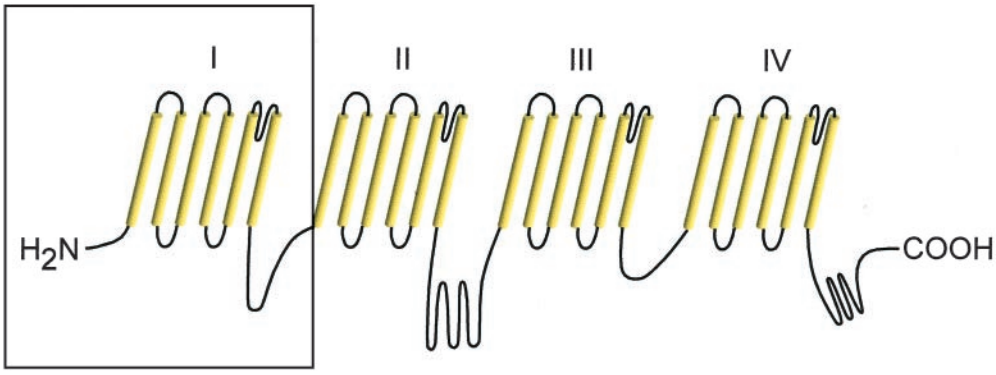


Fig. 4. The bottom panel shows sample currents from pulse 1 (before prepulse) at +10 mV from selected constructs (depicted in the top panel) in the presence of U69593 (a) and norBNI (b). Scale bars represent 500 pA and 100 ms.

Positive Chimeras. Our findings with respect to single and double negative chimeras suggest that a region in domain I extending from S1/S2 to S3/S4 (ND1 region) is involved in mediating inhibition and that the S3/S4 region may be of particular importance. In addition, our data are consistent with the AID-GID region of the I/II loop also playing an important role. To determine whether these regions are in fact sufficient to mediate α1B-like inhibition, we created a series of positive chimeras by transferring regions from α1B into the α1E background. The N terminus alone increased inhibition when transferred into the α1E background (construct B1–93; Table 1; Figs. 2, 3, and 4), consistent with the results of Page et al. (1998) as well as our own previous findings (Simen and Miller, 1998). The further exchange of the AID-GID region of the I/II loop increased inhibition to a small extent (construct B1–93;382–432; Fig. 2; Table 1). The further addition of the S3/S4 region had relatively little effect on inhibition (construct B1–93;185–190;382–432; Fig. 2; Table 1), suggesting that the S3/S4 region from domain I is not by itself sufficient, in contrast to our results with negative chimeras. However, when the ND1 region (domain I S1/S2 to S3/S4) was exchanged along with the N terminus and AID-GID region (construct B1–93;120–190;382–432) the resulting construct showed inhibition that was similar to that of native α1B (Figs. 2, 3, and 4; Table 1). This triple-positive chimera (construct B1–93;120–190;382–432) showed significantly more inhibition ($P < .05$) than the N-terminal chimera (construct B1–93), suggesting that 70 amino acids from domain I in the region from S1/S2 to S3/S4 and the AID-GID region of the I/II loop in addition to the N terminus contribute to inhibition when transferred into the α1E background. Because the domain I fragment transferred in this triple chimera lacked sequence from the α1B S4-S4/S5 region, the lack of involvement of the Val/Ile difference at position 215 (as suggested above) is strongly supported. Facilitation generally paralleled inhibition (Fig. 2; Table 1) and is discussed in detail below.

Mutations to G-Protein Binding Sites. These data together with a number of other studies (e.g., Page et al., 1997; Simen and Miller, 1998; Stephens et al., 1998) suggest that the differences between α1B and α1E in terms of G protein

inhibition do not primarily involve regions thought to directly mediate G protein binding (such as the I/II loop) but are largely determined by regions not thought to mediate direct binding (such as regions in domain I and the N terminus). We were therefore interested in assessing the role of some of the known G protein binding regions in the α1B channel. Mutagenesis of the "QXXER" motif sequence "QQIER" (De Waard et al., 1997; Herlitze et al., 1997) in the AID region of



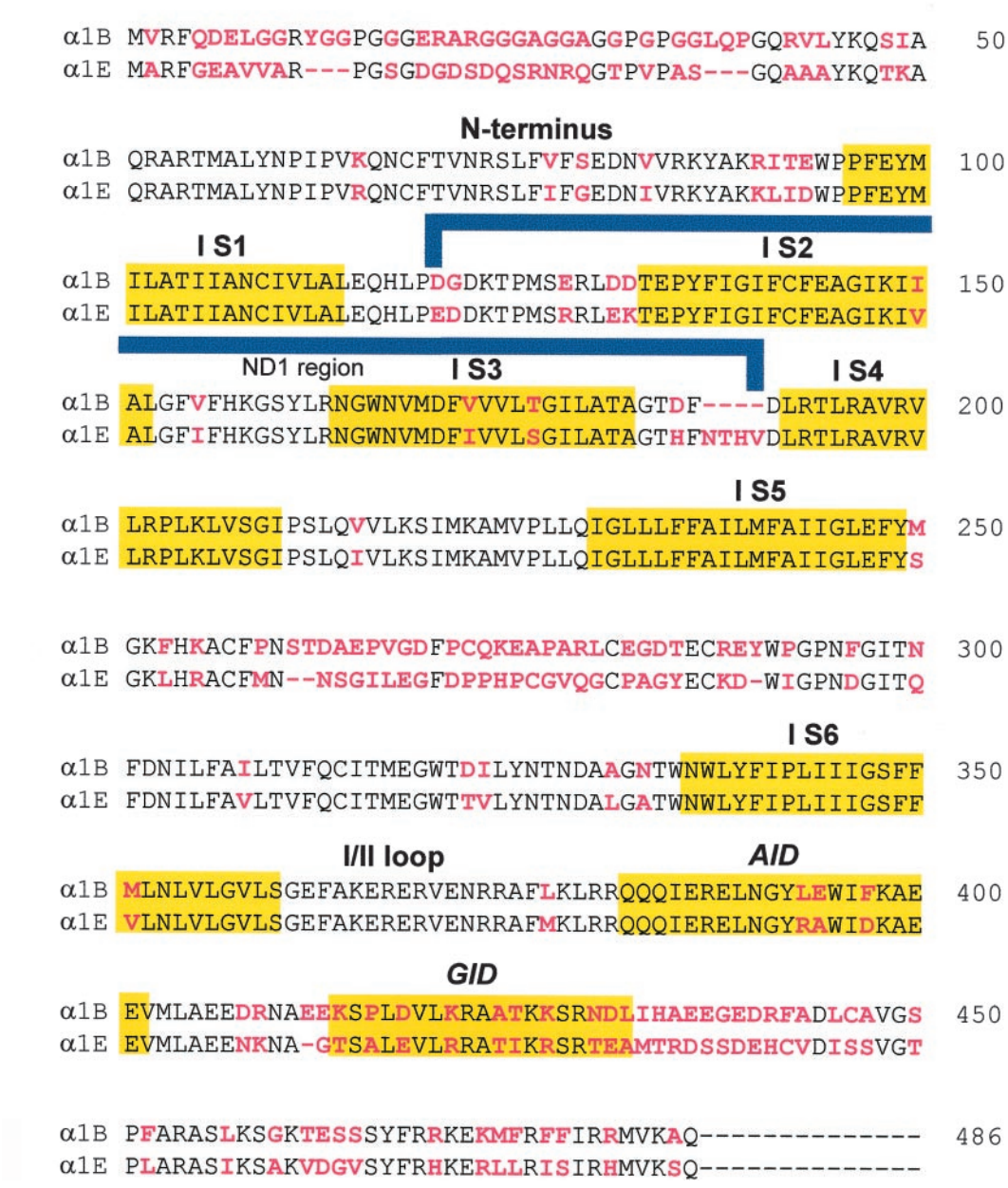


Fig. 5. Alignment of the N terminus, domain I, and I/II loop region of α1B and α1E Ca^{2+} channels. Differences are shown in red, membrane-spanning regions, G$\beta\gamma$ binding regions, and G β subunit binding regions are shown enclosed in gray boxes. The ND1 region (see text) is shown in blue. G$\beta\gamma$ subunits interact with the AID and GID regions, and Ca^{2+} β subunits interact with the AID region (see text). The majority of the differences between the two channels in the domain I region are in the extracellular loops (S1/S2, S3/S4, and S5/S6). Amino acid positions were numbered according to this alignment throughout the text.

the I/II loop of α1B to "AQIAA" (construct BQ1) or "AAAAA" (construct BQ2) had relatively little effect on inhibition (Fig. 2; Table 1). In fact, both constructs showed somewhat increased facilitation compared with α1B, but this difference was not statistically significant (Fig. 2; Table 1). These results are similar to those of Herlitze et al. (1997), who found that mutagenesis of the "QQIER" sequence of α1A to "AQIAA" increased facilitation to some extent. Deletion of the putative C-terminal G$\beta\gamma$ site "CC14" (construct Δ2037–2087; Qin et al., 1997) or truncation of the C terminus to delete the CC14 site as well as the putative Gα site (Furukawa et al., 1998b; construct Δ1875–2339) had relatively little effect on inhibition (Fig. 2; Table 1). These results may partially help to explain our previous observations that exchange of the I/II loop or C-terminal regions of α1B and α1E in isolation had little effect on inhibition (Simen and Miller, 1998). It seems that mutagenesis of these G protein sites in isolation is not sufficient to block inhibition of α1B by G proteins, and exchange of these regions would not be expected, therefore, to affect inhibition by virtue of any one of these particular sites. Because we did not mutate residues within the GID region, we cannot be certain of its role. However, our results are consistent with the notion that the GID region is an important locus of G$\beta\gamma$ interaction with the I/II loop. This is consistent with the high affinity of G$\beta\gamma$ for the C-terminal end of the I/II loop (De Waard et al., 1997) and the clear effects of phosphorylation (Zamponi et al., 1997; Hamid et al., 1999) and splice variants (Bourinet et al., 1999) in this region.

Voltage Dependence of Inhibition. The voltage dependence of inhibition of various Ca^{2+} channel constructs was examined by determining inhibition at various test potentials (Fig. 6). Two differences were noted between α1B and α1E (Fig. 6A). First, α1B channels showed a larger maximum level of inhibition than α1E channels. Second, both channels showed decreasing levels of inhibition at increasingly positive test potentials, but inhibition declined more rapidly for α1E channels than for α1B channels (Fig. 6A). When chimeric constructs were compared in this manner (Fig. 6B), two patterns emerged. The transfer of the N terminus of α1B into α1E (construct B1–93) caused little change in the maximum level of inhibition compared with α1E, but inhibition did not decline with increasing test potential. These results suggest that the N terminus may play a role in determining the voltage dependence of inhibition, and the domain I and I/II loop region may play a role in determining the maximum extent of inhibition. Consistent with this notion, transfer of the N terminus as well as regions from domain I and the I/II loop (ND1 and the AID-GID region) into α1E (construct B1–93;120–190;382–432) resulted in a construct with a maximum extent of inhibition similar to α1B as well as a slow decline of inhibition with increasing test potentials (Fig. 6B).

Facilitation. The degree to which a depolarizing prepulse to +90 mV relieved inhibition (i.e., caused facilitation) for a particular construct generally paralleled inhibition (Fig. 2; Table 1). In fact, the two parameters were highly correlated in an approximately linear fashion (Fig. 7A). However, some constructs showed more or less facilitation than expected given the degree of inhibition obtained and could be identified as "outliers" with respect to the regression relationship between the two parameters (Fig. 7A). One construct showed a disproportionately large amount of facilitation (construct Δ1875–2339), and other constructs showed disproportionately small amounts of facilitation (constructs B1–93;120–190, B1–93;120–190;382–432, and E250–377; Fig. 7A). These differences in facilitation are not caused by differences in current expression levels, because current levels did not differ significantly between the various constructs ($p > .05$). Although sequences from the N terminus, domain I, and the I/II loop effectively accounted for the differences in inhibition between α1B and α1E, the differences between these two Ca^{2+} channels in terms of facilitation were not adequately accounted for by these sequences in our experiments.

There are at least two possible reasons for the small degree of facilitation seen in certain constructs. First, it is possible that the determinants of inhibition and facilitation are at least partially distinct. For example, we have observed previously (Simen and Miller, 1998) that the C terminus may play some small role in determining facilitation but seems to play no role in determining inhibition. A second model involves a "masking" of facilitation by voltage-dependent inactivation from the prepulse. Meza and Adams (1998) have similarly suggested that the small degree of facilitation of α1E current amplitudes may be caused by voltage-dependent inactivation. In this study, we attempted to adjust for the effects of inactivation by calculating inactivation from the prepulse in the presence of norBNI (see *Materials and Methods*) and adjusting facilitation accordingly. However, this

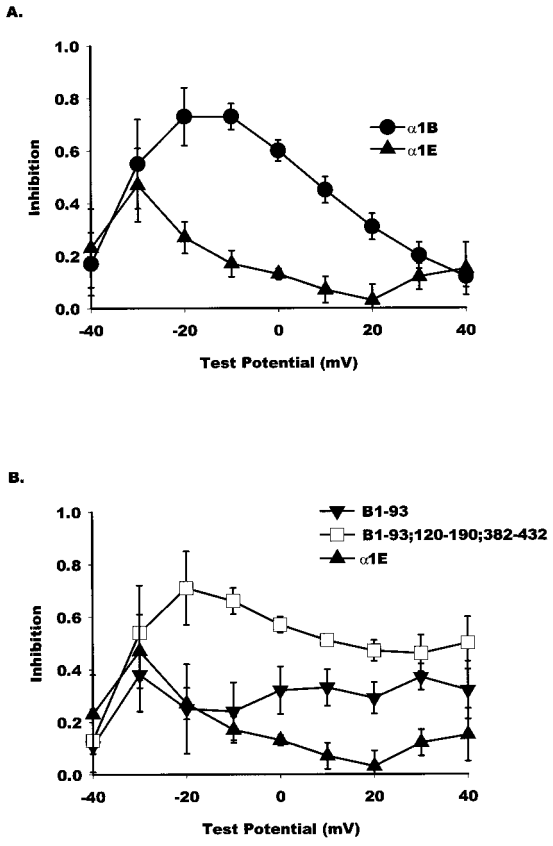


Fig. 6. Inhibition was calculated at each test pulse potential as the fractional difference between the pulse 1 current in the presence of U69593 compared with the pulse 1 current in the presence of norBNI (see *Materials and Methods*). A, inhibition of α1B (see legend) compared with α1E (see legend) at individual test potentials. B, comparison of inhibition of constructs B1–93, B1–93;120–190;382–432, and α1E (see legend) at individual test potentials.

procedure assumes that inactivation and inhibition are independent.

The overall correlation between facilitation and inhibition for the various constructs would suggest that the two have at least similar structural determinants. To explore one possible mechanism responsible for some of these divergent constructs, we examined in greater detail constructs showing similarly large degrees of inhibition with very different levels of facilitation (Fig. 7B). Facilitation of these constructs was inversely correlated with inactivation from the prepulse (Fig. 7B), suggesting that a "masking" of facilitation by inactivation may be at least partially responsible for the disproportionately low facilitation of the B1–93;120–190;382–432 and E250–377 constructs. This "masking" of facilitation by inactivation may have been exacerbated in these studies by coexpression of the β1b isoform of the Ca^{2+} β subunit (discussed below). Inactivation was probably also influenced by exchange of the I S5/S6 to proximal I/II loop region that is thought to play an important role in determining the differences in inactivation between α1B and α1E (discussed below).

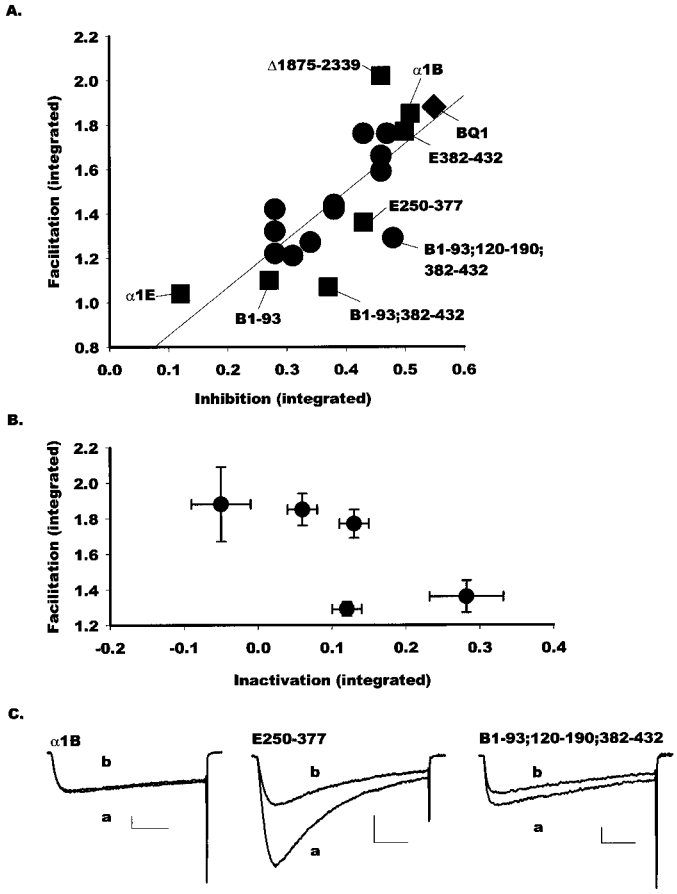


Fig. 7. A, average inhibition and facilitation values from the constructs described in this study are shown with a linear regression fit. B, to assess the potential role of voltage dependent inactivation in "masking" facilitation, facilitation was compared with inactivation across the entire voltage range (see *Materials and Methods*). Averages and bivariate error bars representing standard errors are shown for constructs with high levels of inhibition (Fig. 3; Table 1) with very different levels of facilitation. C, sample currents before a prepulse (a) and after a prepulse (b) from selected constructs in the presence of norBNI are shown to illustrate differences in inactivation. Scale bars represent 500 pA and 100 ms.

Discussion

The results of the present study further define the region in the N-terminal portion of the Ca^{2+} channel responsible for the differences in inhibition between α1B and α1E Ca^{2+} channels to the N terminus, 70 amino acids from the N-terminal portion of domain I between S1/S2 and S3/S4, and 50 amino acids from the I/II loop. The α1B and α1E channels differ in the region from S1/S2 to S3/S4 at only 14 positions, and all but four of these positions are extracellular. Three of the four remaining differences are in transmembrane regions, and the one position localized cytoplasmically is a conservative Val/Ile difference in the S2/S3 loop (Fig. 5). The S2/S3 loop is only 11 amino acids long and therefore seems unlikely to be involved in G$\beta\gamma$ binding, although we cannot rule this out on the basis of our data. These results confirm that domain I plays an important role in determining sensitivity to inhibition by G proteins and that a relatively small region of domain I is sufficient to mediate these effects with regard to the differences between α1B and α1E. It is important to note that the chimera approach used in these studies is limited to identifying structures involved in G protein regulation that differ between the two Ca^{2+} channel isoforms and cannot identify regions that are homologous between the two channels. It is possible, therefore, that a larger portion of the Ca^{2+} channel plays a role in G protein regulation than we can resolve in these experiments.

Our findings with respect to the S3/S4 segment of domain I are complex. Negative chimeras suggested that the S3/S4 segment was sufficient to reduce inhibition significantly only in the "context" of changes to the I/II loop. However, positive chimeras suggested that this region was not sufficient to enhance inhibition. The S3 and S3/S4 segments of domain I have been shown to mediate the differences in activation kinetics between cardiac and skeletal muscle L-type Ca^{2+} channels (Nakai et al., 1994). The S3/S4 segment has also been shown to affect the activation kinetics of Shaker potassium channels (Mathur et al., 1997). It is possible therefore to speculate that this region could play some role in mediating the slowing of activation seen during G protein inhibition, although our data indicate that this region is not sufficient in itself to do so. Interestingly, there is a strong correlation between the length of the S3/S4 segment in domain I and sensitivity to G protein inhibition among Ca^{2+} channels [α1A (8) = α1B (8)] > α1E (12) > α1C (24).

Our results with respect to the N terminus are consistent with those of Page et al. (1998), who have observed that exchange of the N terminus from α1B into α1E increases the inhibition of the resulting construct to a level intermediate between α1B and α1E. These results are also consistent with our previous observations that the exchange of the N terminus of α1B increased the modulation of a chimeric Ca^{2+} channel over and above that seen when domain I and the I/II loop were transferred alone (Simen and Miller, 1998). It is possible that the N terminus physically binds G proteins. However, Qin et al. (1997) observed that the N terminus of α1E channels does not bind G$\beta\gamma$ in vitro, suggesting that the N terminus may not be involved in direct G protein binding. It has recently been demonstrated that the N terminus of α1A has a low affinity Ca^{2+} β4 subunit binding site (Walker et al., 1999). It is possible therefore that the N terminus plays

a role in the kinetic changes produced by G protein binding, perhaps by virtue of differential β subunit interactions.

Theoretically, G protein inhibition must involve an interplay between sequences that physically interact with G proteins (binding) and regions that are involved with producing the conformational changes associated with inhibition (efficacy). Residues in domain I may play some role in the immobilization of gating charge that follows G protein binding. Interestingly, the C-terminal end of the ND1 region (S3/S4 segment) is attached to the S4 voltage-sensing region of domain I. Therefore, this segment of domain I is positioned in such a way that it could directly affect voltage sensing of the channel, although other models are also possible. Garcia et al. (1997) showed that neutralization of single positive charges in the S4 segment of domains I and III but not domains II or IV of L-type Ca^{2+} channels altered activation kinetics. Although these findings have not yet been extended to non-DHP sensitive Ca^{2+} channels, they may suggest that domain I and III of Ca^{2+} channels play a particularly important role in determining channel activation. We therefore propose that regions in domain I may play a role in "efficacy" rather than in directly binding G proteins. Efficacy can be incorporated into models of G protein modulation by positing that Ca^{2+} channels bind G proteins but are not modulated until a distinct conformational change takes place. A recent article on the interpretation of mutagenesis data with respect to this binding/efficacy distinction has appeared (Colquhoun, 1998) and emphasizes the difficulties in distinguishing between effects on binding and effects on efficacy experimentally.

The apparent increase in facilitation caused by alanine mutagenesis of the AID region is consistent with previous findings (Herlitze et al., 1997). It has been demonstrated that alanine mutagenesis of the Q or R residues in the QXXER motif to A blocks binding of G$\beta\gamma$ to AID in vitro (De Waard et al., 1997). The small increase in facilitation resulting from alanine substitutions in the QXXER motif may suggest that G$\beta\gamma$ dissociation from this region is one step in the pathway leading to Ca^{2+} channel facilitation. The overall lack of effect of mutations to certain G protein binding regions with respect to inhibition is consistent with multiple regions on Ca^{2+} channels interacting with G proteins such that disruption of one site does not block inhibition or binding to the channel as a whole. Alternatively, these data can be interpreted as evidence for the notion that the GID region of the I/II loop is primarily responsible for G$\beta\gamma$ binding.

Because we carried out these experiments with the β1b subunit exclusively, we cannot be certain of the importance of the β subunit isoform in determining inhibition in our system. A number of studies have suggested that β subunits affect inhibition by G proteins (e.g., Campbell et al., 1995; Roche et al., 1995; Bourinet et al., 1996; Roche and Treistman, 1998a, 1998b). In addition, it has been shown that different β subunits support voltage-dependent inactivation to varying extents, with the β2a subunit supporting significantly less inactivation than other β subunits (e.g., Olcese et al., 1994; Qin et al., 1996). It is likely that the β1b subunit used in our study contributed to the degree of inactivation that we observed for α1E and some of our chimeric constructs. This inactivation probably exacerbated the degree of "masking" of facilitation that we observed (discussed above), as suggested by Meza and Adams (1998). It has been shown previously that a region extending from the S5/S6 loop of domain I to the proximal portion of the I/II loop contributes to the differences in inactivation between the ray α1E channel and the rabbit α1A channel (Zhang et al., 1994). We have shown previously that this also seems to be the case for human α1B and α1E Ca^{2+} channels (Simen and Miller, 1998). The enhanced inactivation of the E250–377 construct compared with α1B (Fig. 7C) provides further support for the involvement of this region in determining the differences in inactivation between α1B and α1E. Because our positive chimeras did not contain α1B sequence in this region, it is likely that they showed enhanced inactivation compared with native α1B as a consequence. This may account for the fact that we were unable to reconstitute facilitation in our positive chimeras. We cannot be certain whether the determinants of facilitation and inhibition are identical on the basis of our data.

The differences in sensitivity to inhibition by G proteins between α1B and α1E Ca^{2+} channels seem to involve contributions from the N terminus, a region of domain I extending from S1/S2 to S3/S4, and a region of the I/II loop containing Ca^{2+} β and G$\beta\gamma$ binding regions. The differences in sensitivity to G protein inhibition between α1B and α1E channels therefore involves regions involved in G protein binding (the I/II loop) as well as regions that probably do not bind G proteins (N-terminal portion of domain I and the N terminus) that may play a role in determining conformation change as a consequence of G protein binding to other portions of the channel.

Acknowledgments

We are grateful to Dr. R. Harpold for the Ca^{2+} channel subunits used in these studies, to Dr. G.I. Bell for the opioid receptor clones used in this work, and to B. Simen and D. Ren (University of Chicago) for technical assistance with the molecular techniques used in this study.

References

Bourinet E, Soong TW, Sutton K, Slaymaker S, Mathews E, Monteil A, Zamponi GW, Nargeot J and Snutch TP (1999). Splicing of α1A subunit gene generates phenotypic variants of P- and Q-type calcium channels. *Nat Neurosci* **2:**407–415.

Bourinet E, Soong TW, Stea A and Snutch TP (1996) Determinants of the G protein-dependent opioid modulation of neuronal calcium channels. *Proc Natl Acad Sci USA* **93:**1486–1491.

Campbell V, Berrow N, Brickley K, Fitzgerald EM and Dolphin AC (1995) Inhibition of the interaction of G protein G(o) with calcium channel by the calcium beta-subunit in rat neurones. *J Physiol (Lond)* **485:**365–372.

Canti C, Page KM, Stephens GJ and Dolphin AC (1999) Identification of residues in the N-terminus of α1B critical for inhibition of the voltage-dependent calcium channel by G$\beta\gamma$. *J Neurosci* **19:**6855–6864.

Colquhoun D (1998) Binding, gating, affinity and efficacy: The interpretation of structure- activity relationships for agonists and of the effects of mutating receptors. *Br J Pharmacol* **125:**924–947.

De Waard M, Liu H, Walker D, Scott VE, Gurnett CA and Campbell KP (1997) Direct binding of G-protein $\beta\gamma$ complex to voltage-dependent calcium channels. *Nature (Lond)* **385:**446–450.

Dunlap K, Luebke JI and Turner TJ (1995) Exocytotic Ca^{2+} channels in mammalian central neurons. *Trends Neurosci* **18:**89–98.

Furukawa T, Nukada T, Mori Y, Wakamori M, Fujita Y, Ishida H, Fukuda K, Kato S and Yoshii M (1998a) Differential interactions of the C terminus and the cytoplasmic I-II loop of neuronal Ca^{2+} channels with G-protein α and $\beta\gamma$ subunits. I. Molecular determination. *J Biol Chem* **273:**17585–17594.

Furukawa T, Miura R, Mori Y, Strobeck M, Suzuki K, Ogihara Y, Asano T, Morishita R, Hashii M, Higashida H, Yoshii M and Nukada T (1998b) Differential interactions of the C terminus and the cytoplasmic I-II loop of neuronal Ca^{2+} channels with G-protein α and $\beta\gamma$ subunits. II. Evidence for direct binding. *J Biol Chem* **273:**17595–17603.

Garcia J, Nakai J, Imoto K and Beam KG (1997) Role of S4 segments and the leucine heptad motif in the activation of an L-type calcium channel. *Biophys J* **72:**2515–2523.

Hamid J, Nelson D, Spaetgens R, Dubel SJ, Snutch TP and Zamponi GW (1999)

Identification of an integration center for cross-talk between protein kinase C and G protein modulation of N-type calcium channels. *J Biol Chem* **274:**6195–6202.
Herlitze S, Hockerman GH, Scheuer T and Catterall WA (1997) Molecular determinants of inactivation and G protein modulation in the intracellular loop connecting domains I and II of the calcium channel α1A subunit. *Proc Natl Acad Sci USA* **94:**1512–1516.
Mathur R, Zheng J, Yan Y and Sigworth FJ (1997) Role of the S3–S4 linker in Shaker potassium channel activation. *J Gen Physiol* **109:**191–199.
Meza U and Adams B (1998) G-protein-dependent facilitation of neuronal α1A, α1B, and α1E Ca channels. *J Neurosci* **18:**5240–5252.
Miller RJ (1998) Presynaptic receptors. *Annu Rev Pharmacol Toxicol* **38:**201–227.
Nakai J, Adams BA, Imoto K and Beam KG (1994) Critical roles of the S3 segment and S3–S4 linker of repeat I in activation of L-type calcium channels. *Proc Natl Acad Sci USA* **91:**1014–1018.
Olcese R, Qin N, Schneider T, Neely A, Wei X, Stefani E and Birnbaumer L (1994) The amino terminus of a calcium channel beta subunit sets rates of channel inactivation independently of the subunit's effect on activation. *Neuron* **13:**1433–1438.
Page KM, Canti C, Stephens GJ, Berrow NS and Dolphin AC (1998) Identification of the amino terminus of neuronal Ca^{2+} channel α1 subunits α1B and α1E as an essential determinant of G-protein modulation. *J Neurosci* **18:**4815–4824.
Page KM, Stephens GJ, Berrow NS and Dolphin AC (1997) The intracellular loop between domains I and II of the B-type calcium channel confers aspects of G-protein sensitivity to the E-type calcium channel. *J Neurosci* **17:**1330–1338.
Pragnell M, De Waard M, Mori Y, Tanabe T, Snutch TP and Campbell KP (1994) Calcium channel β-subunit binds to a conserved motif in the I-II cytoplasmic linker of the α1-subunit. *Nature (Lond)* **368:**67–70.
Qin N, Olcese R, Zhou J, Cabello OA, Birnbaumer L and Stefani E (1996) Identification of a second region of the β-subunit involved in regulation of calcium channel inactivation. *Am J Physiol* **271:**C1539–C1545.
Qin N, Platano D, Olcese R, Stefani E and Birnbaumer L (1997) Direct interaction of g$\beta\gamma$ with a C-terminal g$\beta\gamma$-binding domain of the Ca^{2+} channel α1 subunit is responsible for channel inhibition by G protein-coupled receptors. *Proc Natl Acad Sci USA* **94:**8866–8871.
Reid CA, Bekkers JM and Clements JD (1998) N- and P/Q-type Ca^{2+} channels mediate transmitter release with a similar cooperativity at rat hippocampal autapses. *J Neurosci* **18:**2849–2855.
Roche JP, Anantharam V and Treistman SN (1995) Abolition of G protein inhibition of α1A and α1B calcium channels by co-expression of the β3 subunit. *FEBS Lett* **371:**43–46.
Roche JP and Treistman SN (1998a) The Ca^{2+} channel β3 subunit differentially modulates G-protein sensitivity of α1A and α1B Ca^{2+} channels. *J Neurosci* **18:**878–886.
Roche JP and Treistman SN (1998b) Ca^{2+} channel β3 subunit enhances voltage-dependent relief of G-protein inhibition induced by muscarinic receptor activation and G$\beta\gamma$. *J Neurosci* **18:**4883–4890.
Simen AA and Miller RJ (1998) Structural features determining differential receptor regulation of neuronal Ca channels. *J Neurosci* **18:**3689–3698.
Stephens GJ, Canti C, Page KM and Dolphin AC (1998) Role of domain I of neuronal Ca^{2+} channel α1 subunits in G protein modulation. *J Physiol (Lond)* **509:**163–169.
Toth PT, Shekter LR, Ma GH, Philipson LH and Miller RJ (1996) Selective G-protein regulation of neuronal calcium channels. *J Neurosci* **16:**4617–4624.
Walker D, Bichet D, Geib S, Mori E, Cornet V, Snutch TP, Mori Y and De Waard M (1999) A new β subtype-specific interaction in α1A subunit controls P/Q- type Ca^{2+} channel activation. *J Biol Chem* **274:**12383–12390.
Wu LG, Borst JG and Sakmann B (1998) R-type Ca^{2+} currents evoke transmitter release at a rat central synapse. *Proc Natl Acad Sci USA* **95:**4720–4725.
Wu LG and Saggau P (1997) Presynaptic inhibition of elicited neurotransmitter release. *Trends Neurosci* **20:**204–212.
Yassin M, Zong S and Tanabe T (1996) G-protein modulation of neuronal class E (α1E) calcium channel expressed in GH3 cells. *Biochem Biophys Res Commun* **220:**453–458.
Zamponi GW, Bourinet E, Nelson D, Nargeot J and Snutch TP (1997) Crosstalk between G proteins and protein kinase C mediated by the calcium channel α1 subunit. *Nature (Lond)* **385:**442–446.
Zhang JF, Ellinor PT, Aldrich RW and Tsien RW (1994) Molecular determinants of voltage-dependent inactivation in calcium channels. *Nature (Lond)* **372:**97–100.
Zhang JF, Ellinor PT, Aldrich RW and Tsien RW (1996) Multiple structural elements in voltage-dependent Ca^{2+} channels support their inhibition by G proteins. *Neuron* **17:**991–1003.

Send reprint requests to: Richard J. Miller, Ph.D., Dept. of Neurobiology, Pharmacology, and Physiology, University of Chicago, 947 East 58th Street, Chicago IL 60637. E-mail: rjmx@midway.uchicago.edu