Mechanism of Auxiliary Subunit Modulation of Neuronal $\alpha_{1E}$ Calcium Channels

Lisa P. Jones, Shao-kui Wei, and David T. Yue

From the Program in Molecular and Cellular Systems Physiology, Departments of Biomedical Engineering and Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract: Voltage-gated calcium channels are composed of a main pore-forming $\alpha_1$ moiety, and one or more auxiliary subunits ($\beta$, $\alpha_2\delta$) that modulate channel properties. Because modulatory properties may vary greatly with different channels, expression systems, and protocols, it is advantageous to study subunit regulation with a uniform experimental strategy. Here, in HEK 293 cells, we examine the expression and activation gating of $\alpha_{1E}$ calcium channels in combination with a $\beta$ ($\beta_1\beta_4$) and/or the $\alpha_2\delta$ subunit, exploiting both ionic- and gating-current measurements. Furthermore, to explore whether more than one auxiliary subunit can concomitantly specify gating properties, we investigate the effects of cotransfecting $\alpha_2\delta$ with $\beta$ subunits, of transfecting two different $\beta$ subunits simultaneously, and of COOH-terminal truncation of $\alpha_{1E}$ to remove a second $\beta$ binding site. The main results are as follows. (a) The $\alpha_2\delta$ and $\beta$ subunits modulate $\alpha_{1E}$ in fundamentally different ways. The sole effect of $\alpha_2\delta$ is to increase current density by elevating channel density. By contrast, though $\beta$ subunits also increase functional channel number, they also enhance maximum open probability ($G_{\text{max}}/Q_{\text{max}}$) and hyperpolarize the voltage dependence of ionic-current activation and gating-charge movement, all without discernible effect on activation kinetics. Different $\beta$ isoforms produce nearly indistinguishable effects on activation. However, $\beta$ subunits produced clear, isoform-specific effects on inactivation properties. (b) All the $\beta$ subunit effects can be explained by a gating model in which subunits act only on weakly voltage-dependent steps near the open state. (c) We find no clear evidence for simultaneous modulation by two different $\beta$ subunits. (d) The modulatory features found here for $\alpha_{1E}$ do not generalize uniformly to other $\alpha_1$ channel types, as $\alpha_{1C}$ activation gating shows marked $\beta$ subunit dependence that is absent for $\alpha_{1E}$. Together, these results help to establish a more comprehensive picture of auxiliary-subunit regulation of $\alpha_{1E}$ calcium channels.

Key words: calcium channels • $\alpha_{1E}$ • gating currents • subunit modulation • heterologous expression

Introduction

Voltage-gated calcium channels are molecular transducers that trigger cellular processes ranging from muscle contraction to neurotransmission. Modulation of these channels thereby constitutes a key potential mechanism for functional adaptation and plasticity. At least three different subunits are believed to comprise native calcium channels: a main, pore-forming $\alpha_1$ subunit, a cytoplasmic $\beta$ subunit, and a disulfide-linked $\alpha_2\delta$ subunit (for review, see Perez-Reyes and Schneider, 1994; De Waard et al., 1996). So far, seven different genes encoding $\alpha_{1A,B,C,D,E,F,G}$ subunits, and four different genes encoding $\beta_{1,2,3,4}$ subunits have been identified, along with multiple splice variants. Given this heterologenic structure, regulation of channel properties by variations in subunit composition have been widely studied as a potential mechanism for tuning channel gating properties to support a given physiologic role.

Despite the potential importance of modulation by subunit combination, fundamental uncertainties remain about the effects of auxiliary subunits (for review see Perez-Reyes and Schneider, 1994; De Waard et al., 1996; Walker and De Waard, 1998). While coexpression studies have demonstrated that the addition of auxiliary subunits ($\beta$, $\alpha_2\delta$) can have striking effects on channel gating and/or channel expression, the specific effects observed vary across studies, even using the same $\alpha_1$ subunit. At least some of the differences in subunit effects may reflect isoform-specific variations in the effects of distinct $\beta$ subunit isoforms on $\alpha_1$ gating. Further variability may arise from the use of diverse expression systems, electrophysiological methods, and experimental solutions. These points underscore the need to explore subunit modulation of each $\alpha_i$ isoform individually, and to undertake comprehensive studies with uniform experimental conditions.

Although most previous work has focused on $\alpha_{1C}$ neuronal $\alpha_{1E}$ channels (Soong et al., 1993) have recently emerged as important channels with which to attempt such comprehensive investigation for several reasons. First, subunit modulation of $\alpha_{1E}$ has potential physiological relevance, as $\alpha_{1E}$ (presumed “R-type”) channels have...
been implicated in neuronal functions including neurotransmitter release (Wu et al., 1998). Second, αIE demonstrates an exceptional capacity for high-level recombinant expression, which permits well-resolved measurements of both ionic and gating currents, even when the αIE subunit is expressed alone (which generally lowers overall expression of current). This capability enables examination of changes in both peak open probability and channel density (Olcense et al., 1996), two critical measures for resolving how auxiliary subunits affect the overall level of calcium current. Third, β subunits may affect αIE expression in a uniquely different manner than observed with other pore-forming α subunits, providing a potentially useful clue as to the underlying mechanism of subunit modulation. Olcense et al. (1994, 1996) provide the most biophysically detailed results in this regard, using Xenopus oocytes. In contrast to other α subunits, β subunits caused little change or even a decrease in overall αIE current density. This outcome resulted from decreased channel density, as assessed by maximal gating charge, countered by increased channel opening. By contrast, in mammalian expression systems, β subunits increased overall αIE current density (Williams et al., 1994; Stephens et al., 1997). Here, however, no αIE gating-current measurements have been made to permit assessment of underlying changes in channel density and open probability. Finally, αIE is one of the channels in which a second β binding site has been explicitly identified (Tarelus et al., 1997; Walker et al., 1998). Characterization of mutant αIE constructs lacking this site would allow determination of the functional importance of the secondary site.

Here, we therefore examine subunit modulation of αIE channels coexpressed with various combinations of auxiliary subunits (β₁, β₂₄, αδ) in mammalian HEK 293 cells. The same recombinant expression system, along with a consistent set of experimental solutions and protocols, is used throughout to facilitate direct comparison of channels with differing molecular composition. Measurements of both ionic and gating currents permits in-depth analysis of subunit modulatory effects. We focus on three key questions. (a) To what degree does modulation of αIE current density reflect modulation of channel gating and/or number of functional channels? (b) How do different auxiliary subunits compare with regard to modulation of activation gating? (c) What is the functional impact of the secondary β binding site in αIE? Through addressing these questions, this study helps to establish a more refined picture of auxiliary-subunit modulation of αIE calcium channels.

**Materials and Methods**

**Expression of N-Type Channels**

HEK 293 cells, obtained from Dr. Jeremy Nathans (Johns Hopkins University; Gorman et al., 1990), were grown at 37°C in Dulbecco’s modified Eagles medium (GIBCO BRL, Grand Island, NY). 10% fetal calf serum (GIBCO BRL), 1% L-glutamine (Sigma Chemical Co., St. Louis, MO), 1% penicillin-streptomycin (P9906; Sigma Chemical Co.), in 5% CO₂. Low-passage number cells were used (<20P). cDNAs encoding channel subunits αIE (Soong et al., 1993), αIE (Wei et al., 1991), β₁b (Pragnell et al., 1991), β₂₄ (Perez-Reyes et al., 1992), β₁ (Castellano et al., 1993c), β₄ (Castellano et al., 1995a), and αδ (Tomlinson et al., 1995) were subcloned into mammalian expression plasmids (pMT2; Genetics Institute, Cambridge, MA, for β₁, pZEM292R; ZymoGenetics, Inc., Seattle, WA, for αδ, pGW1; British Biotechnologies, Cowley, Oxford, UK for all others). α₁ₓ₅ was constructed by replacing the Bst 1107I (αIE; nucleotide 4299, given start codon at nucleotide 1) and SalI (3’ polylinker) region of α₁₃ in pGW1 with a shorter polymerase chain reaction fragment, including a premature stop codon after the codon for amino acid 1871. The portion of the channel derived from PCR was verified in its entirety with the use of the fluorescent deoxydideoxy terminator method of thermocycle sequencing on an automated DNA sequencer (Applied Biosystems Division 373a; Perkin-Elmer Cetus Instruments, Emeryville, CA). HEK 293 cells were transiently transfected using a standard, calcium-phosphate precipitation procedure (Brody et al., 1997) with a total of 30 µg of DNA per 10 cm plate. 10 µg of a plasmid containing a pore forming subunit was included (α₁₃ or α₁₅) and mixed with 10 µg of each desired auxiliary subunit (none, α β subunit, and/or the αδ subunit). If the amount of DNA totaled <30 µg, pBluescript was added to make up the difference. For certain experiments, both β₂₄ and β₁ were simultaneously transfected either in a 1:1 ratio (10 µg of each plasmid) or a 5:1 ratio (15 µg of β₁, 3 µg of β₂₄). More than 20% of cells transfected with a pore forming subunit exhibited detectable high-threshold calcium currents. “Mock-transfected” cells were transfected with 10 µg of β₁b, 10 µg of αδ, and 10 µg of pBluescript. In our usualionic current recording conditions (detailed below), we observed no high threshold, voltage-gated, calcium-channel currents in such cells (n = 32 cells, over two independent rounds of transfection), or in cells transfected with the β₂₄ subunit alone (n > 40 cells; Patil et al., 1998). In mock-transfected cells, we occasionally (~10% of cells) observed endogenous, low threshold calcium channel currents of small amplitude (peak ionic current ~20 pA in 10 mM Ba²⁺), as reported previously by Sun et al. (1994). Although endogenous currents of such small amplitude would contribute negligibly to our results, cells with low threshold activity were nevertheless rejected. At the biochemical level, Western blots performed on total membrane protein (30 µg/lane) from untransfected cells revealed no known high threshold (A, B, C, D, E) or β (1b, 2c, 3a, 4) subunits, and only low levels of αδ (personal communication, Mark Williams, SIBIA Neurosciences Inc., La Jolla, CA). Blots were probed individually with appropriate antibodies, and the lack of subunit proteins was gauged from the absence of bands that were clearly present using cells transfected with corresponding recombinant subunits. The result that coexpression of αδ with αIE potentiated current by approximately threefold suggests that trace expression of endogenous αδ did not significantly influence our results.

**Electrophysiology**

Whole-cell recordings were obtained at room temperature 48–72 h after transfection using an Axopatch 200A (Axon Instruments, Foster City, CA) and standard patch-clamp techniques. Cell capacitance ranged from 10–40 pF. Series resistance was typically <5 MΩ, and compensated 70–85%, resulting in a typical settling time of ~80 µs. Voltage pulses were delivered every 15–20 s from a holding potential of ~110 mV, except for prepulse inactivation.
protocols, where voltage pulses were given every minute from a holding potential of −120 mV to allow recovery from inactivation. Data were typically acquired at 50 kHz and filtered at 10 kHz (−3 dB, four-pole Bessel). Displayed traces have generally been additionally processed with a gaussian digital filter at 2 kHz. Leak and capacity currents were subtracted by a P/8 protocol (ionic currents) or P/−8 protocol (gating currents) from the −110-mV holding potential, unless otherwise noted (Armstrong and Bezanilla, 1974). To allow better resolution of small currents, we often subtracted a smooth curve fitted to the leak currents. In some cases, the first 200 ms after a voltage step contains a large leak subtraction artifact, which was zeroed when present before digital filtering.

The base external solution contained (mM) 155 N-methyl-D-glucamine (NMG) aspartate, 10 HEPES, 10 4-aminopyridine, 0.1 EGTA, pH 7.4 with NMG, 280–300 mM with no added charge carriers. The internal solution contained (mM) 150 NMG-methanesulfonate (MeSO4), 1 MgCl2, 4 MgATP, 10 HEPES, 10 EGTA, pH 7.3, with NMG, typically 280–290 mM. The h(V)-V relations shown in Fig. 1 for αC were obtained with an internal solution in which the 150 mM NMG-MeSO4 was replaced by 150 mM Cesium-MeSO4. For measurement of ionic currents, either 2 or 10 mM BaCl2 was added to the external solution. For typical gating current measurements, 0.2 mM LaCl3/2 mM MgCl2 was added. External solution flowed continuously at a rate of 1–2 ml/min during recording. The bath solution was ground by a 0.5 M KCl agar bridge attached to a Ag-AgCl wire. Measurements were started after >5 min of dialysis with the internal solution. In all cases, the junction potential between external and internal solutions was −5 mV (Nehér, 1992). To determine the true applied potential, this value should be added to the voltages in the figures and text.

For measurement of αC activation curves, 2 mM BaCl2 was the charge carrier throughout. Test depolarizations were 30 ms long and ranged from −70 to +70 mV (see Fig. 2 A, top) with repolarization to −50 mV to allow good resolution of tail currents. For each cell, plots of peak tail current at −50 mV (Itail) vs. test pulse voltage (Vtail) were normalized by an estimate of maximal peak gating current. To assay the magnitude of the error that such corrections might produce, we corrected G-V relations for the contribution of the “OFF” gating current. To determine the magnitude of the error that such OFF gating currents might produce, we corrected G-V relations for six cells transfected with α1β2γδ by subtracting the OFF gating currents measured during repolarization to −50 mV. We found that the average single-Boltzmann fit parameters for the corrected and uncorrected G-V curves were statistically indistinguishable (P < 0.05, Student’s t test, uncorrected: z = 3.51 ± 0.4, V1/2 = 20.1 ± 3.2 mV; corrected: z = 3.57 ± 0.5, V1/2 = 19.7 ± 3.4 mV), although Itail,max was reduced by −5% (−2,638 ± 568 pA [uncorrected] vs. 2,510 ± 542 pA [corrected]). Gmax was calculated according to Gmax = Itail,max/(Vtail-Vrev), where Vrev was +40 mV in 2 mM BaCl2. Therefore, the small error in Itail,max will lead to a slight overestimation of the Gmax/Qmax ratio, which may vary slightly for the different subunit combinations.

For gating currents, ionic currents were blocked by the external solution containing 0.2 mM LaCl3 (Bean and Rios, 1989). The effective free La3+ concentration was 0.1 mM due to the presence of 0.1 mM EGTA in all external solutions. The voltage protocol was the same as for ionic currents, except that the test pulse duration was decreased to 15 ms, and repolarization to −110 mV (see Fig. 5 A, top). Total charge moved during test depolarization (Qon) was obtained by integrating over the entire depolarizing epoch, taking as the zero baseline the average current over the last 3 ms of the test pulse. Total charge moved during repolarization (Qoff) was calculated similarly. For each cell, Qon-V and Qoff-V curves were normalized by an estimate of maximal mobile charge (Qmax), taken as the saturating value of the Boltzmann fit (detailed below) to the Qon-V or Qoff-V curves, as indicated in the text. Such normalized Qon-V and Qoff-V curves were averaged across cells.

To ensure that La3+ does not alter activation gating, we obtained Q-V relations both in the presence and absence of La3+ blockade. Fig. 1 A shows the results of the analysis, in which we compared Qon-V curves acquired in 2 mM MgCl2 (●) and 2 mM MgCl2/0.2 mM LaCl3 (○). The identity of the two curves, absent the expected surface-potential shift, provides additional strong support that La3+ does not perturb activation gating.

To determine explicitly the surface-charge shift between solutions used for ionic and gating currents, we exploited the property that isolated gating currents can actually be measured in the solution for ionic current, so long as the voltage range is negative to the threshold (−65 mV, Fig. 1 B, inset) for ionic-current activation. We could then calculate the surface-charge shift by direct comparison of the rising “foot” of Q-V curves obtained in ionic and gating current solutions. Fig. 1 B shows the results of this approach. Before averaging across cells, Q-V data for a single cell was normalized by the value of Qoff at −65 mV in 2 mM Ba2+. In the ionic-current solution containing 2 mM Ba2+ the Qon-V (Fig. 1 B, inset, ○) and Qoff-V (inset, ●) curves matched at potentials negative to −65 mV, indicating that gating currents were isolated below this potential. The main graph in Fig. 1 B demonstrates that, over this range of voltages, Qon-V relations obtained in 2 mM Ba2+ (○) and 2 mM MgCl2/0.2 LaCl3 (●) are essentially indistinguishable, indicating that there is little if any surface-charge shift between solutions. To quantitate the value of the shift, for each cell the voltage shift required to fit the same dual-Boltzmann to both sets of Qon-V data was taken to be the surface potential difference. Averaging this value across cells gave a value of 3 ± 1 mV (n = 9). These results excluded the need for surface-charge correction between ionic and gating current measurements.

Steady state inactivation curves were approximated using a protocol in which a 20-s prepulse was followed by a step to the current–voltage (I-V) curve (typically −5 mV) to measure the fraction of inactivated current. In some cases, a 10-ms normalizing prepulse at the test pulse potential was included before the 20-s prepulse to assay for the presence of cumulative inactivation or rundown. Steady state inactivation (h(V)-V) curves were derived by normalizing test pulse currents by either the current during the normalizing test pulse, or by the value of the test pulse with no prepulse. All steady state inactivation curves were measured with 10 mM Ba2+ as charge carrier. Voltage commands were given every minute from a holding potential of −120 mV. Typically, prepulse voltages ranged from −120 to −20 mV in 10-mV increments. Normalized h(V)-V relations were averaged across cells. For cells transfected with two β subunits, the h(V)-V relation was fit with a dual-Boltzmann function to obtain parameters for the low and high threshold components, in addition to the relative contribution of each component.

Boltzmann fits to either G-V or Q-V relations were performed with functions of the form B(V) = Bmax/[1 + exp(−(V − V1/2)/RT)], where Bmax is the saturating value, z is the effective charge, and V1/2 is the midpoint of activation. Qon-V data above +40 mV were sometimes unreliable and were therefore excluded.

1Abbreviation used in this paper: I-V, current-voltage.
For dual-Boltzmann fits to $h(\infty)-V$ relations, we used a function of the form $B(V) = f_{\text{low}}[1 + \exp[zs_{\text{low}}(V - V_{1/2,\text{low}})/RT]]^{-1} + f_{\text{high}}[1 + \exp[zs_{\text{high}}(V - V_{1/2,\text{high}})/RT]]^{-1}$, where $V_{1/2,\text{low}}$ and $V_{1/2,\text{high}}$ are midpoints of activation, $zs_{\text{low}}$ and $zs_{\text{high}}$ are the effective valences, and $f_{\text{low}}$ and $f_{\text{high}}$ are amplitudes of low and high threshold components. Fits were obtained using nonlinear, least-squares minimization. All reported values are mean ± SEM.

**RESULTS**

**Enhancement of Expressed Current Density by Auxiliary Subunits**

Transfection of HEK 293 cells with the $\alpha_{1E}$ subunit alone, or in combination with various auxiliary subunits, led to the expression of well-resolved inward barium currents carried by recombinant calcium channels (Fig. 2 A). The relative magnitudes of the various sets of traces illustrate that addition of auxiliary subunits caused striking increases in the level of expressed current. To quantify the relative increase in current density, we calculated the maximum tail current upon repolarization to 50 mV [$G_{\text{max}} = nP_{o,max} g(\text{-}50 \text{ mV})h$], where $g$ is the unitary conductance, $h$ is the fraction of noninactivated channels at the end of the test pulse, $n$ is the number of channels, and $P_{o,max}$ is the maximum open probability. Fig. 2 B compares the average values of $G_{\text{max}}$ for all different subunit combinations examined. The largest effect was the ~12-fold enhancement of expressed current with the coexpression of $\beta$ subunits. All $\beta$ subunits were approximately equipotent in this regard, although the average $B_{3}$ effect was slightly smaller (approximately sevenfold). Addition of $\alpha_{\delta}$ to $\alpha_{1E}$ produced a weaker increase in current (about threefold), and the combination of $\alpha_{\delta}$ and $\beta$ subunits yielded no appreciable current enhancement over the coexpression of $\beta$ subunits alone. Since modulation of $G_{\text{max}}$ values may reflect not only changes in $nP_{o,max}$, but also differences in the number of noninactivated channels ($h$) with different subunits, we examined another measure of current density (Fig. 2 C), $I_{\text{peak}} = nP_{o}[I_{\text{peak}}(V_{\text{peak}})]i(V_{\text{peak}})$, where $P_{o}[I_{\text{peak}}]$ and $i(V_{\text{peak}})$ are the open probability and unitary current at the voltage ($V_{\text{peak}}$) yielding the maximum test-pulse current. This measure ($I_{\text{peak}}$), which is less sensitive to test pulse inactivation, gave similar results. Therefore, we are confident that $G_{\text{max}}$ can henceforth be used as a quantitative indicator of relative changes in current ($nP_{o,max}$).

**Isolation of Gating Currents from Channels Containing the $\alpha_{1E}$ Subunit**

To determine the origin of the increased current density ($nP_{o,max}$), we wished to measure the maximum amount of mobile gating charge ($Q_{\text{max}} = nq$, $q$ is the charge per channel), which provides a convenient assay for the relative number of functional channels ($n$). Measuring $Q_{\text{max}}$ involves good resolution of the cur-

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**Figure 1.** La$^{3+}$ control experiments. (A) La$^{3+}$ does not change the voltage dependence of charge movement. Comparison of average Q-V relations in 2 mM Mg$^{2+}$ ($\bullet$, $n = 6$) and 2 mM Mg$^{2+}$/0.2 mM La$^{3+}$ ($\bigcirc$, same cells). Solid lines represent Boltzmann fits to the data without ($V_{1/2} = 32.8$ mV, $z = 2.4$) and with ($V_{1/2} = 28.5$ mV, $z = 2.4$) La$^{3+}$. (B) Estimate of surface charge shift between 2 mM Ba$^{2+}$ and 2 mM Mg$^{2+}$/0.2 mM La$^{3+}$. (main plot) Comparison of averaged Qo-V relations ($n = 9$) measured in 2 mM Ba$^{2+}$ ($\bigcirc$) and 2 mM Mg$^{2+}$/0.2 mM La$^{3+}$ ($\bigcirc$) to demonstrate the absence of an appreciable surface charge shift. Solid line is a dual-Boltzmann fit by eye to the 2 mM Mg$^{2+}$/0.2 mM La$^{3+}$ data. (inset) Plot of averaged Qo-V ($\bigcirc$) and Qoff-V ($\bullet$) relations measured in 2 mM Ba$^{2+}$ to illustrate the threshold of activation (~65 mV, dotted vertical line). For voltages past the threshold of activation, the presence of ionic current shifts the baseline used in the calculating $Q_{\text{max}}$ as a result, $Q_{\text{off}}$ is overestimated and appears larger than $Q_{\text{on}}$ for these voltages.
rents arising from gating charge movement (gating currents; Armstrong and Bezanilla, 1977; Sigworth, 1994), which in turn requires a blocker that eliminates ionic currents without significantly perturbing channel gating behavior. Previous work indicates that the highly potent block by La\(^{3+}\) can be used to isolate gating currents of calcium channels containing the \(\alpha_{1B}\) subunit (Jones et al., 1997a), but not the \(\alpha_{1C}\) subunit (Kamp et al., 1996). To determine the feasibility of La\(^{3+}\) blockade of calcium channels containing \(\alpha_{1E}\), we examined gating currents with either 2 mM Ba\(^{2+}\) as charge carrier in response to voltage steps ranging from \(-50\) to \(50\) mV in 10-mV increments for the indicated subunit combinations. (Cells 98_16 [\(\alpha_{1E}\)], 174_3 [\(\alpha_{1E} + \alpha_{1O}\)], 296_4 [\(\alpha_{1E} + \beta_{1b}\)], 252_5 [\(\alpha_{1E} + \beta_{1a}\)], 306_21 [\(\beta_{3}\)], and 296_25 [\(\beta_{1c}\)])

**Figure 2.** Modulation of ionic current density by \(\beta\) subunits. (A) Ionic currents in response to the protocol (top) used to measure the voltage dependence of ionic activation. Currents were measured with 2 mM Ba\(^{2+}\) as charge carrier in response to voltage steps ranging from \(-50\) to \(50\) mV in 10-mV increments for the indicated subunit combinations. (Cells 98_16 [\(\alpha_{1E}\)], 174_3 [\(\alpha_{1E} + \alpha_{1O}\)], 296_4 [\(\alpha_{1E} + \beta_{1b}\)], 252_5 [\(\alpha_{1E} + \beta_{1a}\)], 306_21 [\(\beta_{3}\)], and 296_25 [\(\beta_{1c}\)])

With assurance that La\(^{3+}\) does not detectably alter gating or surface-charge properties, we turned to analysis of extensive sets of currents recorded during La\(^{3+}\)
block for $\alpha_{1E} \beta_{2a}$ (Fig. 4 A). These traces represent genuine calcium-channel gating currents for several reasons. First, no such currents are observed in mock-transfected cells (Fig. 4 B). Second, no nonlinear charge movement is present in the range of our leak pulses (Fig. 4 C). Third, the measured charge movement is not affected by the choice of the leak subtraction protocols (data not shown). Finally, these “nonlinear displacement” currents have the standard properties typically associated with gating currents (Fig. 4 D): time integrals of outward ($Q_{\text{on}}$) and inward ($Q_{\text{off}}$) displacement currents saturated with increasing test depolarization; $Q_{\text{off}} \sim Q_{\text{on}}$ in the absence of inactivation (Fig. 4 D); charge movement ($Q_{\text{on-V}}$ or $Q_{\text{off-V}}$ curves) occurs before, and then parallels, ionic-current activation (G-V curve); and finally, the maximal amount of gating-charge ($Q_{\text{max}}$) is linearly correlated with maximal current density ($G_{\text{max}}$) (Fig. 4 E).

**Mechanism of Current Potentiation by Auxiliary Subunits**

With the ability to isolate gating currents, we could now compare auxiliary subunits with regard to their mechanism for current potentiation. Fig. 5 A displays representative gating-current records for most of the different subunit combinations. These traces illustrate that all auxiliary subunits boost the maximum amount of gating charge ($Q_{\text{max}}$), which is taken as the saturating value of the Boltzmann fit to the $Q_{\text{max}}$-$V$ relation. Fig. 5 B compares the average values of $Q_{\text{max}}$ for all different subunit combinations examined. $Q_{\text{max}}$ for channels expressed from $\alpha_{1E}$ alone was characteristically small, with a mean of $0.8 \pm 0.1 \text{ fC/pF} \ (n = 9)$. $\beta$ Subunits induced the strongest enhancement of $Q_{\text{max}}$, ranging from fourfold for $\beta_3$ to sevenfold for $\beta_4$. Coexpression of $\alpha_{1E} \delta$ also produced clear augmentation of $Q_{\text{max}}$, though the effect was less potent than for $\beta$ subunits. Table I summarizes the complete details of the analysis. Given that gating-charge per channel ($q$) does not appear to be affected by auxiliary subunits (Noceti et al., 1996), the rise in $Q_{\text{max}}$ likely reflects an increase in the number of functional channels ($n$). Hence, our results indicate that the enhancement of current density by auxiliary subunits arises, at least in part, from an increase in the number of functional channels. Such an increase in the number of functional channels may reflect either improved processing and trafficking of $\alpha_{1E}$ channels (increasing total amount of $\alpha_{1E}$ protein), or an increase in the fraction of functional $\alpha_{1E}$ protein in the membrane (with no increase in total amount of $\alpha_{1E}$ protein) by the $\alpha_{1E} \delta$ and $\beta$ subunits.

To determine whether an increase in the maximal open probability ($P_{\text{a,max}}$) also contributes to higher ionic-current densities, we calculated the ratio $G_{\text{max}}/Q_{\text{max}}$, which is directly proportional to $P_{\text{a,max}}$, so long as auxiliary subunits do not alter permeation properties of the channel (as in Fig. 6 D and Noceti et al., 1996). Fig. 5 C shows that all $\beta$ subunits approximately doubled $G_{\text{max}}/Q_{\text{max}}$, but $\alpha_{1E} \delta$ left the ratio unchanged. Table I reports further details of the calculations. The data in Fig. 5 suggest that $\beta$ subunits enhance $\alpha_{1E}$ current density by jointly increasing the number of functional channels (as reported by $Q_{\text{max}}$) and the maximal open probability (as reflected by $G_{\text{max}}/Q_{\text{max}}$). The enhancement of current by the $\alpha_{1E} \delta$ subunit appears to be fundamentally different: there may be a pure increase in the number of functional channels, without change in $P_{\text{a,max}}$.
A second goal of this study was to compare auxiliary subunit effects on the kinetics and voltage dependence of channel activation. To qualitatively compare activation kinetics for channels with different $\beta$ subunits, we normalized the rising phases of exemplar ionic-current records (Fig. 2A) evoked by voltage steps to $-20, 0, 20, 100$, and $40$ mV (Fig. 6A). The identical trajectories of traces from all four $\beta$ subunits suggest that $\beta$ subunits produce channels with similar activation kinetics. Fig. 6B shows the identical analysis for channels expressed from $\alpha_{1E}$ alone (solid trace) or from $\alpha_{1E} + \alpha_2\delta$ (dashed trace). The records for $\alpha_{1E} + \beta_1b$ (gray traces) are reproduced for comparison. Here again, the close correspondence between traces suggests that auxiliary subunits do not significantly modulate activation kinetics.

To examine whether auxiliary subunits affect the steady state voltage dependence of activation (G-V), we tested for subunit-dependent changes in G-V curves derived from peak tail currents (see MATERIALS AND METHODS) (Fig. 6C). Coexpression of the $\alpha_2\delta$ subunit had little effect on the G-V (Fig. 6C) or I-V (Fig. 6D) relations. The lack of effect of $\alpha_2\delta$ on the kinetics and voltage dependence of activation, as well as on $G_{max}/Q_{max}$ (Fig. 5C), suggests that this subunit is functionally uncoupled from any aspect of activation in $\alpha_{1E}$. In contrast, single-Boltzmann function analysis (Fig. 6C, solid curves, and Table II) clearly demonstrates that coex-
pression of β subunits produces an ~7-mV hyperpolarizing shift and a modest increase in the steepness of G-V relations (e.g., the Boltzmann valence (z) increases from 2.4 for α1E to 3.6 for α1Eβ2a). As expected from the shift in the G-V relation, coexpression of β subunits shifted the peak of the I-V relation leftward (Fig. 6D) without altering the reversal potential.

The results in Fig. 6, C and D, are compatible with

**Figure 5.** Comparison of gating currents for α1E in combination with different auxiliary subunits. (A) Representative gating currents were obtained using the same voltage protocol and block solutions as in Fig. 4. Traces are for cells transfected with the indicated subunit combinations in response to test pulse voltages of −40, −20, 0, 20 and 40 mV, respectively. Same cells as in Fig. 2. (B) Comparison of the average Qmax values for different subunit combinations. The maximum amount of mobile charge (Qmax) was calculated from the saturating values of Boltzmann fits to Qon-V. (C) Comparison of the average Gmax/Qmax values for different subunit combinations. The maximum conductance (Gmax) was determined from the saturating value of the Boltzmann fit to the tail-activation curves measured in 2 mM Ba2+. Average Gmax, Qmax, and Gmax/Qmax values are summarized in Table I.

**Table I**

| Subunit Combination | Gmax | Ipeak | Qon,max | Qoff,max | Gmax/Qon | Gmax/Qoff |
|---------------------|------|-------|---------|---------|----------|-----------|
| α1E                 |      |       |         |         |          |           |
| α1Eα2               | 2.9  | 2.6   | 0.8     | 0.8     | 100      | 110       |
| α1Eβ2a              | 2.3  | 2.6   | 0.8     | 0.8     | 90       | 120       |
| α1Eβ2b              | 2.3  | 2.6   | 0.8     | 0.8     | 100      | 120       |
| α1Eβ2c              | 2.3  | 2.6   | 0.8     | 0.8     | 100      | 120       |
| α1Eβ2d              | 2.3  | 2.6   | 0.8     | 0.8     | 100      | 120       |

Gmax, Qon, and Qoff are derived as the saturating values of the single-Boltzmann fits to Itail-V, Qon-V, and Qoff-V data, respectively, normalized by the cell capacitance. Itail corresponds to the peak tail current during repolarization to −50 mV with 2 mM Ba2+. Qon and Qoff are given by the integral of the ON and OFF gating current transients, respectively, measured in 2 mM Mg2+/0.2 mM La3+.
earlier work on β subunit effects in Xenopus oocytes (Olcese et al., 1994), in which G-V relations were fitted with dual-Boltzmann functions. In agreement with the earlier report, application of dual-Boltzmann analysis to our G-V data (Fig. 6, dashed curves) suggests that the apparent hyperpolarization and steepening of activation by β subunits could arise from an increase in the proportion of the low threshold Boltzmann component from ~30 to ~70%, without change in valence or midpoint parameters of individual Boltzmann functions. More in-depth interpretation of the data, like that introduced by dual-Boltzmann analysis, is deferred to the discussion, where explicit fits of a multistate kinetic model will be employed. For simplicity, in the remainder of the results, we retain single-Boltzmann analysis for first-order characterization of experimentally resolvable changes in activation. Regardless of the particular analytical functions used to describe the data, the
results thus far (Figs. 5 and 6, A–D) clearly indicate that β subunits increase ionic current by simultaneously modulating the G-V relation and doubling the G max/Q max ratio, in agreement with the findings of Olcese et al. (1994, 1996).

To explore the mechanistic basis of the β subunit effects on activation, we investigated how auxiliary subunits influenced Q-V curves derived from gating currents (Fig. 6 E). The rising phase of Q-V curves is very sensitive to modulation of the early events in the activation pathway, and the interrelation of Q-V and G-V curves lends insight into steps that couple voltage sensor movement to channel openings (Jones et al., 1997a). Fig. 6 E illustrates that all β subunits produced essentially identical effects on the Q-V relation: a small hyperpolarizing shift in the midpoint (≈5 mV) with little change in the steepness (Boltzmann valence $z$ ranges from 2.5 to 2.8, Table II). The effects of β subunits on Q-V curves are smaller than on G-V curves, thereby narrowing the gap between Q-V and G-V relations along the voltage axis. As expected from previous null results, $\alpha_\delta$ had no effect on the Q-V relation. All the β subunit effects on gating (Figs. 5 C and 6, C–E), particularly the contraction between Q-V and G-V curves, fit nicely with the idea that all β subunits act primarily to modulate a single locus of weakly voltage-dependent steps late in the activation pathway (see Discussion).

### Functional Stoichiometry of β Subunit Interaction

Previous reports in *Xenopus* oocytes indicate that $\alpha_{1E}$ channels containing different β subunits have strikingly different inactivation characteristics, despite very similar activation gating (Olcese et al., 1994). Here, we sought to confirm this effect in HEK 293 cells so that we could exploit this property to test whether multiple β subunits can simultaneously define the functional behavior of a calcium channel. To assay inactivation properties, we used a 20-s prepulse followed by a test pulse to the peak of I-V relations (Fig. 7 A). Typical currents for $\alpha_{1E}\beta_{2a}$ and $\alpha_{1E}\beta_3$ channels illustrate the extremes of inactivation behavior observed with the different subunit combinations. $\beta_{2a}$ dramatically slowed inactivation, while $\beta_3$ accelerated inactivation. $\beta_{1b}$ and $\beta_3$ subunits also accelerated inactivation during the test pulse (not shown), though not as strongly as $\beta_3$. To provide a robust indication of the differences in inactivation properties, we used such records to calculate steady state inactivation curves ($h(\infty)$ V curves; Fig. 7 B). While addition of $\alpha_\delta$ did not affect the $h(\infty)$ V relation, coexpression of β subunits induced striking modulation of steady state inactivation: $\beta_{1b}$, $\beta_3$, and $\beta_4$ all left-shifted $h(\infty)$ V curves by $\approx$10, 15, and 10 mV, respectively; $\beta_{2a}$ imparted a right shift of $\approx$15 mV. The profound distinction between the effects of $\beta_{2a}$ and the other β subunits has been reported in previous studies of $\alpha_{1E}$ (Olcese et al., 1994), and of other neuronal calcium channels, including $\alpha_{1A}$ (Stea et al., 1994) and $\alpha_{1B}$ (Patil et al., 1998).

Table III summarizes the Boltzmann analysis of $h(\infty)$ V data.

To investigate whether multiple β subunits can concomitantly specify the functional properties of a single calcium channel, we took advantage of the vast difference between the $h(\infty)$ V relations for $\alpha_{1E}\beta_3$ and $\alpha_{1E}\beta_{2a}$ channels. If there are multiple β subunit sites on $\alpha_{1E}$ that specify inactivation properties, then cotransfection of both $\beta_{2a}$ and $\beta_3$ subunits should result in mixed-composition channels (e.g., $\alpha_{1E}\beta_2\beta_3$) whose inactivation behavior should be distinct from that of pure $\alpha_{1E}\beta_{2a}$ or $\alpha_{1E}\beta_3$-like channels. However, if there is only one functionally active β subunit site per channel, the aggregate $h(\infty)$ V relation should possess only two components. Fig. 8, A and B, shows the results for one such experiment in which $\beta_3$ and $\beta_{2a}$ were cotransfected in a 1:1 weight ratio. This example demonstrates that inactivation is clearly biphasic, with a low threshold, readily inactivating component, as well as a high threshold, inactivation-resistant component. Only two Boltzmanns are

### Table II

Comparison of Average Boltzmann Parameters for both the Voltage Dependence of Ionic Activation (G-V) and Charge Movement (Q-V)

| Channel | G-V | I off | G-V $V_{1/2}$ | G-V | Q-V | Q off | Q-V $V_{1/2}$ | Q off |
|---------|-----|-------|---------------|-----|-----|-------|---------------|-------|
| $\alpha_{1E}$ | 9   | -210 ± 20 | -14.9 ± 2.6 | 2.4 ± 0.1 | 8 | 80 ± 40 | -23.4 ± 2.2 | 2.8 ± 0.2 |
| $\alpha_{1E}\beta_3\delta$ | 15  | -738 ± 155 | -12.8 ± 1.7 | 2.5 ± 0.2 | 12 | 119 ± 18 | -23.3 ± 1.9 | 2.6 ± 0.2 |
| $\alpha_{1E}\beta_{2a}$ | 19  | -2419 ± 249 | -21.3 ± 1.0 | 3.6 ± 0.1 | 25 | 200 ± 25 | -29.0 ± 1.2 | 2.8 ± 0.1 |
| $\alpha_{1E}\beta_3$ | 11  | -2283 ± 382 | -22.6 ± 1.2 | 3.1 ± 0.1 | 9 | 184 ± 26 | -31.0 ± 1.1 | 2.5 ± 0.2 |
| $\alpha_{1E}\beta_{1b}$ | 22  | -1731 ± 223 | -20.9 ± 0.4 | 3.1 ± 0.1 | 10 | 157 ± 42 | -27.6 ± 1.7 | 2.8 ± 0.2 |
| $\alpha_{1E}\beta_4$ | 17  | -1436 ± 179 | -21.2 ± 0.6 | 3.2 ± 0.1 | 7 | 165 ± 14 | -31.6 ± 1.2 | 2.5 ± 0.1 |
| $\alpha_{1E}\beta_2\alpha\beta_3\delta$ | 31  | -2572 ± 221 | -20.4 ± 0.9 | 3.3 ± 0.1 | 27 | 279 ± 52 | -29.5 ± 1.2 | 2.8 ± 0.1 |

G-V relations were derived by measuring peak tail currents in 2 mM Ba$^{2+}$ and Q-V relations were derived by integrating the ON gating current transient measured in 2 mM Mg$^{2+}$/0.2 La$^{3+}$. The fit function is described in materials and methods.
Figure 7. Auxiliary subunits modulate inactivation. (A, top) Voltage protocol used to approximate the steady state inactivation ($h(\infty)$)-$V$ relation. Voltage commands were given from a holding potential of $-120$ mV every 60 s. The test pulse potential was chosen to be the peak of the I-V relation, typically $-5$ mV. All $h(\infty)$-$V$ measurements were made with $10$ mM Ba$^{2+}$ as a charge carrier unless otherwise indicated. (bottom) Representative whole-cell records for a cell transfected with $\alpha_{1E}\beta_{2a}$ (cell 325_19) and a cell transfected with $\alpha_{1E}\beta_{3a}$ (cell 327_11) for prepulse potentials of $-100$ to $-30$ mV ($\beta_{2a}$) and $-100$ to $-60$ mV ($\beta_{3a}$), respectively. Only the first $200$ ms of the prepulse were acquired and displayed. (B) Average $h(\infty)$-$V$ relations derived by normalizing the peak test pulse current data from the protocol in A by the peak test pulse current in the absence of a prepulse and averaging across cells. All subunit combinations are plotted, with the identical legend as in Fig. 6, with the addition of $\alpha_{1E}\beta_{2a}\delta$ ($\times$). Solid lines represent single-Boltzmann fits with values (subunit combinations [$z$, $V_{1/2}$ (mV)]): $\alpha_{1E}$ (2.4, $-66$), $+\alpha_{1E}\delta$ (2.3, $-62$), $+\beta_{1b}$ (3.9, $-74$), $+\beta_{2a}$ (2.8, $-46$), $+\beta_{3}$ (3.6, $-81.3$), $+\beta_{4}$ (3.7, $-75$), $+\beta_{2a}\alpha_{1E}\delta$ (3.2, $-47$), and $+\beta_{2a}\alpha_{1E}\delta$ (3.5, $-74$). Average fit values are summarized in Table III.

Table III

| $n$ | $V_{1/2}$ | $z$ |
|-----|----------|-----|
| $\alpha_{1E}$ | 10 | $-65.7 \pm 2.8$ | $-3.0 \pm 0.1$ |
| $\alpha_{1E}\beta_{2a}\delta$ | 9 | $-64.2 \pm 4.7$ | $-3.2 \pm 0.1$ |
| $\alpha_{1E}\beta_{3a}$ | 12 | $-50.6 \pm 1.5$ | $-3.7 \pm 0.1$ |
| $\alpha_{1E}\beta_{4}$ | 6 | $-75.7 \pm 1.4$ | $-3.7 \pm 0.1$ |
| $\alpha_{1E}\beta_{1b}$ | 9 | $-73.8 \pm 1.2$ | $-4.0 \pm 0.2$ |
| $\alpha_{1E}\beta_{2a}$ | 7 | $-81.4 \pm 1.2$ | $-3.7 \pm 0.2$ |
| $\alpha_{1E}\beta_{2a}\alpha_{1E}\delta$ | 11 | $-48.7 \pm 1.3$ | $-3.4 \pm 0.2$ |
| $\alpha_{1E}\beta_{2a}\alpha_{3E}\delta$ | 7 | $-74.3 \pm 2.1$ | $-3.3 \pm 0.2$ |
| $\alpha_{1E}\beta_{2a}\alpha_{3E}\delta$ | 13 | $-56.5 \pm 2$ | $-3.2 \pm 0.1$ |
| $\alpha_{1E}\beta_{2a}\delta$ | 6 | $-38.9 \pm 2.1$ | $-3.2 \pm 0.3$ |
| $\alpha_{1E}\beta_{2a}\delta$ | 7 | $-71.0 \pm 1.2$ | $-3.8 \pm 0.2$ |
| $\alpha_{1E}\beta_{2a}$ | 4 | $-75.8 \pm 2.3$ | $-4.4 \pm 0.1$ |

For each cell, a single-Boltzmann fit was to the $I_{\text{test}}$-$V_{\text{pre}}$ relation, where $I_{\text{test}}$ is the test pulse current after a 20-s prepulse to $V_{\text{pre}}$ (Fig. 7). Measurements were made in 10 mM Ba$^{2+}$. The fit function is given in MATERIALS AND METHODS.

required to produce an excellent fit of the data since the average residual for the dual-Boltzmann fit is close to zero (Fig. 8 C). Furthermore, the average fit parameters to the $h(\infty)$-$V$ data correspond closely to the steady state inactivation properties of pure $\alpha_{1E}\beta_{2a}$ and $\alpha_{1E}\beta_{3a}$ channels (Fig. 7 B and Tables III and IV). Cotransfection of $\beta_{3a}$ and $\beta_{2a}$ in a 5:1 weight ratio merely decreased the relative amplitude of the low threshold component (Fig. 8 D), while preserving the intrinsic properties of the two components (Table IV). The only apparent deviation from parameters obtained with pure-composition channels is a small 7–9-mV increase in the $V_{1/2}$ for the high threshold component (compare Tables III and IV). Although this increase could reflect a minor contribution of mixed-composition channels, the overall results are consistent with the functional dominance of pure $\alpha_{1E}\beta_{2a}$ and $\alpha_{1E}\beta_{3a}$ channels.

As a further test for the possible functional role of a second $\beta$ subunit site (Tareilus et al., 1997), we examined how auxiliary subunits modulated the properties of a COOH-terminal truncation of the $\alpha_{1E}$ construct ($\alpha_{1E}\Delta_{3}$, amino acids 1–1871 of $\alpha_{1E}$ [1–2251]) that lacks the secondary binding site. Fig. 9 A displays ionic current curves for channels composed of $\alpha_{1E}\Delta_{3} + \alpha_{1E}\delta$ or $\alpha_{1E} + \beta_{2a} + \alpha_{1E}\delta$ subunits. Coexpression of $\beta_{2a}$ with $\alpha_{1E}\Delta_{3}$ increased $G_{\text{max}}$ from $-306 \pm 115$ pS/pF $(n = 11)$ to $-1,708 \pm 251$ pS/pF $(n = 4)$, a 5.6-fold increase similar to the 4.1-fold increase in $G_{\text{max}}$ seen for wild-type $\alpha_{1E}$ (Fig. 2 B). Similarly, modulation of activation by $\beta_{2a}$ is unchanged by the COOH-terminal deletion, as demonstrated in Fig. 9 B by the identical subunit modulation of G-V relations for $\alpha_{1E}\Delta_{3}$ (circles) and wild-type $\alpha_{1E}$ (squares). Finally, we compared $\beta$ subunit modulation of the steady
state inactivation properties of $\alpha_{1E}$ (Fig. 9, C, traces, and D, symbols; Table III) with data obtained with wild-type $\alpha_{1E}$ (Fig. 9 D, lines). The $\alpha_{1E}$ data have been shifted uniformly by $-7$ mV in the Fig. 9 D overlay to account for a difference in inactivation that is present even without $\beta$ subunit coexpression (e.g., $\alpha_{1E} + \alpha_{1D}$ in Fig. 9 D); this small shift likely reflects a difference in the intrinsic inactivation behavior of the $\alpha_{1}$ backbone (Soldatov et al., 1997), rather than a change in the modulatory action of $\beta$ subunits. The close correspondence between $h(\psi)$-$V$ relations for $\alpha_{1E}$ (lines) and $\alpha_{1E} + \alpha_{1D}$ (symbols) in the Fig. 9 D overlay illustrates that $\beta$ subunit modulation of inactivation is similar for the two constructs. Although the small difference between modulation of $\alpha_{1E} + \alpha_{1D}$ and $\alpha_{1E}$ inactivation (most apparent for $\alpha_{1E} + \alpha_{1D}$, Fig. 9 D, V) could reflect a minor contribution of a second $\beta$ subunit binding site, all the results in Figs. 8 and 9 support the view that a single $\beta$ subunit binding site predominates in specifying inactivation properties. If present, the potential contribution of a second site appears to be small by comparison.

**Discussion**

Although auxiliary subunits clearly have a role in defining channel properties, specific modulatory effects vary widely across studies, underscoring the need to examine comprehensively the modulation of each $\alpha_{1}$ subunit under the same experimental conditions. Here, we have performed a systematic evaluation of auxiliary subunit regulation of expression and gating of $\alpha_{1E}$ calcium channels in HEK 293 cells. The experiments lead to three main conclusions. (a) The $\alpha_{1D}$ and $\beta$ auxiliary subunits differ fundamentally in the manner by which they induce an overall increase in current density. Coexpression of $\alpha_{1D}$ with the pore-forming $\alpha_{1E}$ moiety produced a clear-cut enhancement of current, arising purely from an increase in the number of functional channels ($n$), without significantly affecting channel gating behavior. By contrast, coexpression of $\beta$ subunits induced stronger potentiation of current by joint elevation of channel number ($n$) and maximal open probability ($P_{o,max}$), suggesting effects on both channel assembly and gating. (b) While $\alpha_{1D}$ had no appreciable effect on activation gating, $\beta$ subunits produced significant hyperpolarizing shifts in the voltage dependence of ionic-current activation and gating-charge movement, all without discernible change in activation kinetics. Importantly, different $\beta$ isoforms produced nearly indistinguishable effects in regard to both current potentiation and activation gating. (c) Little functional evi-

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Figure 8. Simultaneous cotransfection of two $\beta$ subunits ($\beta_{2a}$ and $\beta_{3}$) leads to two distinguishable channel populations. (A) Current traces recorded in $10$ mM Ba$^{2+}$ in response to the same $h(\psi)$-$V$ protocol as in Fig. 7 (top), for a cell (330,18) transfected with $\alpha_{1E}$ in combination with equal amounts of $\beta_{2a}$ and $\beta_{3}$. Traces displayed are for prepulse potentials ranging from $-120$ to $-30$ mV in $10$-mV increments. (B) Plot of peak test-pulse currents (○) for the cell in A, illustrating the bimodal nature of inactivation. The thick line corresponds to a dual-Boltzmann fit to the data with parameters (low: $-397$ pA, $z = -3.4$, $V_{1/2} = -81.2$ mV; high: $-164$ pA, $z = -4.7$, $V_{1/2} = -39$ mV), in good correspondence with the average steady state inactivation properties of $\alpha_{1E} + \beta_{2a}$ and $\alpha_{1E} + \beta_{3}$ channels, respectively (Table III). The dotted and dashed lines illustrate the two components of the dual-Boltzmann fit. (C) Plot of average residuals ($n = 26$). The difference between the data and the fit value was normalized by the peak test-pulse current before averaging across cells. (D) Bar graph illustrating the dependence of the fraction of the low threshold Boltzmann component on the ratio of transfected $\beta_{2a}$ and $\beta_{3}$. For the 1:1 ratio, $n = 17$, and for the 5:1 ratio, $n = 9$. (E) Comparison of average $V_{1/2}$ derived from dual-Boltzmann fits to the $h(\psi)$-$V$ for $\beta_{2a} + \beta_{3}$-transfected cells (diagonal striped), with the average fit values derived from single-Boltzmann fits to $h(\psi)$-$V$ data for $\beta_{2a}$- or $\beta_{3}$-transfected cells (gray). Fit values and cell numbers are summarized in Table III (for $\beta_{2a}$ or $\beta_{3}$ alone) and Table IV (for $\beta_{2a} + \beta_{3}$-transfected cells).
Table IV
Average Values for Dual-Boltzmann Fits to h(∞)-V Data for Cells Transfected with both β2a and β3 Simultaneously

| Construct                        | n  | Percent low (V) | V1/2,low (mV) | z1/2,low | V1/2,high (mV) | z1/2,high |
|----------------------------------|----|----------------|--------------|----------|---------------|----------|
| α1Eβ2aβ3 (1:1)                   | 17 | 21 ± 5         | -85.6 ± 2.1  | -3.7 ± 0.3| -44.5 ± 1.2   | -4.0 ± 0.3|
| α1Eβ3β2a (1:5)                   | 8  | 57 ± 8         | -85.1 ± 1.3  | -3.9 ± 0.2| -41.6 ± 1.4   | -3.8 ± 0.3|
| α1Eβ2aβ3 (all)                   | 25 | 32 ± 5         | -85.4 ± 1.3  | -3.8 ± 0.2| -43.6 ± 1.0   | -3.9 ± 0.2|

The h(∞)-V relation was derived from peak test pulse currents after a 20-s prepulse. Fit function is described in Materials and Methods.

Figure 9. Activation and inactivation properties of a COOH-terminal truncated version of α1E (α1E3, amino acids 1–1871) that lacks a putative β binding site. All measurements for both α1E and α1E3 are in 10 mM Ba2+. (A) Traces illustrating activation of ionic currents for α1Eβ2aδ (cell 375_19) or α1Eβ2bδ (cell 370_6) using the same voltage protocol as in Fig. 2. Data are shown for test pulse potentials of -30, -20, -10, 0, 10, and 50 mV. (B) Comparison of G-V for α1E3 and α1E indicates that modulation of α1E3 activation by β subunits is preserved. Symbols correspond to data for α1E3 and wild-type α1E (α1Eβ2aδ, ○, n = 4; α1Eβ2bδ, ●, n = 11; α1E3β2aδ, □, n = 11; and α1E3β2bδ, ▽, n = 8). Lines are single-Boltzmann fits to the α1E3β2aδ data with fit parameters z = 2.4, V1/2 = -10.1; and z = 3.4, V1/2 = -18.2, respectively. The shift in V1/2 values relative to Fig. 6 corresponds to a surface potential shift between 10 mM (used here) and 2 mM Ba2+ (Fig. 6). (C) Steady state inactivation. Traces after a 20 s prepulse (as in Fig. 7) to the indicated potentials are shown for α1E3 in combination with αδδ (cell 376_9), β3 (cell 375_4), β2aδδ (cell 370_11), and β2aδδ (cell 371_10). (D) Comparison of h(∞)-V relations for α1E3 (symbols) and α1E (same data as in Fig. 7, shown as lines connecting mean data values, without explicit reproduction of data points as symbols). Symbols correspond to the following constructs: α1E3αδδ, ●; α1E3β2aαδδ, ○; α1E3β3, ○; α1E3β2bαδδ, ●; α1E3β3, □. Data for α1E3 is shifted by -7 mV to overlay the α1E3 data. Average fit values and cell numbers are summarized in Tables V (G-V) and III [h(∞)-V] for both α1E and α1E3.
In the sections to follow, we will first relate each of the conclusions to previous studies of $\alpha_{1E}$ channels. Together, these findings represent an important contribution to clarifying both the mechanism and structural determinants of auxiliary-subunit modulation of calcium channels.

In the sections to follow, we will first relate each of the conclusions to previous studies of $\alpha_{1E}$ and, where relevant, other calcium channels. For clarity, we will discuss $\alpha_\delta$ and $\beta$ subunit effects sequentially, as independent parts. A kinetic mechanism is then developed to explain how $\beta$ subunits can produce all the observed changes in gating, simply by alteration of the equilibrium between a single, weakly voltage-dependent transition near the open state. Finally, we consider the generality of our conclusions to other $\alpha_1$ isoforms.

**Modulation of $\alpha_{1E}$ by $\alpha_\delta$**

The $\alpha_\delta$ subunit produced an approximately threefold increase in $\alpha_{1E}$ current, which arose almost exclusively from elevated channel expression ($Q_{\text{max}}$). The $\alpha_\delta$ subunit had no other clear modulatory effects, except to slightly antagonize the effect of $\beta_3$ on inactivation (Fig. 7 B). All measures of activation gating, including the maximal open probability ($G_{\text{max}}/Q_{\text{max}}$), the voltage dependence of charge movement ($Q/V$), and the voltage dependence of ionic activation ($G/V$) were similar to $\alpha_{1E}$ alone. Similar effects on inactivation and expression were reported for doe1 (marine ray analog of $\alpha_{1E}$) expressed in Xenopus oocytes (Ellinor et al., 1993). However, in contrast to our results, in studies of rat $\alpha_{1E}$ in COS-7 cells (Stephens et al., 1997) and human $\alpha_{1E}$ in Xenopus oocytes (Wakamori et al., 1994), coexpression of $\alpha_\delta$ was found to produce a depolarizing shift in the G-V without modifying expressed current levels; however, these studies agree with our findings concerning the slight antagonism of $\beta_3$ effects on inactivation (Fig. 7 B).

The applicability of our results to other channel types is unclear. However, it is interesting to note that the reported effects of the $\alpha_\delta$ subunit on other $\alpha_1$ subunits also varies widely, sometimes agreeing with our findings, other times not. For example, with regard to modulation of $\alpha_{1C}$ channel density, the $\alpha_\delta$ subunit was found to increase ligand binding sites (Welling et al., 1993), protein levels (Shistik et al., 1995), and gating currents (Bangalore et al., 1996). By contrast, in other studies (Wei et al., 1995; Gurnett et al., 1997) of $\alpha_{1C}$ maximal dihydropyridine binding is not increased by $\alpha_\delta$ coexpression. Similarly, activation kinetics of $\alpha_{1C}$ accelerate in some studies (Singer et al., 1991; Bangalore et al., 1996), but not others (Mikami et al., 1989; Welling et al., 1993). The sources of this variability have yet to be determined.

**$\beta$ Subunits Act Differently than the $\alpha_\delta$ Subunit**

By contrast to the $\alpha_\delta$ subunit, coexpression of $\beta$ subunits ($\beta_1$-$\beta_5$) enhanced current density ($G_{\text{max}}$) by increasing not only the number of functional channels ($Q_{\text{max}}$) but also the maximum open probability ($G_{\text{max}}/Q_{\text{max}}$). Similar effects on current density have been reported in COS 7 (Stephens et al., 1997) and HEK 293 cells (Williams et al., 1994), but not in Xenopus oocytes (Soong et al., 1993; Olcese et al., 1994, 1996). Furthermore, in a study of $\alpha_{1E}$ gating currents in Xenopus oocytes (Olcese et al., 1996), coexpression of $\beta_2a$ with $\alpha_{1E}$ actually decreased the number of functional channels ($Q_{\text{max}}$), although surprisingly they found a twofold increase of $G_{\text{max}}/Q_{\text{max}}$ that is qualitatively similar to our result. Additional support for the role of the $\beta$ subunit in modulating $P_{\alpha_{\text{max}}}$ comes from a separate study that used fluctuation analysis to determine the effects of $\beta_2a$ and $\beta_{1E}$ on $\alpha_{1E}$ open probability (Noceti et al., 1996).

Fitting with the doubling of the $G_{\text{max}}/Q_{\text{max}}$ ratio, the $\beta$ subunit also induced hyperpolarizing shifts of both G-V and the Q-V relations and slightly reduced the gap between the two, all while producing little effect on activation kinetics. Here, the action of $\beta$ subunits is also somewhat controversial. Although most studies of $\alpha_{1E}$ report effects on G-V relations and activation kinetics that are similar to ours (Witcher et al., 1993; Olcese et al., 1994; Stephens et al., 1997), in one case (Wakamori et al., 1994), $\beta_{1C}$ coexpression with the human $\alpha_{1E}$ in Xenopus oocytes was found to slow activation kinetics substantially. With respect to gating currents, the only other study of $\alpha_{1E}$ charge movement (Olcese et al., 1996) also found that the $\beta$ subunit reduced the gap between the G-V and Q-V. However, in contrast to the small but statistically significant ($P < 0.01$, Student’s $t$ test, comparing cells with and without a $\beta$) shift in the Q-V reported here, Olcese et al. (1996) found that the $\beta$ subunit produced no significant change in the Q-V. Again, these discrepancies may reflect differences between clones (human versus rat $\alpha_{1E}$) or expression systems (Xenopus oocytes versus HEK 293 cells).

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**Table V**

Comparison of Average Boltzmann Fit Values to G-V Relations

for $\alpha_{1E}$ and $\alpha_{1E1C}$ Subunits

| $n$ | $I_{\text{tail,max}}$ | G-V $V_{1/2}$ | G-V $z$ |
|---|---|---|---|
| $\alpha_{1E}\alpha_\delta$ | 11 | $-550 \pm 14$ | $-10.2 \pm 0.3$ | $2.5 \pm 0.1$ |
| $\alpha_{1E1C}\alpha_\delta_{1C}$ | 8 | $-3365 \pm 437$ | $-16.0 \pm 1.2$ | $3.7 \pm 0.2$ |
| $\alpha_{1E}\alpha_\delta$ | 11 | $-643 \pm 203$ | $-9.0 \pm 1.8$ | $2.7 \pm 0.2$ |
| $\alpha_{1E1C}\alpha_\delta_{1C}$ | 4 | $-4622 \pm 1066$ | $-16.4 \pm 0.9$ | $3.5 \pm 0.2$ |

G-V relations were derived by measuring peak tail currents in 10 mM Ba$^{2+}$ upon repolarization to $-50$ mV. The fit function is described in materials and methods.
these minor differences, all the results indicate a role for β subunits in modulating activation gating.

In other calcium channels, the reported effects of the β subunits vary even more widely than for the αββ subunit. However, at least in some respects, β subunit modulation of other αβ subunits appears similar to what we find for α1E. For example, there are reported shifts in the voltage dependence of ionic activation for α1A (Stea et al., 1994; De Waard and Campbell, 1995) and α1C (Wei et al., 1991; Neely et al., 1993). Increased current density has also been observed for many of the αβ subunits including α1A (Mori et al., 1991), α1B (Williams et al., 1992a), α1C (Perez-Reyes et al., 1992), α1D (Williams et al., 1992b), and α1S (Ren and Hall, 1997). Furthermore, for α1C, studies of gating currents in both HEK 293 cells (Kamp et al., 1996; Josephson and Varadi, 1996) and Xenopus oocytes (Neely et al., 1993) find that β subunit modulation of ionic current activation is not associated with much shift in the Q-V, similar to what we find for α1E. On the other hand, even these few gating current studies disagree in other regards. While coexpression of β1s (Kamp et al., 1996) or β3 (Josephson and Varadi, 1996) with α1C increased both current density and Qmax in HEK 293 cells similar to our results for α1E, β2 increased current without changing Qmax in Xenopus oocytes (Neely et al., 1993). Therefore, as with α1E, the specific effects observed with β coexpression appear to depend on as yet unknown distinctions between expression systems, perhaps the endogenous expression of βXO subunits in Xenopus oocytes (Tareilus et al., 1997).

Different β Isoforms Have Similar Effects on Activation and Expression, but Not on Inactivation

There was little isoform dependence to the modulation of all the above measures of activation gating, suggesting that different β subunits act by a similar mechanism to modulate activation and expression of α1E, despite very different effects on inactivation. While no other study has compared gating currents of α1E channels containing different β subunits, measurements of α1E ionic-current G-V curves in Xenopus oocytes support this finding (Olc ese et al., 1994). However, for other α subunits, modulation of expression and activation may differ across β subunits. For example, there clearly is isoform specificity in the β subunit modulation of current potentiation in α1A (Stea et al., 1994; De Waard et al., 1994) and α1S (Ren and Hall, 1997). This fits with the binding affinity differences in vitro of various β subunits to α1A (De Waard et al., 1995). However, binding of various β subunits to the I-II linker of α1A occurs with the same affinity (Scott et al., 1996). Whether differences in in vitro binding affinities translates into discernible gradations of functional effects remains to be established.

One β Subunit May Predominate in Directing Baseline Channel Properties

Most previous studies have implicitly assumed that only one β subunit is involved in modulating channel properties, consistent with biochemical evidence for a 1:1 stoichiometry of α1 and β subunits for skeletal (De Waard et al., 1996) and N-type (Witcher et al., 1993) channels. However, a recent report by Tareilus et al. (1997) identified a second β subunit binding site on the COOH terminus of α1E, raising the possibility that two or more β subunits might collectively determine channel gating properties. Here, we found little evidence that two β subunits modulate the properties of the α1E channel, either in regard to expression or gating.

Mechanism of β Subunit Modulation of α1E Gating

To account for β subunit effects on G-V and Q-V curves, previous studies have proposed that the β subunit acts mainly on the weakly voltage-dependent steps that “couple” channel opening to voltage sensor movement (Neely et al., 1993; Olcese et al., 1996). Here, we demonstrate that this mechanism may explain not only the modulation of G-V and Q-V curves, but also the doubling of maximum open probability. Fig. 10 depicts a channel gating model that closely resembles those previously used in the study of potassium channel gating (Zagotta and Aldrich, 1990; Schoppa et al., 1992). There are three independent, voltage-dependent transitions between the closed states (C0, C1, C2, and C3), each associated with an appropriately scaled equilibrium constant Ki. These transitions are followed by a weakly voltage-dependent transition (C2→C3) with equilibrium constant Kf and a final voltage-independent step with equilibrium constant K2. Kf and K2 are voltage dependent according to a Boltzmann distribution, Ki = exp(zi(F(V − V0, i))/RT). To obtain baseline model parameters (zi, V0, V1, Kf), we fit α1E alone Q-V and G-V data (Fig. 10 B). Then, to simulate the observed twofold change in the maximum open probability, we modified only K2, the equilibrium constant for the last voltage-independent transition leading to channel opening. This simple change reproduced well both the shift in the G-V relationship and the shift in the Q-V relationship (Fig. 10 C). In fact, such simulations indicate that modifying the coupling of charge movement to channel opening (K2) usually also perturbs the Q-V relation and, therefore, charge movement. Yet in several studies (Kamp et al., 1996; Josephson and Varadi, 1996; Neely et al., 1993; Olcese et al., 1996), shifts in ionic activation have been seen with little or no modification of charge movement. In these studies, it may be that the shift in charge movement is too small to be well resolved.

To determine the effect of modifying K2 on the time course of activation, we modeled the kinetics of activa-
The expected time course of activation kinetics, these ionic currents also represent equilibrium constants, according to the rate of the transition. The choice of rate constants is constrained by the equilibrium constant, \( K \). The movement of the three gating particles is followed by a weakly voltage-dependent step, which has a separate equilibrium constant, \( K_2 \). Finally, there is a voltage-independent transition, with \( K_3 \) constant. This change in \( K_2 \) corresponds to a 1.96-fold increase in the maximum open probability \( P_{n_{max}} = K_2/(K_2 + 1) \).

**Figure 10.** (A) Scheme used to model steady state activation. The equilibrium constant between two states is assumed to have the form \( K_i = \alpha_i(V)/\beta_i(V) = \exp(\Delta G_i/(RT)) \), where \( \alpha_i(V) \) and \( \beta_i(V) \) are rate constants (millisecond\(^{-1}\)), \( z \) gives the valence of the gating charge moved in the transition, and \( V_i \) is related to the zero-potential free energy difference between the two states. Transitions through states \( C_0-C_5 \) correspond to Hodgkin-Huxley-like behavior of three identical gating particles, each characterized by the same equilibrium constant, \( K_i \). The solid line is the model fit with kinetic parameters \( K_2 \) that we could vary to fit the activation kinetics. Fig. 10 shows a model fit (thin solid line) consistent with the steady state model parameters from Fig. 6 for \( \alpha_{iE} \) alone. The solid lines correspond to model fits produced by changing \( K_2 \) for \( \alpha_{iE} \) to 3.14, leaving all other equilibrium parameters the same. This change in \( K_2 \) corresponds to a 1.96-fold increase in the maximum open probability \( P_{n_{max}} = K_2/(K_2 + 1) \).

**Figure 10.** (D) Model fits for kinetics of activation. Pure ionic currents (thick gray line) were obtained for \( V_{test} = -30, -10, \) and 10 mV by subtracting gating currents measured in 2 mM Mg\(^{2+}/0.2 \mM \) La\(^{3+} \) from the currents measured in 2 mM Ba\(^{2+} \) (Fig. 10 A). The solid line is the model fit with kinetic parameters \( (f_1 = 1.8, d_4 = 0.4, f_2 = 1.75, d_1 = 0.5, f_3 = 1.75) \) consistent with the steady state model parameters from Fig. 6 for \( \alpha_{iE} \) alone. The dashed line illustrates the kinetics that result when \( K_2 \) is changed from 0.63 to 3.14, while otherwise maintaining the same model parameters as in D. This effect may be offset by setting \( f_1 \) to 6 and \( f_2 \) to 0.8 (solid line). Note that it is not necessary to modify parameters for the early transitions \( (f_1, d_2) \) to obtain a reasonable fit.

The choice of rate constants is constrained by the equilibrium constants, according to \( K_i(V) = \alpha_i(V)/\beta_i(V) \), where \( \alpha_i(V) \) is the forward rate constant and \( \beta_i(V) \) is the backward rate constant (seconds\(^{-1}\)). We chose \( \alpha_i(V) = f_i \exp(\Delta G_i/(RT)) \), and \( \beta_i(V) = \alpha_i(V)/K_i(V) \), giving us free parameters \( f_i, d_i, f_\alpha, f_\beta, \) and \( f_\delta \), which we could vary to fit the activation kinetics. Fig. 10 D shows a model fit (thin solid line) consistent with \( \alpha_{iE} \) alone steady state model parameters \( (z_0, z_1, V_0, V_1, K_2) \) (Fig. 10 B). Representative ionic current data (Fig. 10 B, thick gray line) are derived by subtracting the gating currents (Fig. 5 A) from the \( \alpha_{iE} \beta_{b1} \) whole cell records in Fig. 6 A. Because of the subunit invariance of activation kinetics, these ionic currents also represent the expected time course of \( \alpha_{iE} \) alone. Changing \( K_2 \) to accord with the twofold increase in maximal open probability produced by \( \beta \) subunits can modify the activation kinetics (Fig. 10 E, dashed line), but this may be compensated for by altering only \( f_1 \) and \( f_2 \). This amounts to subtle changes in the absolute rate constants of the last two transitions, but only an alteration of the equilibrium constant of the last transition. Although no change in parameters corresponding to the more voltage-dependent steps \( (z_0 \) and \( f_\delta) \) is necessary, we found that we could not well reproduce the invariance of activation kinetics by just modifying \( f_2 \), corresponding to the last voltage-independent step. Therefore, this simulation argues that all the effects of \( \beta \) subunit modulation of \( \alpha_{iE} \) (increased open probability, hyperpolarization of G-V and Q-V curves, and invariant activation kinetic-
ics) can be attributed to actions on one or a few weakly voltage-dependent steps before opening.

**Generalizability of Results to Other α₁ Isoforms?**

From the previous discussion, it is clear that generalization across α₁ subtypes is a difficult proposition. Nevertheless, we wondered whether some of the most robust properties of α₁E modulation by subunits would translate to a different α₁ subunit. In particular, there was striking adherence to two “rules” for α₁E modulation by subunits: (a) all β subunits produce no change in the kinetics of activation, but induce identical but relatively small hyperpolarizing shifts of the G-V curve, and (b) distinct β subunits impart vastly different steady state inactivation curves. Do these features of subunit modulation hold true as general tenets for other α₁ subunits?

Fig. 11 tests this proposition for the α₁C calcium channel. The results indicate a complete reversal of the behavior found with α₁E. Now the kinetics of activation are clearly different for β₃ and β₂a subunits. In addition, the different β isoforms led to large differences in G-V curves. On the other hand, steady state inactivation curves show only small isoform-dependent distinctions.

The diametrically opposite behaviors exhibited by α₁E and α₁C subunits have interesting implications for the structure–function relations underlying α₁–β modulation. The leading candidates for structural interaction between these two subunits are a small motif on the I-II linker of α₁ subunits known as the “alpha interaction domain” or AID (Pragnell et al., 1994), and another small motif in the middle of β subunits known as the “β interaction domain” or BID (De Waard et al.,

![Figure 11](#)
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