Structural Features Determining Differential Receptor Regulation of Neuronal Ca Channels

Arthur A. Simen and Richard J. Miller

Department of Pharmacological and Physiological Sciences, Committee on Neurobiology, University of Chicago, Chicago, Illinois 60637

Dihydropyridine-insensitive Ca channels are subject to direct receptor G-protein-mediated inhibition to differing extents. α_{1B} channels are much more strongly modulated than α_{1E} channels. To understand the structural basis for this difference, we have constructed and expressed various α_{1B} and α_{1E} chimeric Ca channels and examined their regulation by κ-opioid receptors. Replacement of the first membrane-spanning domain of α_{1E} with the corresponding region of α_{1B} resulted in a chimeric Ca channel that was modulated by κ-opioid receptors to a significantly greater extent than α_{1E}. Transfer of the N terminus and I/II loop from α_{1B} in addition to domain I resulted in a chimeric channel that was modulated to the same extent as α_{1B}. Other regions of the molecule do not appear to contribute significantly to the degree of inhibition obtained, although the C terminus may contribute to facilitation.

Key words: calcium channels; G-proteins; opioid receptors; modulation; structure–function; inverse agonism

Voltage-sensitive Ca channels are one of the most important classes of molecules used by excitable cells for the transduction of cellular excitability into the intracellular Ca signals that regulate events such as muscle contraction and the release of neurotransmitters. Inhibition of Ca influx through these channels by G-protein-linked receptors is an important means by which neurotransmitters and drugs can regulate the strength of synaptic transmission (Miller, 1990; Rhim et al., 1994). Elucidation of the primary structure of Ca channels has demonstrated that they consist of a family of related proteins that has two branches. Each branch consists of three different proteins that differ in their pharmacology and, in particular, in their sensitivity to dihydropyridine (DHP) drugs. It is thought that the subfamily of DHP-insensitive Ca channels can be regulated by receptors through the direct interaction of G-protein $\beta\gamma$ subunits with the major pore-forming (α_1) subunit of the channel (Herlitze et al., 1996; Ikeda, 1996; De Waard, 1997; Zamponi et al., 1997). Expression studies have indicated that all three types of DHP-insensitive Ca channels (α_{1A}, α_{1B}, and α_{1E}) are regulated by G-proteins, although there are large differences in their susceptibility to modulation. Thus, α_{1B} is the most sensitive to G-protein modulation, and α_{1E} is the least sensitive (Toth et al., 1996; Yassin et al., 1996).

The structural basis for the G-protein regulation of Ca channels has received considerable attention, and recent studies have started to reveal the sites of interaction between G-protein $\beta\gamma$ subunits and Ca channels. Initially, it was shown that G-protein $\beta\gamma$ subunits could interact with two distinct sites in the intracellular loop connecting the first two domains of the α_1 subunit (I/II loop) (De Waard et al., 1997; Qin et al., 1997; Zamponi et al., 1997). One of these sites contains the QXXER motif, which has been shown to be important in the binding of $\beta\gamma$ subunits to other effectors (Pragnell et al., 1994; Chen et al., 1995). However, although there is little doubt that $\beta\gamma$ subunits can interact with this region of the channel, controversy exists as to the significance of this interaction. Experiments designed to demonstrate the importance of this interaction for G-protein regulation of Ca channels have yielded mixed results. Further studies have indicated that $\beta\gamma$ subunits can also bind to a region in the C-terminal tail of sensitive Ca channels (Qin et al., 1997), and there are data suggesting that this interaction may be important in G-protein modulation (Qin et al., 1997; Zhang et al., 1996). In either case, because binding to these sites is a common element to all three DHP-insensitive Ca channels, further structural features are likely to be required to determine differential sensitivity to G-proteins. To identify structural elements involved in determining the differential sensitivity of α_{1B} compared with α_{1E} channels, we have created a variety of chimeric Ca channels in which different parts of α_{1B-1} were transferred into an α_{1E-3} background. Exchange of domain I alone yields a chimeric channel with considerably increased sensitivity to modulation, but the further exchange of the I/II linker and the N terminus is required to obtain a chimeric channel with sensitivity to modulation equivalent to native α_{1B-1}. Transfer of the C terminus of α_{1B-1} appears to enhance facilitation but not inhibition.

MATERIALS AND METHODS

Cell culture. tsA-201 cells (a gift from Dr. W. A. Horne, Stanford University) were cultured in MEM (Life Technologies, Gaithersburg, MD), 10% fetal bovine serum (Life Technologies), and 1% penicillin-streptomycin (Life Technologies) at 37°C in 5% CO_2. When cells reached 40–70% confluency they were replated in 35 mm dishes and were transfected 4–10 hr later with a total of 5–7 μg of DNA (2–5 μg of α_1, 1.7 μg of α_{2B}, 0.8 μg of β_{1B}, 1.5 μg of κ_1R or δR, and 1 μg of CD8-α) by polyethyleneimine-mediated transfection (Boussif et al., 1996). Cells were washed with culture medium 1–2 hr after transfection, and fresh 10% serum-containing medium was added. Cells were detached from

dishes with HBSS (Ca- and Mg-free; Life Technologies) 30–40 hr later and were replated on poly-L-lysine-coated coverslips. Cells were recorded from 36 to 80 hr after transfection. cDNA for CD8α was a gift from Dr. J. Bluestone (University of Chicago). The κ_1R and δR constructs were gifts from Dr. G. I. Bell (Howard Hughes Medical Institute, University of Chicago) and have been described previously (Yasuda et al., 1993). Expression of α_{2B} and β_{1B} were verified by reverse transcription-PCR and Northern blotting (data not shown).

Electrophysiology. Currents were recorded in a solution containing 15 mM BaCl$_2$ (5 mM BaCl$_2$ in the case of the current–voltage data), 150 mM TEA-Cl, and 10 mM HEPES, pH 7.4, with TEA-OH or *N*-methyl-D-glucamine. Pipette solutions were made according to the method of Bean (1992). U69593 (Sigma, St. Louis, MO; or Research Biochemicals, Natick, MA) and norbinaltrophimine (norBNI; Research Biochemicals) were made as concentrated stock solutions in 100% ethanol. ICI-174,864 was made as a 10 mM stock solution in DMSO. [D-Pen^{2,5}]-enkephalin (DPDPE) was made as a 10 mM stock solution in sterile water. U69593, DPDPE, and ICI-174864 were added to the bath solution at concentrations of 1 μM, and norBNI was added at 100 nM. Data were recorded under the control of pClamp6 software (Axon Instruments, Foster City, CA) with an Axon 200A amplifier at an acquisition rate of 10 kHz and were filtered with a four-pole Bessel filter at 2 kHz. Pipettes were typically 1–3 mΩ in resistance, and series resistance was compensated >80% in all cases. To assay G-protein modulation, currents were elicited by a dual-pulse protocol consisting of two 50 msec depolarizations to 0 mV from a holding potential of −90 mV, separated by 800 msec at the holding potential, with a 30 msec, 90 mV depolarization (prepulse) ending 5 msec before the second pulse. Cells expressing CD8α were identified by incubation with anti-CD8α-coated beads (Dynal, Great Neck, NY), and bead-decorated cells were chosen exclusively for recordings.

Data analysis. Data were analyzed off-line using Clampfit (Axon Instruments), Systat (SPSS Inc., Chicago, IL), MATLAB (MathWorks Inc.), and custom-written software. Current inhibition was estimated as follows. Peak current amplitudes were measured and were adjusted to remove any slow linear "runup" or "rundown" by subtraction of linear function fits to the baselines. A series of adjacent current amplitudes was averaged just before drug exposure, during U69593 exposure at the peak of the drug effect, and during the peak of the norBNI response. Currents in the presence of U69593 were taken as an estimate of current in the presence of maximally activated receptor $I(R^*)$. When norBNI caused amplitudes to increase beyond baseline values before drug exposure, currents were taken as an estimate of current in the presence of maximally inactive receptor $I(R)$. Otherwise, baseline values were used to estimate $I(R)$. "Total modulation" was then defined as $[I(R) - I(R^*)]/I(R)$.

Relief of inhibition by the depolarizing prepulse was quantified by calculating a "corrected prepulse ratio" to allow for comparisons between constructs while correcting for differences in inactivation caused by the prepulse. Current ratios in the presence of U69593, $I(+pp)/I(-pp)$, were calculated and corrected for differences in inactivation between constructs by multiplying by the ratio $I(-pp)/I(+pp)$ in the presence of norBNI. Facilitation was expressed as a simple current ratio, $I(+pp)/I(-pp)$, for the purpose of describing the intrinsic receptor activity of the κ_1R with respect to α_{1B-1} currents.

Unless otherwise noted, comparisons between the constructs in terms of current inhibition and relief of inhibition were conducted by means of a one-way ANOVA followed by *post hoc* analysis by the Tukey multiple-comparison procedure (Neter et al., 1985). Averages were expressed as mean ± SEM.

Construction of cDNAs. The chimeric contructs were created using overlap extension PCR (Ho et al., 1989; Horton et al., 1989, 1990) and PCR-mediated site-directed mutagenesis. PCR products were cloned into pGEM-T (Promega, Madison, WI), pGEM-T-EZ (Promega), or pT7-Blue (Novagen, Madison, WI) and sequenced using semiautomated DNA sequencing (ABI 377; Perkin-Elmer, Oak Brook, IL). All α_1 subunit expression contructs were created by ligation of recombinant and native sequences into the pcDNA3.1 expression vector (Invitrogen, San Diego, CA). Final constructs were confirmed by a combination of restriction analysis and DNA sequencing. The native α_{1B-1} construct bBbBbBbBb consisted of residues 1–2340 of GenBank accession number 284339 (a gift from Dr. R. Harpold, SIBIA Neurosciences). The native α_{1E-3} construct eEeEeEeEe consisted of residues 1–2271 of GenBank accession number 1082919 (a gift from Dr. R. Harpold, SIBIA Neurosciences). The construct bBbBbEeEb consisted of α_{1B-1} 1–1145, α_{1E-3} 1149–1724, and α_{1B-1} 1710–2340. The construct bBbBbEeEe consisted of

α_{1B-1} 1–1145 and α_{1E-3} 1149–2271. The construct bBbEeEeEe consisted of α_{1B-1} 1–482 and α_{1E-3} 477–2271. The construct eBbEeEeEe consisted of α_{1E-3} 1–89, α_{1B-1} 96–482, and α_{1E-3} 477–2271. The construct eBeEeEeEe consisted of α_{1E-3} 1–89, α_{1B-1} 96–355, and α_{1E-3} 351–2271. The construct eEbEeEeEe consisted of α_{1E-3} 1–350, α_{1B-1} 356–482, and α_{1E-3} 477–2271. The construct eEbBbEeEe consisted of α_{1E-3} 1–350, α_{1B-1} 356–1145, and α_{1E-3} 1149–2271. The construct eEeEbEeEe consisted of α_{1E-3} 1–703, α_{1B-1} 710–1145, and α_{1E-3} 1149–2271. The construct eEbEbEeEe consisted of α_{1E-3} 1–350, α_{1B-1} 356–482, α_{1E-3} 477–703, α_{1B-1} 710–1145, and α_{1E-3} 1149–2271. The construct e(E$_{90-309}$ B$_{315-355}$)bEeEeEe consisted of α_{1E-3} 1–309, α_{1B-1} 315–482, and α_{1E-3} 477–2271. The α_{2B}/δ and β_{1B} Ca channel subunit cDNAs were gifts from Dr. R. Harpold (Sibia Neurosciences) and were subcloned into the pCMV6c expression vector.

RESULTS

Intrinsic activity of the κ-opioid receptor and effects on α_{1B} and α_{1E}

After transfection of tsA201 cells (Margolskee et al., 1993) with the Ca channel subunits α_{1-B1}, β_{1B}, and α_{2B}, the κ_1-opioid receptor (κ_1R), and CD8α, we observed that α_{1-B1} currents often showed a degree of prepulse facilitation, suggestive of G-protein-mediated inhibition, even without previous exposure to κ_1R agonists (Fig. 1*A,B*). Overall, 62% of α_{1B-1}- and κ_1R-transfected cells responsive to κ_1R agonists showed some degree of facilitation before drug exposure. These effects were not seen in cells incubated overnight with 100 ng/ml pertussis toxin ($n = 5$) or in cells that were not transfected with the κ_1R receptor ($n = 6$), suggesting a receptor-mediated, G-protein (G$_i$ and/or G$_o$)-dependent mechanism (data not shown). Similar G-protein receptor-dependent modulation of Ca channels in the absence of agonist has been described by other groups (Ikeda, 1992; Zhang et al., 1996). The effects seen in the present study did not diminish when cells remained in a rapidly flowing bath for longer than 20 min, suggesting that agonist-independent modulation was not caused by the secretion of opioid agonists by the cells or the presence of opioid agonists in the culture media (Doupnik et al., 1994). U69593 is a κ_1R-selective agonist with an affinity for κ_1 receptors in the range of 1–2 nM (Avidor-Reiss et al., 1995; Simonin et al., 1995) and an EC$_{50}$ for adenylyl cyclase inhibition of ~8 nM (Avidor-Reiss et al., 1995). When U69593 was added to the bath solution at a supramaximal concentration of 1 μM, α_{1-B1}-expressing cells showed a marked reduction in current (Fig. 1*A*) ($47 \pm 6.4\%$; $n = 13$), similar to the results of a number of other groups (Kaneko et al., 1994; Tallent et al., 1994; Carpenter et al., 1996; Toth et al., 1996). When the κ_1R-selective "antagonist" norBNI (Portoghese et al., 1987) was added directly after U69593 exposure at a concentration of 100 nM, the inhibition produced by the agonist was always relieved within 3–4 min (Fig. 1*A,B*), and currents often increased in magnitude beyond those seen before U69593 exposure (Fig. 1*A,B*) ($n = 13$). When U69593 was added to cells without subsequent addition of norBNI, current inhibition and facilitation washed out in ~10 min, and no enhancement of current amplitudes was observed (data not shown) ($n = 5$). When norBNI was added to κ_1R expressing cells that showed facilitation without previous agonist exposure, this facilitation was always reversed, and currents were always observed to increase in amplitude (Fig. 1*C*) ($n = 5$). However, when the opioid receptor antagonist naloxone (20 μM) was added to such cells, no current increase was observed ($n = 3$) (data not shown), suggesting that naloxone, unlike norBNI, is a neutral antagonist. norBNI is known to bind at κ_1 receptors with affinities in the range of 0.5–1.2 nM (Prather et al., 1995; Simonin et al., 1995) and completely blocks opioid agonist stimulation of adenylyl cyclase at 100
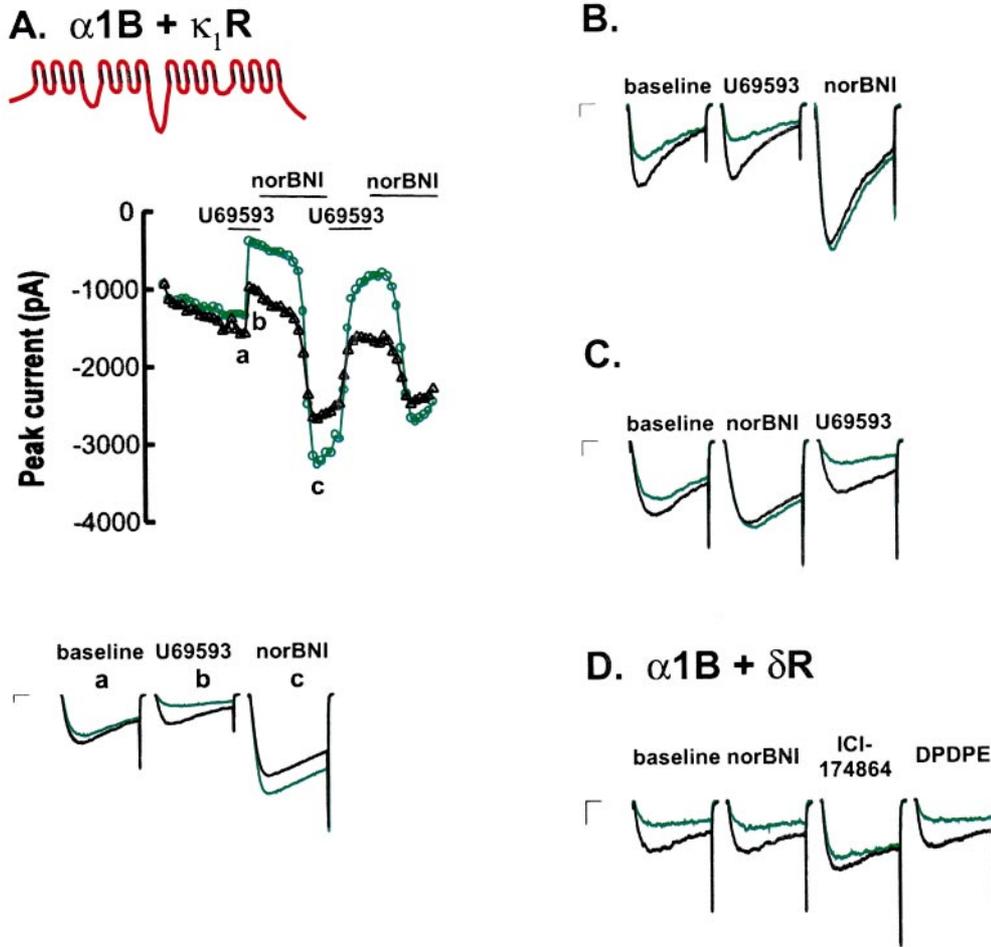
Figure 1. Opioid agonist and inverse agonist effects on Ba currents for $\alpha_{1B\text{-}1}$- and $\kappa_1 R$-expressing tsA-201 cells. *A*, Peak current amplitudes and representative currents before prepulse (*green*) and after a 30 msec depolarizing prepulse (*black*), illustrating the effects of the addition to the bath of 1 μM U69593 or 100 nM norBNI. Currents were taken from the points indicated. Currents were evoked by a 50 msec depolarization to 0 mV from a holding potential of -90 mV. Calibration: 250 pA, 10 msec. *B*, Representative currents illustrating relatively small current inhibition after U69593 exposure and relatively large current enhancement after norBNI exposure in cells with a large degree of modulation (as indicated by a relatively large increase in current amplitudes after a prepulse) before agonist exposure. *C*, Representative currents illustrating the effects of 100 nM norBNI added before 1 μM U69593. *D*, Currents evoked from a $\alpha_{1B\text{-}1}$- and δR-expressing cell in the presence of 100 nM norBNI, 1 μM ICI-174864, and 1 μM DPDPE.

nM (Avidor-Reiss et al., 1995). Because norBNI was applied in the absence of agonist and reduced prepulse ratios to that seen in untransfected cells, we believe that maximal relief of agonist-independent modulation was obtained with 100 nM norBNI.

Both the U69593 (inhibitory) and norBNI (enhancing) effects in these experiments could be completely blocked by pertussis toxin ($n = 5$), and cells responsive to one drug were always responsive to the other. When 50 μM GF109203, a specific PKC inhibitor, was included in the intracellular solution ($n = 3$), the response of $\alpha_{1B\text{-}1}$- and $\kappa_1 R$-expressing cells to U69593 and norBNI was unaltered relative to cells without GF109203 in the intracellular solution, suggesting that PKC-mediated phosphorylation plays no role in the observed effects on the currents (data not shown).

To further verify that norBNI does in fact act by relieving agonist-independent G-protein modulation, the effects of receptor expression, U69593, and norBNI on current facilitation as determined by current ratios were examined (Ikeda, 1991) and were defined as the ratio of the current amplitude after a prepulse, $I(+pp)$ to that before a prepulse, $I(-pp)$. Baseline current ratios (before drug exposure) for $\alpha_{1B\text{-}1}$- and $\kappa_1 R$-expressing cells (ratio, 1.05 ± 0.08; $n = 13$) were significantly greater than for cells not expressing $\kappa_1 R$ (data pooled from $\kappa_1 R$-untransfected cells and U69593- and norBNI-unresponsive cells; ratio, 0.89 ± 0.05; $n = 13$; $p < 0.05$, one-tailed t test). These ratios were significantly correlated with the degree of current enhancement obtained after the addition of norBNI (baseline relative to peak of norBNI effect; $r = 0.84$; $p < 0.001$; $n = 13$) (Fig. 1, compare *A,B*),

suggesting that cells most responsive to norBNI expressed the highest levels of agonist independent modulation. The effects of U69593 and norBNI on current amplitudes were mirrored in terms of the current ratios. At baseline, before drug exposure, a small degree of facilitation was seen (ratio, 1.05 ± 0.08), facilitation increased in the presence of U69593 (ratio, 1.70 ± 0.13), and currents inactivated to a small extent after a prepulse in the presence of norBNI (ratio, 0.78 ± 0.03; $n = 13$ in all cases) (Fig. 1). This current inactivation after a prepulse, as a consequence of a reduction in active G-protein, is similar to the results of Ikeda (1992), who found that internal perfusion of rat sympathetic neurons with GDPβS caused inactivation after a prepulse at most prepulse potentials. In summary, these results suggest that the currents were inhibited to an intermediate degree before drug exposure, that the agonist U69593 rapidly caused additional current inhibition, and that norBNI is actually an inverse agonist, relieving both the agonist-independent and agonist-dependent inhibition of the current.

Because this agonist-independent receptor activity is likely to complicate estimates of receptor effects on Ca channels, we used U69593 to observe currents in the presence of maximum receptor activity, $I(R^*)$, and norBNI to observe currents in the presence of inactive receptor, $I(R)$. An index of total modulation was estimated from the peak of the U69593 response [an estimate of $I(R^*)$] and the peak of the norBNI response (in the case of cells showing agonist-independent modulation) or baseline currents (in the case of cells without agonist-independent modulation) to estimate $I(R)$. Total modulation was then defined as

$[I(\text{R}) - I(\text{R}^*)]/I(\text{R})$. In the case of $\alpha_{1\text{B-1}}$- expressing cells, modulation was $68.9 \pm 3.7\%$ ($n = 13$) (see Fig. 5) when defined in this way and $47 \pm 6.4\%$ ($n = 13$) when defined as $[I(\text{baseline}) - I(\text{R}^*)]/I(\text{baseline})$, suggesting a nearly 50% reduction in the SEM of the estimated effect size. Consistent with the involvement of heterotrimeric G-proteins (Marchetti et al., 1986; Ikeda, 1991; Pollo et al., 1992), this current reduction was greater with respect to currents evoked before a voltage prepulse than for currents evoked after a prepulse (total modulation, $38.6 \pm 3.7\%$ after prepulse; $n = 13$) (Fig. 1). Kinetic slowing was evident in the majority of cells (Fig. 1) but was not examined in detail for the purpose of this study.

To further explore the specificity of agonist-independent opioid receptor effects on Ca currents, we transfected cells with $\alpha_{1\text{-B1}}$, $\beta_{1\text{B}}$, $\alpha_{2\text{B}}$, CD8α, and the mouse δ-opioid receptor (δR). This receptor has well characterized agonist-independent activity; ICI174,864 is known to act as an inverse agonist, and DPDPE (enkephalin, [D-Pen^{2,5}]) is known to act as an agonist (Chiu et al., 1996; Mullaney et al., 1996; Merkouris et al., 1997). These cells showed inhibition and prepulse facilitation qualitatively similar to κ_1R-expressing cells before drug exposure (Fig. 1*D*). When norBNI was added to the bath, δR-expressing cells showed no alteration in their $\alpha_{1\text{B-1}}$ currents (Fig. 1*D*; $n = 3$). However, when ICI174,864 was added to the bath, an increase in current amplitudes was noted ($28.0 \pm 14.0\%$; $n = 3$), and facilitation decreased (current ratio, 1.4 ± 0.2 at baseline; current ratio, 1.0 ± 0.1 in the presence of ICI174,864). When DPDPE was subsequently added to the bath, current amplitudes rapidly decreased and facilitation increased (current ratio, 1.5 ± 0.4), and the DPDPE-mediated inhibition could be reversed by reapplication of ICI174,864 over a time frame of minutes (total modulation, $37.0 \pm 13.0\%$; $n = 3$). The response of these cells to agonist and inverse agonist was therefore qualitatively similar to cells expressing κ_1R but selective for δR-specific agents.

When κ_1R- and $\alpha_{1\text{E-3}}$-expressing cells were exposed to U69593 and norBNI, much less current inhibition ($25.3 \pm 1.9\%$; $n = 11$) (Fig. 2; see Fig. 5) was observed than for $\alpha_{1\text{B-1}}$-expressing cells ($p < 0.001$), consistent with previous findings (Toth et al., 1996; Yassin et al., 1996). $\alpha_{1\text{E-3}}$-expressing cells also showed partial voltage-dependent relief of inhibition such that inhibition after a prepulse was less than before a prepulse (total modulation, $21.9 \pm 1.6\%$ after prepulse), but cells fell far short of the ~50% relief seen in $\alpha_{1\text{B-1}}$-expressing cells. Prepulse ratios in the presence of U69593 corrected for inactivation (see Materials and Methods) were 1.052 ± 0.02 for $\alpha_{1\text{E-3}}$-expressing cells but 2.184 ± 0.02 for $\alpha_{1\text{B-1}}$-expressing cells ($p < 0.001$; see Fig. 5).

Structural determinants of G-protein regulation

To elucidate the structural basis for differences in the degree of G-protein regulation of $\alpha_{1\text{B-1}}$ and $\alpha_{1\text{E-3}}$ Ca channels, we constructed Ca channels in which portions of $\alpha_{1\text{B-1}}$ were inserted into an $\alpha_{1\text{E-3}}$ background. When the entire N-terminal half of $\alpha_{1\text{B-1}}$ was transferred into the $\alpha_{1\text{E-3}}$ background to create the bBbBbEeEe chimera (where lowercase letters indicate intracellular sequence, and uppercase letters indicate transmembrane domains, written in the sequence N terminus, domain I, I/II loop, etc.), currents were markedly reduced by U69593 and markedly enhanced by norBNI (Fig. 3; see Fig. 5) ($n = 7$). Overall modulation was $69.4 \pm 4.3\%$ and not significantly different from modulation seen with $\alpha_{1\text{B-1}}$. Inhibition was partially relieved by a voltage prepulse (to $46 \pm 7.9\%$), and somewhat less relief was
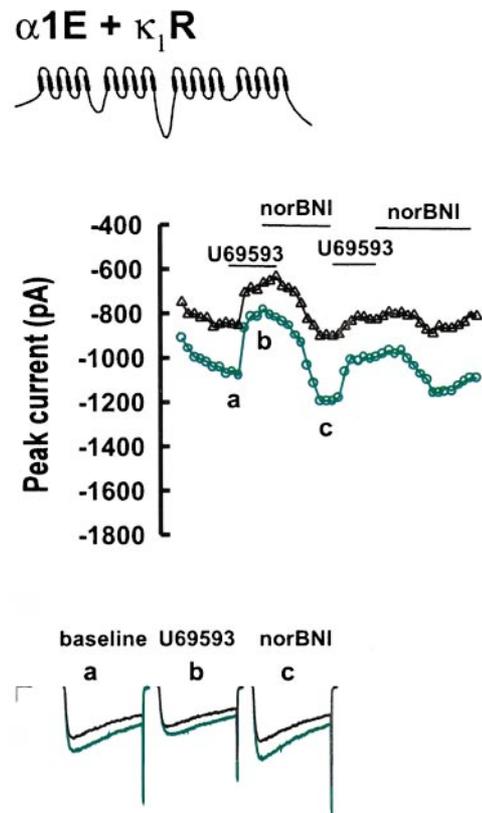
Figure 2. Opioid agonist and inverse agonist effects on Ba currents for $\alpha_{1\text{E-3}}$- and κ_1R-expressing tsA-201 cells. Currents were taken from the points indicated. Calibration: 250 pA, 10 msec. See Figure 1 for details.

seen than for $\alpha_{1\text{B-1}}$ (corrected prepulse ratio, 1.78 ± 0.20; $p > 0.05$; see Fig. 5).

To determine the minimal sequence responsible for these observations, we transferred two of the intracellular loops contained in the bBbBbEeEe chimera into the $\alpha_{1\text{E-3}}$ background, individually and in combination. The I/II intracellular loop has been shown by GST fusion studies to bind $\beta\gamma$ G-protein subunits (De Waard et al., 1997) and to posses a consensus sequence QXXER that it shares with adenyl cyclase and a number of other G-protein effectors (Pragnell et al., 1994). Somewhat consistent with the results of Page et al. (1997), the eEbEeEeEe chimera (Figs. 4–6) showed current inhibition that was slightly greater but not significantly different from $\alpha_{1\text{E-3}}$ ($33.8 \pm 2.3\%$; $n = 9$) and much less than that of $\alpha_{1\text{B-1}}$ ($p < 0.001$) or the bBbBeEeE chimera ($p < 0.001$). When the II/III intracellular loop of $\alpha_{1\text{B-1}}$ was transferred into the $\alpha_{1\text{E-3}}$ background (Figs. 4, 5, *eEeEbEeEe* chimera), inhibition similar to that of $\alpha_{1\text{E-3}}$ was seen ($23.1 \pm 4.8\%$; $n = 7$). When the II/III loop was transferred into the $\alpha_{1\text{E-3}}$ background, in combination with the I/II loop (Figs. 4, 5, *eEbEbEeEe* chimera), the resulting construct also showed inhibition similar to that of $\alpha_{1\text{E-3}}$ ($24.6 \pm 1.1\%$; $n = 8$). All three constructs showed prepulse facilitation similar to $\alpha_{1\text{E-3}}$ ($p > 0.05$ in each case) and significantly less facilitation than $\alpha_{1\text{B-1}}$ ($p < 0.001$ for each).

Because the I/II loop alone failed to significantly enhance modulation but may play some role in mediating the binding of $\beta\gamma$ subunits to Ca channels (De Waard et al., 1997), a series of additional constructs was created to assess the role of sequences near the I/II loop in determining effector sensitivity. When domain II, the I/II loop, and the II/III loop from $\alpha_{1\text{B-1}}$ were

Figure 3. Representative currents from various α_{1B-1} and α_{1E-3} chimeric Ca channels with sensitivity to modulation approaching that of native α_{1B-1} channels (Fig. 1) before prepulse (*green*) and afte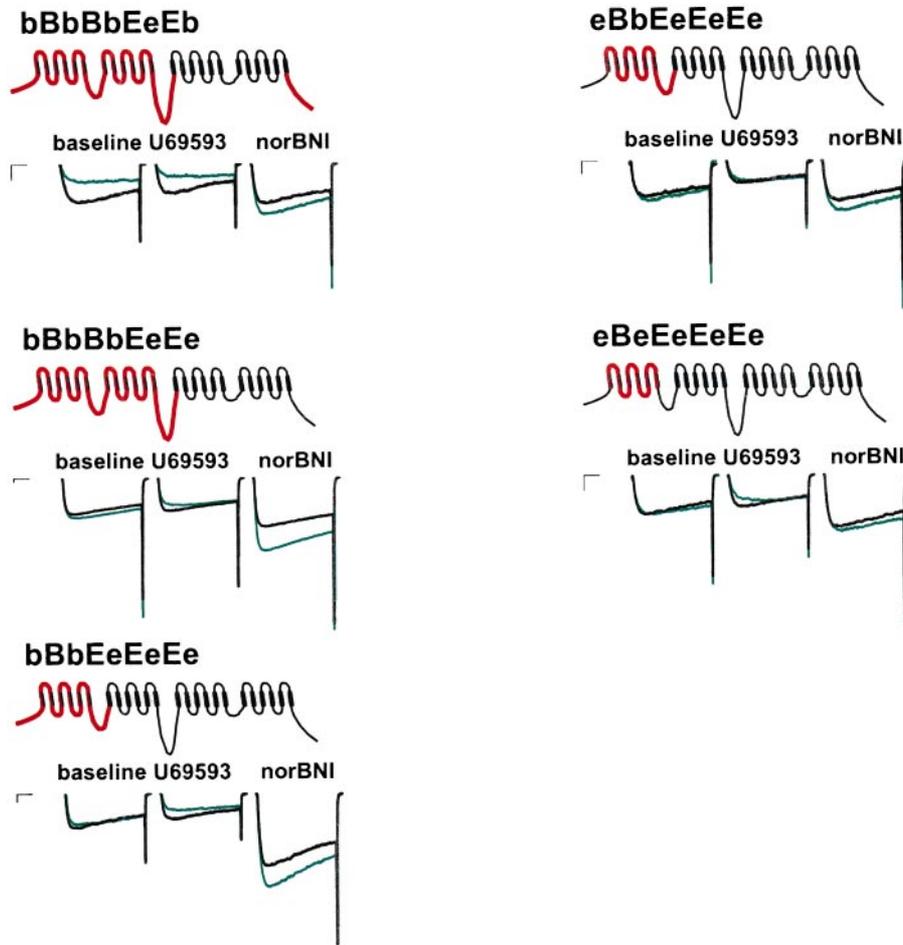r a 30 msec depolarizing prepulse (*black*). Maximal effects of U69593 and norBNI are illustrated. Calibration: 250 pA, 10 msec.

transferred into the α_{1E-3} background (eEbBbEeEe chimera), modulation and facilitation similar to that of α_{1E-3} was obtained (total modulation, 24.6 ± 1.1%; $p > 0.05$; corrected ratio, 1.03 ± 0.02; $p > 0.05$; $n = 8$). The addition of α_{1B-1} sequence from the N terminus to the C-terminal end of the I/II loop into the α_{1E-3} background (Figs. 3, 5, 6, *bBbEeEeEe* chimera) resulted in a channel showing inhibition not significantly different from α_{1B-1} (66.6 ± 4.1%; $n = 8$). However, as in the case of the bBbBbEeEe chimera, this inhibition was less completely relieved by a prepulse (to 46.0 ± 7.9%; corrected prepulse ratio, 1.55 ± 0.11; $p < 0.001$) than in the case of α_{1B-1}, although facilitation was greater than that seen in the case of α_{1E-3} ($p < 0.05$). When domain I and the I/II loop from α_{1B-1} were transferred alone into the α_{1E-3} background (Figs. 3, 5, *eBbEeEeEe* chimera) modulation that was not significantly different from α_{1B-1} was also observed (56.8 ± 4.4%; $n = 7$; $p > 0.05$). These channels showed less facilitation (corrected prepulse ratio, 1.27 ± 0.05) than α_{1B-1}-expressing cells ($p < 0.001$) and not significantly more facilitation than α_{1E-3}-expressing cells ($p > 0.05$). We also attempted to examine the contribution of the N terminus in isolation by the chimera bEeEeEeEe but were unable to obtain adequate current expression from this chimera. When domain I of α_{1B-1} was transferred alone into the α_{1E-3} background (Figs. 3, 5, *eBeEeEeEe* chimera), modulation significantly greater than α_{1E-3} was observed (44.6 ± 0.03%; $n = 10$; $p < 0.001$), but this was significantly less than that observed for α_{1B-1} ($p < 0.05$). Facilitation (corrected ratio, 1.3 ± 0.02) was not significantly different from α_{1E-3} ($p > 0.05$).

Domain I of Ca channels is thought to be partly responsible for determining the voltage dependence of Ca channel inactivation (Zhang et al., 1994), although the C terminus (Klockner et al., 1995; Soldatov et al., 1997) and I/II loop (Herlitze et al., 1997) are also thought to play a role. The regions in domain I responsible for determining inactivation are thought to be a C-terminal portion of the $I_{s5\ sec6}$ linker as well as I_{s6} (Zhang et al., 1994). When this sequence alone was added along with the I/II loop (Figs. 4, 5, *eE*$_{(90–309)}$*B*$_{(315–355)}$*bEeEeEe chimera*), the resulting construct showed modulation (30.0 ± 3.9%; $n = 7$) and facilitation (corrected ratio, 1.04 ± 0.01) similar to α_{1E-3} and the eEbEeEeEe chimera ($p > 0.05$ in both cases), although inactivation was reduced compared with the eEbEeEeEe chimera ($p < 0.05$).

Recently Qin et al. (1997) have provided biochemical and functional evidence that the C terminus of non-DHP-sensitive Ca channels is involved in $\beta\gamma$ binding and current inhibition. To assess the role of the C terminus in our system, we exchanged the C terminus of α_{1B-1} into the bBbBbEeEe chimera to produce the bBbBbEeEb chimera (Figs. 3, 5). These channels showed inhibition (65.6 ± 4.2%; $n = 7$) not significantly different from α_{1B-1} or the bBbBbEeEe chimera ($p > 0.05$ in both cases). However, facilitation more closely approximated that of α_{1B-1} than was seen in the case of the bBbBbEeEe chimera (corrected ratio, 2.04 ± 0.12).

Because Ca channel modulation by G-proteins is voltage-dependent, we collected current–voltage data in the presence of norBNI and U69593 for a number of the constructs (Fig. 6). The bBbBbEeEb chimera ($n = 7$) closely resembled wild-type α_{1B-1} ($n = 5$) both in terms of inhibition and facilitation (Fig. 6). As we
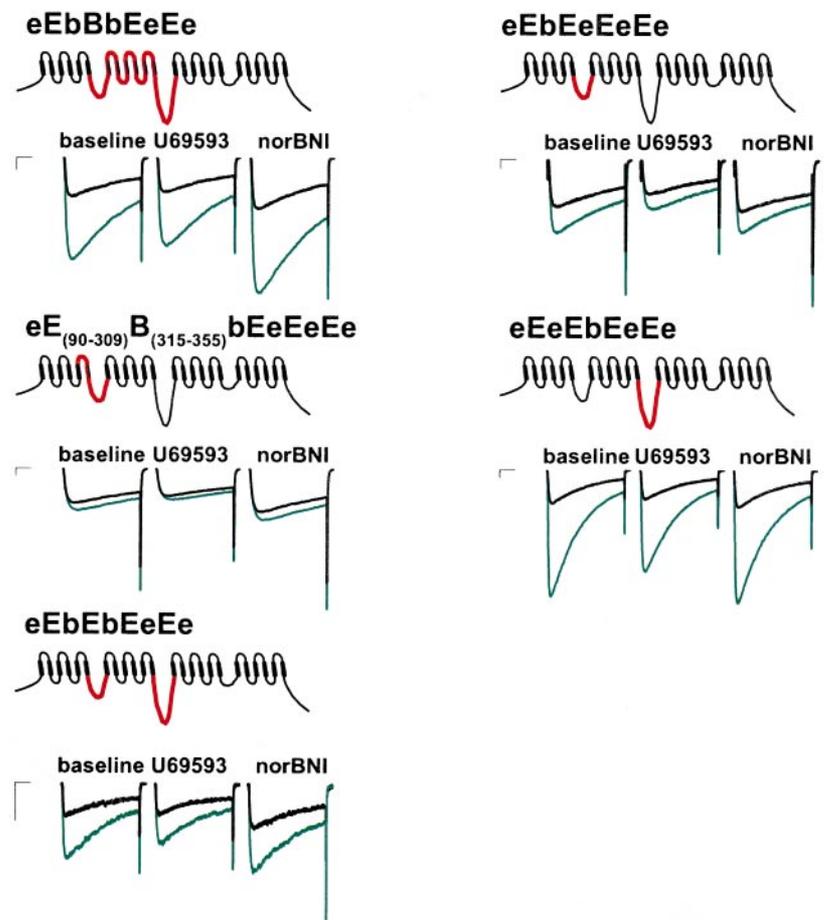
Figure 4. Representative currents from various $\alpha_{1B\text{-}1}$ and $\alpha_{1E\text{-}3}$ chimeric Ca channels with sensitivity to modulation approaching that of native $\alpha_{1E\text{-}3}$ channels (Fig. 2) before prepulse (*green*) and after a 30 msec depolarizing prepulse (*black*). Maximal effects of U69593 and norBNI are illustrated. Calibration: 250 pA, 10 msec.

observed using a single test potential of 0 mV, the bBbBbEeEe construct ($n = 5$) showed less facilitation than bBbBbEeEb across a range of activating potentials (Fig. 6). These results suggest that the C terminus may be partly responsible for the differences in facilitation between $\alpha_{1B\text{-}1}$ and $\alpha 1_{E\text{-}3}$, but it does not appear to mediate the differences in inhibition between the two channels. The bBbEeEeEe chimera ($n = 5$) showed strong inhibition, similar in magnitude to $\alpha_{1B\text{-}1}$, but showed little facilitation at any activating potential. The eEbEeEeEe chimera (Fig. 6; $n = 8$) showed relatively little facilitation or inhibition at any activating potential. For example, at a potential of -10 mV, the current ratio in the presence of U69593 for $\alpha_{1B\text{-}1}$ (2.28 ± 0.34) was not significantly different from the bBbBbEeEb chimera (2.00 ± 0.20; $p > 0.05$) but was significantly greater than the ratios for the bBbEeEeEe (1.22 ± 0.11), eEbEeEeEe (0.92 ± 0.04), and bBbBbEeEe (1.40 ± 0.11) chimeras (all $p < 0.05$). These data further reinforce the conclusion that facilitation and inhibition are mediated by different structural elements.

We defined agonist-independent modulation as the difference in the current amplitude in the presence of norBNI compared with that at baseline. The degree of such modulation would be expected to correlate with the index of total modulation for a particular construct if both indices reflect sensitivity to inhibition by G-proteins. We computed this correlation across all of our observations and found that the correlation was large and highly significant ($r = 0.74$; $p < 0.00001$). In addition, the magnitude of the agonist-independent modulation seen for each construct was well correlated with the magnitude of the total modulation seen for the construct ($0.03 \pm 0.01\%$, $\alpha_{1E\text{-}3}$; $0.02 \pm 0.01\%$, eE-bEeEeEe; $0.22 \pm 0.03\%$, eBeEeEeEe; $0.38 \pm 0.06\%$, $\alpha_{1B\text{-}1}$) This provides additional support for the notion that the index of total modulation is indeed a valid index of sensitivity to current inhibition by G-proteins.

We were concerned that the observed differences in facilitation between the constructs were attributable to differences in the kinetics of G-protein association with the channels. We measured the G-protein reassociation rates for a number of our constructs using a variable interval between the prepulse and the second test pulse. Facilitation decayed as a single exponential with time constants between the limits of 15.9 ± 2.3 msec (eEbEeEeEe; $n = 4$) (data not shown) and 41.6 ± 2.4 msec ($\alpha_{1B\text{-}1}$; $n = 6$) (data not shown). Facilitation will have decayed to 73–89% of its maximum after a 5 msec interval for our constructs. Therefore, the large differences in facilitation observed (Figs. 5, 6) cannot be accounted for by differences in reassociation kinetics.

As noted above, current ratios (Fig. 5) were corrected for inactivation to facilitate comparisons between constructs that differed markedly in terms of inactivation. An adequate kinetic model that can account for modulation, activation, and inactivation of Ca channels has not yet been elaborated, and the relationship between inactivation and modulation therefore remains unclear. However, with respect to the most important constructs used in the present study, it is clear that modulation and inactivation are relatively independent. For example, the constructs bBbBbEeEb, bBbBbEeEe, bBbEeEeEe, eBbEeEeEe, eBeEeEeEe, and e($E_{90-309}B_{315-355}$)bEeEeEe did not differ in terms of inactivation compared with bBbBbBbBb (all $p > 0.9$). However, these constructs differed markedly from one another in
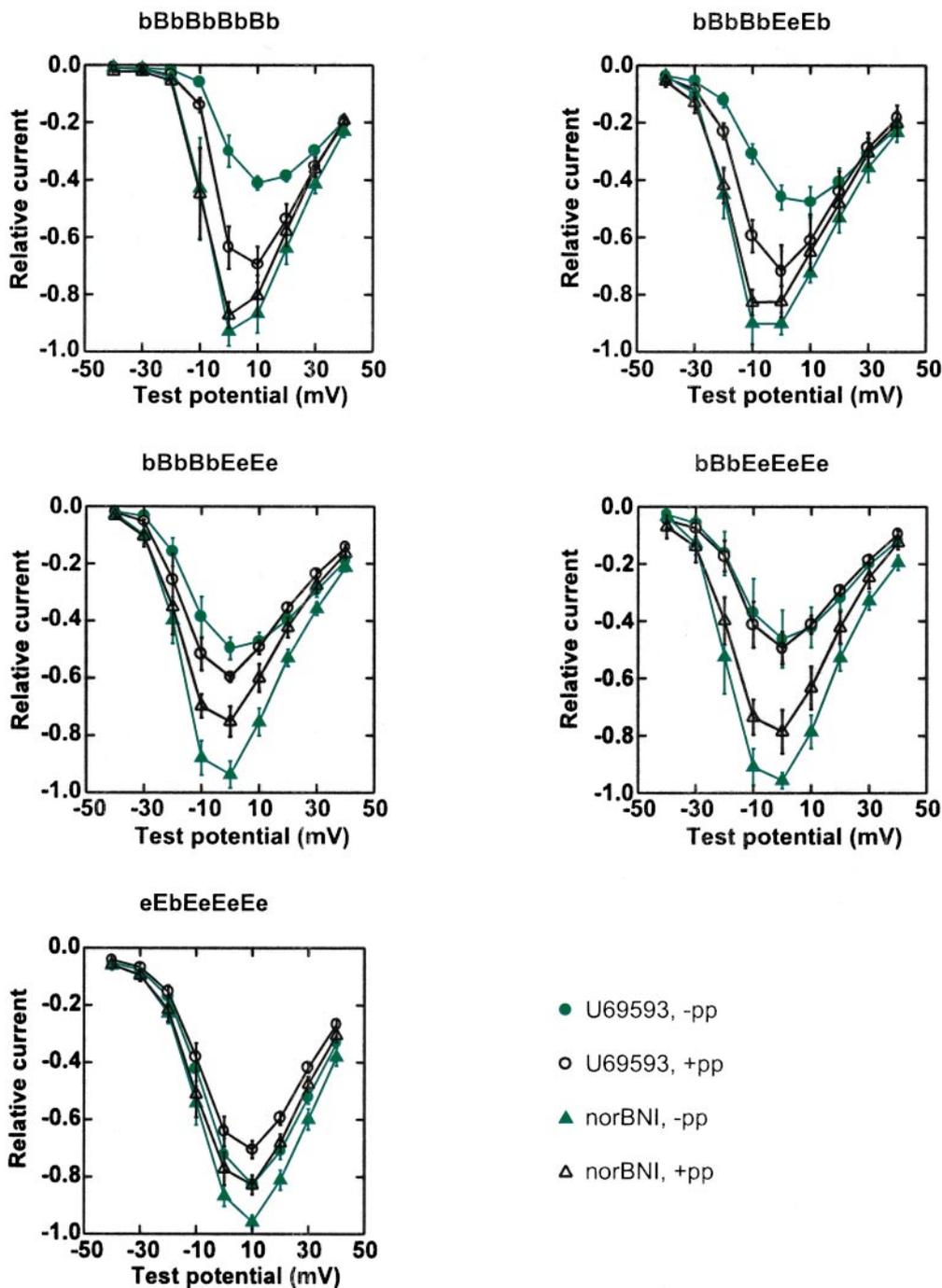
Figure 5. Summary of inhibition and facilitation seen in native α_{1B-1}- and α_{1E-3}-expressing cells as well as for various chimeric Ca channels. *A*, Summary of κ_1R inhibition obtained for Ba currents evoked before a depolarizing prepulse from native α_{1B-1}- and α_{1E-3}-expressing cells, as well as cells expressing various α_{1B-1} and α_{1E-3} chimeras. Values are plotted as mean ± SEM. Sample sizes for each construct were ≥6 as detailed in Results. *Asterisks* indicate modulation significantly different from native α_{1E-3} ($p < 0.05$). *B*, Summary of corrected κ_1R-mediated prepulse facilitation ratios from native α_{1B-1}- and α_{1E-3}-expressing cells, as well as cells expressing various α_{1B-1} and α_{1E-3} chimeras. Values are plotted as mean ± SEM. Ratios were calculated by multiplying prepulse ratios in the presence of U69593 [$I(+pp)/I(-pp)$] by an index of inactivation [$I(-pp)/I(+pp)$] calculated from currents in the presence of norBNI to adjust for differences in inactivation between the various constructs. Sample sizes for each construct were ≥6 as detailed in Results. *Asterisks* indicate facilitation significantly different from native α_{1E-3} ($p < 0.05$).

terms of facilitation. The chimeras bBbBbEeEb and bBbBbEeEe did not differ significantly from α_{1B-1} in terms of facilitation ($p > 0.1$), but bBbEeEeEe, eBbEeEeEe, eBeEeEeEe, and e(E-B)bEeEeEe did differ significantly (all $p < 0.0001$). Because these constructs differ greatly in terms of facilitation but not in terms of inactivation, it seems clear that the two are not highly correlated.

DISCUSSION

We have identified a domain in the family of DHP-insensitive Ca channels that appears to largely dictate the efficacy of G-protein-induced channel inhibition. The results of the present study also suggest that the κ_1R, like the δR (Chiu et al., 1996; Mullaney et al., 1996; Merkouris et al., 1997), has agonist-independent activity that can be suppressed by drugs with inverse-agonist properties. Therefore, it appears that these pharmacological properties may be general characteristics of the opioid receptor family. Agonist-independent activity of the κ_1R has not been previously reported. It is likely that we observed such activity in our experiments because of the high transient expression levels obtainable in tsA-201 cells or because of some other cell-dependent variables. The observation of this agonist-independent activity allowed us to identify norBNI as an inverse agonist at the κ_1R. The use of inverse agonists in combination with agonists appears to have important utility in that receptor states near maximal and minimal activity can be readily generated, allowing for more precise estimates of receptor effects on G-protein regulated effectors. This consideration would theoretically be of greatest importance for systems with the highest levels of receptor expression, that would be expected to exhibit significant agonist-independent receptor activity. We achieved substantial improvements in the precision of our estimates of G-protein modulation by using this approach.

With regard to the structural basis for the differences in G-protein sensitivity between α_{1B-1} and α_{1E-3} Ca channels, the results of the present study suggest that domain I of α_{1-B1} is the single most important structural feature required for determining the higher sensitivity to opioid receptor modulation of α_{1B-1} compared with α_{1E-3}. Whereas κ_1R-mediated modulation of α_{1E-3} was only 37% of that obtained for native α_{1B-1} channels, exchange of domain I alone yielded a chimeric channel with 65% of the modulation of α_{1B-1}. The further addition of the I/II linker increased modulation to 83% of native α_{1B-1} channels, and the further addition of the N terminus increased modulation to 100% of α_{1B-1} (Fig. 5). These same regions appear largely responsible for facilitation, although the determinants of facilitation appear to be complex and may also include the C terminus (Figs. 5, 6). The lack of significant differences in modulation between the eEbEeEeEe chimera and native α_{1E-3} (Fig. 5) suggests that the I/II loop is not sufficient to determine the differential sensitivity to modulation between α_{1B-1} and α_{1E-3}. Likewise, the lack of significant differences in inhibition between the bBbBbEeEe and bBbBbEeEb chimeras (Fig. 5) suggests that the C terminus is not involved in determining differences in sensitivity to modulation. Therefore, the two structural elements shown to be involved in G-protein $\beta\gamma$ binding to Ca channels (De Waard et al., 1997; Qin et al., 1997; Zamponi et al., 1997) do not appear to account for differences in sensitivity to inhibition between α_{1B-1} and α_{1E-3}.

The role of the I/II loop in G-protein modulation of Ca channels is controversial. A number of other studies have also suggested that the I/II loop is not sufficient to account for high sensitivity to G-protein modulation. Page et al. (1997) showed that transfer of the I/II loop from α_{1B} to α_{1E} or from α_{1B} to α_{1A}

Figure 6. Current–voltage relations for selected Ca channel constructs in the presence of U69593 (*circles*) and norBNI (*triangles*) before (*green*) and after (*black*) a depolarizing prepulse (*pp*). Sample sizes were ≥5 as detailed in Results.

conferred kinetic slowing but not inhibition on the recipient channel. Qin et al. (1997) showed that transfer of the I/II loop from α_{1C} to α_{1E} yielded a chimeric channel that was still modulated like α_{1E}. Zhang et al. (1996) showed that transfer of the I/II loop from α_{1A} or α_{1C} into α_{1B} yielded a channel that was still modulated like α_{1B}. In contrast, Zamponi et al. (1997) showed that transfer of the I/II loop from α_{1B} into α_{1A} yielded a chimeric channel with significantly more modulation than native α_{1A}. Herlitze et al. (1997) and De Waard et al. (1997) showed that mutations in the sequence QXXER of α_{1A} can disrupt modulation, and peptides made according to sequences in the I/II loop

can block modulation of both channels (Zamponi et al., 1997). In addition, a number of groups have demonstrated that the I/II loop can indeed bind $\beta\gamma$ subunits (De Waard et al., 1997; Qin et al., 1997; Zamponi et al., 1997), and this may be important in mediating the effects of $\beta\gamma$ subunits in some general way. The results of Qin et al. (1997) and Zhang et al. (1996) also suggest that the C terminus plays a critical role in mediating modulation, and the latter study suggests a role of the N terminal portion of the Ca channel that is N-terminal to the I/II loop. Overall, the results of the present study are most consistent with those of Zhang et al. (1996) in terms of the involvement of the N terminal

portion of the channel, although we find that the C terminus plays little if any role in determining inhibition.

There are at least three models that could account for the findings of the present study. First, differences in the kinetics of the two channels, probably determined by multiple and complex structural features, could account for at least some of the differences in modulation. For example, we numerically integrated the kinetic model of G-protein modulation proposed by Patil et al. (1996) while systematically varying the rate constants of the model a small amount near their fitted values and have observed alterations in predicted inhibition for each of the rate constants in the model (data not shown). Apart from rate constants directly introduced to model modulation (e.g., "willing" to "reluctant" transitions), predicted inhibition is particularly sensitive to the values of the rate constants for exit from the inactivated state and for transitions between "willing" closed states.

A second model that could account for these data is an allosteric model in which the I/II loop and/or C terminus binds G-protein $\beta\gamma$ subunits and changes conformation but then must induce conformational changes in the gating apparatus of the channel. The C terminus and I/II loop may interact directly with the gating apparatus or may be coupled to these domains through other structures. According to this view, it is possible that domain I of α_{1B-1}, which may itself contain sequence involved in voltage-dependent channel gating, is more susceptible to structural alteration by $\beta\gamma$ binding to the I/II loop and/or C terminus than is domain I of α_{1E-3}, and that the N terminus somehow facilitates such interactions. Alternatively, domain I may play some role in linking binding to the C terminus and/or I/II linker to regions of the channel involved in gating to generate modulation.

Finally, it is possible that Ca channel regions other than the I/II loop and C terminus play a role in $\beta\gamma$ binding. Biochemical binding studies performed to date (Qin et al., 1997; De Waard et al., 1997) have demonstrated that the I/II loop and C terminus have affinities that are by themselves sufficient for tight binding but have not shown that other sequences do not play an important role in $\beta\gamma$ binding to the intact channel. Although domain I (and the N terminus) may not be competent to bind $\beta\gamma$ subunits in isolation, it is possible that it does contribute to the affinity of $\beta\gamma$ subunit binding to the intact channel and that domain I of α_{1B-1} is differentially capable of stabilizing $\beta\gamma$ binding in comparison to domain I of α_{1E-3}.

The notion that different structural elements account for facilitation and inhibition is similar to the results of Zhang et al. (1996), who showed that both the first domain and the C terminus of α_{1B} were required within an α_{1A} background to confer facilitation and inhibition, but N- or C-terminal halves of α_{1B} in an α_{1A} background were sufficient for inhibition alone. A physical interaction of the C terminus of α_{1B-1} with G$\beta\gamma$ subunits may play some role in the functional uncoupling of G$\beta\gamma$ and Ca channels after a depolarizing pulse.

Whatever the precise mechanism by which domain I and the N terminus contribute to Ca channel modulation, the findings of the present study suggest that Ca channel sensitivity to G-protein modulation not only is determined by sequences with high-affinity interactions with $\beta\gamma$ subunits but also requires another sequence that is perhaps not directly involved in high-affinity $\beta\gamma$ binding. Domain I and the N terminus of the channel appear largely responsible for these differences. The N-terminal tails of the two channels are fairly dissimilar (60% identity), especially in their N terminal halves. The only significant regions of dissimilarity between α_{1B-1} and α_{1E-3} in domain I fall within the extracellular loops (s1–s2, s3–s4, and s5–s6). One or both of these regions of dissimilarity may therefore account for the observed effects of exchanging domain I between α_{1B-1} and α_{1E-3}.

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