Cooperative regulation of Ca,1.2 channels by intracellular Mg²⁺, the proximal C-terminal EF-hand, and the distal C-terminal domain

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L-type Ca²⁺ currents conducted by Ca,1.2 channels initiate excitation–contraction coupling in cardiac myocytes. Intracellular Mg²⁺ (Mgi) inhibits the ionic current of Ca,1.2 channels. Because Mg is altered in ischemia and heart failure, its regulation of Ca,1.2 channels is important in understanding cardiac pathophysiology. Here, we studied the effects of Mg on voltage-dependent inactivation (VDI) of Ca,1.2 channels using Na⁺ as permeant ion to eliminate the effects of permeant divalent cations that engage the Ca²⁺-dependent inactivation process. We confirmed that increased Mg reduces peak ionic currents and increases VDI of Ca,1.2 channels in ventricular myocytes and in transfected cells when measured with Na⁺ as permeant ion. The increased rate and extent of VDI caused by increased Mg were substantially reduced by mutations of a cation-binding residue in the proximal C-terminal EF-hand, consistent with the conclusion that both reduction of peak currents and enhancement of VDI result from the binding of Mg to the EF-hand (Kₐ = 0.9 mM) near the resting level of Mg in ventricular myocytes. VDI was more rapid for L-type Ca²⁺ currents in ventricular myocytes than for Ca,1.2 channels in transfected cells. Coexpression of Ca,β₂n subunits and formation of an autoinhibitory complex of truncated Ca,1.2 channels with noncovalently bound distal C-terminal domain (DCT) both increased VDI in transfected cells, indicating that the subunit structure of the Ca,1.2 channel greatly influences its VDI. The effects of noncovalently bound DCT on peak current amplitude and VDI required Mg binding to the proximal C-terminal EF-hand and were prevented by mutations of a key divalent cation-binding amino acid residue. Our results demonstrate cooperative regulation of peak current amplitude and VDI of Ca,1.2 channels by Mg, the proximal C-terminal EF-hand, and the DCT, and suggest that conformational changes that regulate VDI are propagated from the DCT through the proximal C-terminal EF-hand to the channel-gating mechanism.

INTRODUCTION

Intracellular Mg²⁺ (Mgi) is not used as a signaling molecule in normal cellular function, and its concentration is thought to be nearly constant under physiological conditions. However, Mg increases after transient ischemia in the heart (Murphy et al., 1989; Headrick and Willis, 1991) and decreases in heart failure (Haigney et al., 1998). Altered Mg is also observed in pathophysiological conditions in the brain (Resnick et al., 2004; Mendez et al., 2005) and skeletal muscle (Resnick et al., 2004). Elucidation of the regulatory effects of Mg under these pathophysiological conditions would be an important advance toward understanding the impairments of cell function in these disease states.

L-type Ca²⁺ currents initiate excitation–contraction coupling in cardiac muscle cells (Reuter, 1979; Bers, 2002). Mg inhibits the L-type Ca²⁺ currents in ventricular myocytes at physiologically relevant concentrations in the range of 0.8 mM (White and Hartzell, 1988; Agus et al., 1989; Yamaoka and Seyama, 1996a; Pelzer et al., 2001; Wang et al., 2004). L-type Ca²⁺ currents in ventricular myocytes are conducted by Ca,1.2 channels consisting of a pore-forming α₁.2 subunit in association with β and α₂δ subunits (Catterall, 2000). The α₁ subunits are composed of four homologous domains (I–IV) with six transmembrane segments (S1–S6) and a reentrant pore loop in each. Multiple regulatory sites are located in the large C-terminal domain (De Jongh et al., 1996; Peterson et al., 1999; Zuhlke et al., 1999; Hulme et al., 2003), which is subject to in vivo proteolytic processing near its center (De Jongh et al., 1991, 1996; Hulme et al., 2005). A nearby IQ motif in the proximal C terminus is implicated in Ca²⁺-dependent inactivation mediated by Ca²⁺/calmodulin (Peterson et al., 1999; Zuhlke et al., 1999). Noncovalent interaction of the distal C terminus with the proximal C-terminal domain has an autoinhibitory effect by reducing coupling efficiency of gating charge movement to channel opening and positively shifting the voltage dependence of activation (Hulme et al., 2006). The proximal C-terminal domain also contains an EF-hand motif, a potential divalent...
cation-binding site and a prime candidate for mediating inhibition by Mg.

Upon maintained depolarization, L-type calcium currents in neurons, cardiac myocytes, and other cell types inactivate by a dual mechanism dependent on both Ca\(^{2+}\) and voltage (Brehm and Eckert, 1978; Tillotson, 1979; Ashcroft and Stanfield, 1981; Lee et al., 1985; Nilius and Benndorf, 1986). Inactivation plays an important role in the control of the action potential duration and excitation–contraction coupling (Kleiman and Houser, 1988; Keung, 1989; Ahmmed et al., 2000). The physiological significance of voltage-dependent inactivation (VDI) is illustrated by the dramatic effects of missense mutations that impair VDI in Timothy syndrome (Splawski et al., 2004), which is characterized by prolonged QT interval, prolonged action potential duration, and severe ventricular arrhythmias in the heart, as well as by developmental abnormalities in other tissues and autism spectrum disorder in the brain (Splawski et al., 2004, 2005).

Our previous work showed that Mg, inhibits Ca\(_{\text{v}}\)1.2 channels in transfected cells in the same concentration range as in cardiac myocytes, and implicated the proximal C-terminal EF-hand motif in the reduction of peak L-type Ca\(^{2+}\) currents of Ca\(_{\text{v}}\)1.2 channels by Mg (Brunet et al., 2005a,b). In the experiments described here, we have examined whether the autoinhibitory action of the distal C-terminal domain interacts functionally with the inhibition of Ca\(_{\text{v}}\)1.2 channel activity by the binding of Mg to the EF-hand. Using Na\(^{+}\) as permeant ion to eliminate effects of permeant divalent cations, we confirm that Mg reduces the amplitude and increases VDI of L-type Ca\(^{2+}\) currents in ventricular myocytes. With these recording methods, we find that Mg also increases VDI of transfected Ca\(_{\text{v}}\)1.2 channels through interaction with the proximal C-terminal EF-hand. In addition, we show that an autoinhibitory complex containing the distal C-terminal domain noncovalently bound to the proximal C-terminal domain greatly enhances VDI of Ca\(_{\text{v}}\)1.2 channels in transfected tsA-201 cells. The binding of Mg to the proximal C-terminal EF-hand is required for the distal C-terminal domain to exert its autoinhibitory effects. Our results indicate that the EF-hand with bound Mg\(^{2+}\) is required for inhibition of Ca\(_{\text{v}}\)1.2 channel activity by the distal C-terminal domain, and suggest cooperative regulation of channel function by the distal C-terminal domain and Mg, binding to the proximal C-terminal EF-hand.

**MATERIALS AND METHODS**

**Ventricular myocyte isolation**

Left ventricular myocytes were isolated from 8–12-wk-old female adult C57/BL6 mice as described previously (Brunet et al., 2004) and maintained at 37°C until use. All protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

cDNA constructs

The C-terminal truncation of α\(_{1}\)1.2a Δ1821 and Δ1800 were generated by introducing a stop codon after amino acid residues 1,821 and 1,800, respectively; using cDNA encoding the rabbit α\(_{1}\)1.2a (Mikami et al., 1989) as template; Ca\(_{\text{v}}\)1.2 EF-hand single (D1546A/N/S/R/K) and double (E1537Q, D1546N) mutants; the mutant α\(_{1}\)1.2a Δ1800 (D1546R); and the triple mutant distal (E2103Q,E2106Q,D2110Q), abbreviated EED, were constructed using PCR overlap extension (Brunet et al., 2005b; Hulme et al., 2006). The mutant sequence, orientation, and reading frame of all constructs were confirmed by DNA sequencing.

**Cell culture**

TsA-201 cells were grown to 80% confluence and transfected with an equimolar ratio of cDNA encoding full-length, truncated, or mutant α\(_{1}\)1.2a, Ca\(_{\text{v}}\)1.2β (Ca\(_{\text{v}}\)1.2β\(_{\alpha}\), Ca\(_{\text{v}}\)1.2β\(_{\beta}\), or Ca\(_{\text{v}}\)1.2β\(_{\gamma}\)); Ca\(_{\text{v}}\)1.2α\(_{\alpha}\), and CD8 as a cell surface marker (EBO-pCD-Leu2; American Type Culture Collection) using Fugene (Roche). 15–24 h after transfection, cells were suspended, plated at low density in 35-mm dishes, and incubated at 37°C in 10% CO\(_{2}\) for at least 17 h before recording using the whole cell configuration of the patch clamp technique. Transiently transfected cells were visualized with latex beads conjugated to an anti-CD8 antibody (Invitrogen).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Effects of Ba\(^{2+}\) and Na\(^{+}\) ion as charge carrier on VDI of Ca\(_{\text{v}}\)1.2 current expressed in tsA-201 cells. (A) Relationship between \(I_{\text{CaV1.2}}\) and current amplitude of Ca\(_{\text{v}}\)1.2 currents \((n = 52)\) with Ba as charge carrier \(I_{\text{CaV1.2}}\) (Ba). The straight line represents a linear regression \((r = -0.61; P < 0.001)\). (B) Inactivation of typical current traces from cells with low and high current amplitudes (cells a and b from A). (C) Relationship between \(I_{\text{CaV1.2}}\) and current amplitude of Ca\(_{\text{v}}\)1.2 currents \((n = 19)\) with Na\(^{+}\) as charge carrier \(I_{\text{CaV1.2}}\) (Na). The straight line represents a linear regression \((r = -0.11; P = \text{NS})\). (D) Inactivation of typical current traces from cells with low and high current amplitudes (cells a and b from C). Currents were elicited by a depolarization to 0 mV for 1,000 ms from a HP of −80 mV and normalized to their peak amplitudes. The Mg concentration was 0.8 mM. Dotted lines in this and all figures represent the zero current level.
Electrophysiology

Patch pipettes (2.5–3.5 MΩ) were pulled from micropipette glass (VWR Scientific) and fire-polished. Currents were recorded with an Axopatch 200B amplifier (MDS Analytical Technologies) and sampled at 5 kHz after anti-alias filtering at 2 kHz. Data acquisition and command potentials were controlled by Pulse (Pulse 8.50; HEKA), and data were stored for off-line analysis. Voltage protocols were delivered at 10 s intervals unless otherwise noted, and leak and capacitive transients were subtracted using a P/4 protocol. Approximately 80% of series resistance was compensated with the voltage clamp circuitry.

For whole cell voltage clamp recordings of CaV1.2 current in tsA-201 cells with Ba2+ as a charge carrier (ICa,L(Ba)), the extracellular bath solution contained (in mM): 10 BaCl2, 140 Tris, 2 MgCl2, and 10 d-glucose, titrated to pH 7.3 with MeSO4. The normal intracellular Mg (0.8 mM free Mg) solution contained (in mM): 130 N-methyl-d-glucamine, 60 HEPES, 5 MgATP, 1 MgCl2, and 10 EGTA, titrated to pH 7.4 with MeSO4. For coupling ratio determination, the intracellular solution contained (in mM): 130 CsCl, 100 CsOH, 1 MgCl2, and 10 EGTA titrated to pH 7.3 with CsOH (Hulme et al., 2006). When Na+ was used as charge carrier (ICaV1.2(Na)), the extracellular solution contained (in mM): 100 NaCl, 20 TEA-Cl, 1 MgCl2, 10 d-glucose, and 10 HEPES, pH 7.3 with CsOH. The normal Mg intracellular solution (0.8 mM free Mg) contained (in mM): 150 CsOH, 110 glutamate, 20 HCl, 10 HEPES, 5 MgATP, 1 MgCl2, and 10 EGTA titrated to pH 7.6 with CsOH (Ferreira et al., 1997).

For whole cell voltage clamp recordings of ventricular myocyte L-type Ca2+ currents with Ca2+ as charge carrier (ICa,L(Ca)), the extracellular solution contained (in mM): 1.8 CaCl2, 140 HEPES, 2 MgCl2, 10 d-glucose, and 10 HEPES, pH 7.3 with CsOH. The normal Mg intracellular solution (0.8 mM Mg) contained (in mM): 100 CaCl2, 20 TEA-Cl, 10 EGTA, 10 HEPES, 5 MgATP, and 1 MgCl2, titrated to pH 7.3 with CsOH. When Na+ was the charge carrier (ICa,L(Na)), the extracellular recording solution contained (in mM): 100 NaCl, 40 TEA-Cl, 0.7 MgCl2, 5 CsCl, 10 HEPES, 10 d-glucose, and 0.250 µM EDTA, pH 7.3 with CsOH. The intracellular solution (0.8 mM Mg) contained (in mM): 110 CsOH, 40 TEA-Cl, 110 glutamate, 20 HCl, 1 MgCl2, 10 HEPES, 5 MgATP, and 10 EGTA titrated to pH 7.6 with CsOH (Ferreira et al., 1997). The Mg concentration was altered by changing the amount of MgCl2 added to the intracellular solution. Free Mg was calculated by the MaxChelator program (Bers, 2002).

Data analysis

Voltage clamp data were compiled and analyzed using IGOR Pro (WaveMetrics Inc.) and Excel (Microsoft). Peak currents were measured during 300-ms (for L-type Ca2+ current) or 1,000-ms (for CaV1.2 current) depolarization to potentials between −50 and 70 mV for L-type Ca2+ currents, and −80 to 20 mV for CaV1.2 currents. To quantify inactivation, peak currents elicited by 300- or 1,000-ms depolarizations to 0 mV were normalized to 1.0, and the fraction of peak current remaining at the end of the voltage pulse (F300 or F1000) was measured. For steady-state inactivation parameters, tsA-201 cells were depolarized from a holding potential (HP) of −80 mV for 4 s to membrane potentials from −80 to 20 mV in 10 mV increments. Na+ currents were then elicited by a 30-ms depolarization to 30 mV, followed by repolarization to −40 mV to measure tail currents. Pulses were applied every 30 s. L-type Ca2+ current density (pA/pF) was defined as the peak current elicited by the voltage depolarization normalized to the whole cell membrane capacitance (within the same myocyte).

All data are presented as mean ± SEM. The statistical significance of differences between the various experimental groups was evaluated using the Student’s t test or one-way ANOVA, followed by the Newman-Keuls post-test; p-values are presented in the text.

RESULTS

VDI of CaV1.2 channels

In our previous studies, we found that increased Mg reduces peak Ba2+ currents conducted by CaV1.2 channels expressed in tsA-201 cells (Brunet et al., 2005b). To determine whether Mg modulates VDI of cloned CaV1.2 channels, we initially examined the effect of Mg on the inactivation properties of CaV1.2 channels.

Figure 2. Mg dependence of L-type Ca2+ current density in mouse ventricular myocytes with Ca2+ and Na+ as charge carriers. (A) Effect of Mg on ICa,L(Ca) density. Currents were elicited by 300-ms depolarizations from an HP of −50 mV to the indicated potentials from −50 to 70 mV. (B) Mean peak ICa,L(Ca) density with different Mg concentrations. n = 13, 8, and 11 myocytes for 0.8, 2.4, and 7.2 mM Mg, respectively. (C) Effect of Mg on ICa,L(Na) density. (D) Mean peak ICa,L(Na) density measured at different Mg concentrations. n = 7, 6, and 6 myocytes for 0.8, 2.4, and 7.2 mM Mg, respectively. Error bars are ± SEM in this and all subsequent figures. In this and all figures, * and ** indicate P < 0.05 and P < 0.01, respectively.

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expressed in tsA-201 cells using Ba\(^{2+}\) as the charge carrier. This approach reduces Ca\(^{2+}\)-dependent inactivation because Ba\(^{2+}\) does not bind with high affinity to calmodulin, which mediates Ca\(^{2+}\)-dependent inactivation by binding to an IQ motif in the C-terminal domain (Peterson et al., 1999; Zuhlke et al., 1999). Ba\(^{2+}\) currents inactivated slowly as expected (Fig. 1, A and B). However, the rate of inactivation depended on the size of the Ba\(^{2+}\) current (Fig. 1, A and B). Using the ratio of inward current at the end of a 1,000-ms test depolarization to the peak current (\(r_{1000}\)) as an index of inactivation, we found a significant increase in inactivation with larger peak Ba\(^{2+}\) current amplitude (\(r = -0.61; P < 0.001\)) (Fig. 1, A and B). Because of this Ba\(^{2+}\)-dependent effect on inactivation, we could not use Ba\(^{2+}\) as a charge carrier to examine the effect(s) of Mg\(_i\) on VDI of Ca\(_{V1.2}\) channels unambiguously. To avoid such effects of permeant divalent cations, we used Na\(^+\) as charge carrier to measure VDI independently of divalent cation–dependent inactivation.

When extracellular Ca\(^{2+}\) is reduced below micromolar level, Ca\(_{V1.1}\) and Ca\(_{V1.2}\) channels become permeable to monovalent cations (Almers et al., 1984; Hess and Tsien, 1984; Hadley and Hume, 1987), allowing measurements of Ca\(_{V1.2}\) channel activity in the absence of permeant divalent cations. As anticipated, no correlation between the peak current amplitude and \(r_{1000}\) was observed with Na\(^+\) as charge carrier (\(r = -0.11; P = \text{NS}\)) (Fig. 1, C and D). These results show that large Ba\(^{2+}\) currents induce cation-dependent inactivation, presumably caused by low affinity binding of Ba\(^{2+}\) to calmodulin. Similar effects of Ba\(^{2+}\) have been observed previously (Ferreira et al., 1997; Sun et al., 2000). Except where indicated, Na\(^+\) was used as charge carrier to examine the effects of Mg\(_i\) on VDI of Ca\(_{V1.2}\) channels in our subsequent experiments.

Reduction of peak L-type Ca\(^{2+}\) currents in ventricular myocytes by Mg\(_i\).

Mg\(_i\) inhibits L-type Ca\(^{2+}\) currents of cardiac myocytes (White and Hartzell, 1988; Yamaoka and Seyama, 1996a; Wang et al., 2004). As a baseline for our experiments, we examined the impact of changing Mg\(_i\) on the L-type Ca\(^{2+}\) currents of mouse ventricular myocytes (Fig. 2). With Ca\(^{2+}\) as a charge carrier, increasing Mg\(_i\) from the normal resting value of 0.8 to 2.4 mM, a pathophysiologically relevant concentration (Murphy et al., 1989), decreased mean ICa,L(Ca) density from -8.0 ± 0.6 pA/pf (\(n = 13\)) to -5.6 ± 0.3 pA/pf (\(n = 8\); \(P < 0.05\)) (Fig. 2, A and B). Further reduction was observed with 7.2 mM Mg\(_i\) (Fig. 2, A and B). The reduction in current density when Mg\(_i\) was increased from 0.8 to 2.4 mM was greater with Na\(^+\) as charge carrier (61 ± 5%; \(n = 6\)) compared with Ca\(^{2+}\) as charge carrier (30 ± 3%; \(P < 0.001\)), and an additional decrease was observed at 7.2 mM Mg\(_i\) (Fig. 2, C and D).

Enhancement of VDI of L-type Ca\(^{2+}\) currents of ventricular myocytes by Mg\(_i\).

In ventricular myocytes, the rate of inactivation of ICa,L(Na) currents was reduced compared with ICa,L(Ca) currents, as observed previously (Sun et al., 2000) (Fig. 3 A). The value of \(r_{300}\) for ICa,L(Na) decreased from 0.44 ± 0.02 (\(n = 9\)) with 0.8 mM Mg\(_i\) to 0.30 ± 0.02 (\(n = 13\)) with 2.4 mM Mg\(_i\) (\(P < 0.01\)), and 7.2 mM Mg\(_i\) caused a further reduction (Fig. 3, B and C). The reduction of peak Ca\(_{V1.2}\) current (Fig. 2) plus the acceleration of VDI (Fig. 3) would work together to markedly reduce Ca\(^{2+}\) entry when Mg\(_i\) is elevated in cardiac myocytes.

![Figure 3](https://example.com/figure3.png)
Effects of Mg on VDI of CaV1.2 channels in tsA-201 cells

To determine whether Mg modulates VDI of CaV1.2 channels expressed in tsA-201 cells, we measured I_{Ca,L(Na)} with a range of Mg concentrations (0.26–7.2 mM). Increased Mg enhanced the rate and extent of VDI of CaV1.2 current (Fig. 4 A), with an apparent K_d of 0.9 mM (Fig. 4 B). Coexpression of the calmodulin mutant CaM_{1234}, in which mutations of all four EF-hands prevent Ca^{2+} binding and C-terminal inactivation (Peterson et al., 1999), had no effect on the measured inactivation (Fig. 4 C). These results indicate that only VDI is observed under our recording conditions.

Effects of EF-hand mutations on VDI

To further explore the role of Mg, and the EF-hand in modulation of VDI, we tested the EF-hand mutations D1546A/N/S/K/R, which reduce the effects of Mg on peak Ba^{2+} currents compared with wild-type (WT) CaV1.2 channels (Brunet et al., 2005b). These mutations significantly reduced VDI, as assessed from the ratio of r_{1000} values at 0.26 and 2.4 mM Mg, (Fig. 4 D).

The rank order of effects (K>R>S>N>A) was the same as previously observed for reduction of peak currents by Mg (Brunet et al., 2005b), consistent with the conclusion that Mg^{2+} binds to the proximal C-terminal EF-hand and causes both effects.

To further explore the role of Mg, and the EF-hand in modulation of VDI, we studied steady-state inactivation of WT CaV1.2 or EF-hand mutant (D1546K) channels expressed in tsA-201 cells. For WT CaV1.2 channels at low Mg concentration, steady-state inactivation was incomplete compared with higher Mg concentrations (Fig. 5 A and Table I). Elevation of Mg resulted in a negative shift in the voltage dependence of inactivation and an increase of maximal inactivation at positive potentials (Fig. 5 A). These effects are similar to those observed when Ba^{2+} was used as charge carrier for transfected CaV1.2 channels (Brunet et al., 2005b) and for myocyte L-type Ca^{2+} currents (Hartzell and White, 1989). Complete inactivation at depolarized potentials was observed with 2.4 and 7.2 mM Mg in these experiments using Na^+ as a charge carrier (Fig. 5 A and Table I), but not when Ba^{2+} was the permeant ion (Brunet et al., 2005b).

To test the role of the proximal C-terminal EF-hand in the enhancement of steady-state inactivation by Mg, we examined the effects of Mg on the mutant D1546K. Mg caused a similar negative shift of the voltage dependence of inactivation for this mutant compared with WT, suggesting that the negative shift of the voltage dependence of inactivation does not require binding to the EF-hand. However, steady-state inactivation of mutant D1546K was less complete at the most positive prepulse potential (+20 mV) than WT at each concentration of Mg (Fig. 5 B and Table I, VD_inact). When plotted as a concentration–response curve, the dependence of the extent of inactivation at +20 mV on Mg is shifted to higher concentrations by the mutation D1546K with a half-maximal effect at 0.78 ± 0.04 mM Mg in WT compared with 3.6 ± 1.4 mM Mg for the mutant, a ratio of 4.6 ± 0.39 (Fig. 5 C). This decrease in apparent affinity for Mg in enhancing the extent of VDI caused by the mutation D1546K is similar to the decrease of 3.6-fold in the apparent affinity for Mg in increasing the rate of VDI that is caused by the same mutation (Fig. 4 D). These results are consistent with the conclusion that both the effect of the mutation on the rate of VDI and the effect on the extent of VDI arise from the same mechanism-impaired affinity for Mg binding to the proximal C-terminal EF-hand.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Effect of Mg on VDI of WT CaV1.2 and C-terminal EF-hand mutant channels expressed in tsA-201 cells using Na^+ as charge carrier. Currents were elicited by 1,000-ms steps to 0 mV from an HP of −80 mV. (A) Dependence of VDI of CaV1.2(Na) current on Mg. n = 8, 12, 19, 15, and 15 for 0.26, 0.38, 0.8, 2.4, and 7.2 mM Mg, respectively. (B) Plot of r_{1000} versus log[Mg]. Curve represents the best fit of a binding isotherm to the data. The apparent K_d was 0.9 ± 0.1 mM. (C) Effects of Mg on VDI of CaV1.2 with the coexpression of CaM_{1234}. A calmodulin (CaM) construct containing alanine substitutions for critical aspartate residues that impair Ca^{2+} coordination in the paired EF-hand of both lobes of CaM (CaM_{1234}) was expressed in molar excess relative to the Ca^{2+} channel subunits. n = 3 and 4 for 0.26 and 7.2 mM Mg, respectively. (D) Dependence of VDI of C-terminal EF-hand mutant of CaV1.2 on Mg. Mean ratio of r_{1000} with 2.4 mM Mg to r_{1000} with 0.26 mM Mg (r_{1000} at 2.4 mM/Mg/r_{1000} at 0.26 mM) for each CaV1.2 C-terminal EF-hand mutant. Number of cells at 0.26 and 2.4 mM Mg were WT: 9 and 11; D1546A: 7 and 9; D1546S: 6 and 4; D1546N: 6 and 4; D1546R: 5 and 7; and D1546K: 4 and 6.
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Figure 5. Mg modulation of the voltage dependence of inactivation of WT CaV1.2 and C-terminal EF-hand mutant (CaV1.2(D1546K)) with Na⁺ as charge carrier. Comparison of mean inactivation–voltage relationships for ICa,L(Na) of WT CaV1.2 (A) and C-terminal EF-hand mutant (D1546K) (B) exposed to the indicated Mg. (C) Plot of fraction I_inact versus log [Mg] for WT CaV1.2 and C-terminal EF-hand mutant (D1546K). Curves represent the best fit of a binding isotherm to the data, with K_d = 0.78 ± 0.04 mM for WT and K_d = 3.6 ± 1.4 mM Mg for D1546K.

Comparison of VDI for native and transfected CaV1.2 channels
Under recording conditions with reduced Mg (0.26 mM) and Na⁺ as charge carrier, the level of inactivation of ICa,L(Na) in ventricular myocytes was substantially greater than that of ICa,L(Na) in tsA-201 cells (Fig. 6A). At 0.26 mM Mg, we observed a value of V_0 of 31.6 ± 0.8 (n = 4) for ICa,L(Na) versus 8.9 ± 0.7 (n = 8) for CaV1.2(Na) (P < 0.0001) (Fig. 6, A and B). Several factors could contribute to the observed difference between the VDI of L-type Ca²⁺ current in ventricular myocytes and CaV1.2 expressed in tsA-201 cells, including differences in expressed CaVβ subunits (Colecraft et al., 2002) and/or in association with the distal C-terminal regulatory domain (DCRD) (Hulme et al., 2006; see below).

Effect of CaVβ subunits on VDI
CaVβ subunits affect the inactivation properties of CaV1.2 channels (Catterall, 2000; Colecraft et al., 2002). In agreement with previous work, we found that VDI of CaV1.2 channels is greater with the CaVβ₁b subunit than with the CaVβ₂ₐ subunit in the presence of 2.4 mM Mg, (Fig. 6, C and D). It remains uncertain which CaVβ subunit is predominant in the heart (Foell et al., 2004; Pitt et al., 2006; Ter Keurs and Boyden, 2007), but increasing evidence points to the CaVβ₂ family (Gao et al., 1997; Foell et al., 2004; Weissgerber et al., 2006). Previous studies of Ca²⁺ and Ba²⁺ currents suggested that CaVβ₂b recapitulates the inactivation profile of L-type currents of ventricular myocytes (Colecraft et al., 2002). We tested the impact of CaVβ₁b on VDI of CaV1.2 expressed in tsA-201 cells, using 0.26 mM Mg to minimize its effect on VDI of CaV1.2 and Na⁺ as charge carrier to eliminate any effects of divalent ions on the inactivation process. Under these conditions, CaVβ₂b significantly increased VDI compared with CaVβ₁b and CaVβ₂a (Fig. 6, E–H). Although the expression of CaVβ₂a enhanced the inactivation of CaV1.2(Na) currents, the level of inactivation is less than previously reported for CaVβ₁b and CaVβ₂b (Colecraft et al., 2002; Takahashi et al., 2003). This difference is caused by the use of 0.26 mM Mg in our experiments because a much larger effect of the CaVβ₁b subunit is observed at 2.4 mM Mg (Fig. 6, C and D). Nevertheless, the results at 0.26 mM Mg show that the CaVβ subunits do not have a profound effect on

### Table 1

| [Mg] (mM) | V_1/2 (mV) | k (mV) | I_inact* | n | V_1/2 (mV) | k (mV) | I_inact* | n |
|---|---|---|---|---|---|---|---|---|
| 0.26 | −31.6 ± 0.8 | 9.6 ± 0.7 | 0.26 ± 0.04 | 13 | −29.8 ± 0.5 | 9.2 ± 0.5 | 0.32 ± 0.04 | 5 |
| 0.8 | −31.2 ± 0.7 | 9.4 ± 0.7 | 0.15 ± 0.04 | 9 | −31.7 ± 1.0 | 10.2 ± 1.1 | 0.28 ± 0.04* | 5 |
| 2.4 | −36.0 ± 0.2* | 8.7 ± 0.2 | 0.05 ± 0.02* | 4 | −36.9 ± 0.8* | 7.3 ± 0.7 | 0.22 ± 0.07* | 8 |
| 7.2 | −47.3 ± 0.2* | 9.0 ± 0.1 | 0.02 ± 0.01* | 10 | −43.4 ± 0.3* | 6.1 ± 0.3 | 0.05 ± 0.01* | 5 |

*Non-inactivating normalized CaV1.2 (Na) current after a 4-s depolarization at 20 mV.

Values are significantly different from WT, P < 0.05.

Values are significantly (P < 0.01) different than parameters at 0.26 mM Mg.

Values are significantly (P < 0.05) different than parameters at 0.26 mM Mg.
VDI in the presence of low Mg^2+ when divalent cation-dependent inactivation is prevented by use of Na^+ as charge carrier, and therefore indicate that CaVβ subunits cannot fully account for the difference in the rate of VDI between L-type Ca^2+ currents in ventricular myocytes and CaV1.2 channels expressed in tsA-201 cells illustrated in Fig. 6A.

Effect of the distal C terminus of CaV1.2 on VDI
The C termini of CaV1.1 and CaV1.2 channels are proteolytically processed in skeletal and cardiac muscle tissues, respectively (De Jongh et al., 1991, 1996; Gerhardstein et al., 2000; Hulme et al., 2005). The proteolytically processed distal C terminus is thought to remain tethered to the proximal C terminus via noncovalent interactions between the DCRD and the proximal C-terminal regulatory domain (PCRD) (Hulme et al., 2006). This interaction has potent autoinhibitory effects on CaV1.2 currents when the distal C terminus is expressed as a separate protein with truncated CaV1.2 channels (Hulme et al., 2006). To determine the effect of formation of this complex on VDI, we expressed the truncated CaV1.2 channel (CaV1.2Δ1821) in tsA-201 cells with and without the distal C terminus composed of amino acid residues 1,822–2,171 (distal1822–2171).

Figure 6. Effects of CaVβ subunits and Mg^2+ on VDI of CaV1.2 with Na^+ as charge carrier. (A and B) Comparison of VDI of mouse ventricular myocyte CaV1.2 current (I_{Ca,L}) and current due to CaV1.2 expressed in tsA-201 cells I_{CaV1.2}. (A) Normalized and averaged current traces with Na^+ as charge carrier. (B) Mean r_{1000} for I_{Ca,L} (n = 4) and I_{CaV1.2} (n = 7). Mg^2+ was 0.26 mM. (C and D) CaV1.2 and the indicated CaVβ subunits expressed in tsA-201 cells and studied with high (2.4 mM) Mg^2+. Mean normalized current traces in response to 1,000-ms depolarizations to 0 mV. (D) Mean r_{1000} for CaV1.2 expressed with CaVβ_{1b} (n = 15) and CaVβ_{2a} (n = 6). (E–H) CaV1.2 and the indicated CaVβ subunits expressed in tsA-201 cells at low (0.26 mM) Mg^2+ with pulse durations of 300 ms (E and F) and 1,000 ms (G and H). (E and G) Mean normalized currents in response to depolarizations to 0 mV. (E) Mean r_{1000} for CaV1.2 expressed with the indicated β subunits. CaVβ_{2a}: n = 16; CaVβ_{2b}: n = 10. Data from ventricular myocytes I_{Ca,L}(Na) are replotted for comparison. (H) Mean r_{1000} for CaV1.2 expressed with CaVβ_{1b}, CaVβ_{2a}, or CaVβ_{2b}.
contribute to the more rapid rate of VDI observed in ventricular myocytes, where we estimated that at least 48% of CaV1.2 channels are in the form of proteolytically processed channels with associated C-terminal domain (Hulme et al., 2006).

Because the effect of the distal C terminus on the activity of CaV1.2 channels is mediated via an electrostatic interaction between two positively charged amino acid residues in the PCRD (RR) and three negatively charged residues (EED) in the DCRD (Hulme et al., 2006), we tested the effect of the DCRD mutant EED­QQQ (Distal1822–2171), which was shown to significantly reduce the effect of the distal C terminus on the coupling ratio of CaV1.2 channels (Hulme et al., 2006). Our results show that the EED­QQQ distal C-terminal mutant does not enhance VDI of CaV1.2 (Fig. 7, B and C), suggesting that the electrostatic interaction between the PCRD and DCRD is important in mediating the effect of the distal C terminus on VDI of CaV1.2 channels. This effect of the C terminus was not dependent on the CaVb subunit expressed, as similar observations were made with CaVb1d subunit (unpublished.

Figure 7. Effect of the distal C terminus of CaV1.2 on VDI. (A) Mean normalized currents due to expression of the truncated CaV1.2Δ1821 α1 subunit alone or with coexpression of the separate distal C terminus (distal1822-2171). Data for ICa,L from ventricular myocytes is shown for comparison. (B) Mean normalized currents due to the truncated CaV1.2Δ1821 α1 subunit coexpressed with a mutant distal C terminus containing the triple mutation EED­QQQ compared with currents due to the channel constructs shown in A. (C) Mean r1000 for truncated CaV1.2 expressed alone or with WT or mutant distal C termini (EED­QQQ). CaVb2b was used in the experiments in this and all subsequent figures. n = CaV1.2Δ1821 alone: 10; CaV1.2Δ1821 + distal1822-2171: 16; CaV1.2Δ1821 + EED­QQQ: 10. Mg0 was 0.26 mM.

Figure 8. Effects of the distal C terminus on modulation of VDI by Mg. Effect of Mg on VDI in mean normalized current traces recorded from expression of (A) full-length CaV1.2, (B) truncated CaV1.2Δ1821, and (C) CaV1.2Δ1821 coexpressed with distal1822-2171. In A, n = 10 and 8 for 0.26 and 7.2 mM Mg0, respectively. In B, n = 10 and 7. In C, n = 5, 16, and 11 for 0.1, 0.26, and 7.2 mM Mg0, respectively.
The dialysis of 0.1 mM Mg reduced inactivation \( (r_{1000} = 0.60 \pm 0.05; P < 0.01) \) compared with 0.26 and 7.2 mM Mg (Fig. 8 C). This result suggests that the noncovalent interaction of the distal C terminus with the CaV1.2 channel enhances regulation of VDI by Mg, possibly by increasing the affinity for binding of Mg to the proximal C-terminal EF-hand.

**Requirement for the EF-hand of CaV1.2 for regulation of VDI by the distal C terminus**

The effect of the distal C-terminal domain on the rate of VDI can be clearly observed by comparing the rate of inactivation for CaV1.2\( _{1821} \) without and with distal\( _{1822–2171} \) at 0.26 mM Mg (Fig. 7, A and B). A similar effect on VDI is observed for truncation at position 1,800 (Fig. 9 A), the probable point of in vivo proteolytic processing determined by mass spectrometric analysis of the related CaV1.1 channel (Hulme et al., 2005), and more extensive analysis has shown that these two forms of the autoinhibitory CaV1.2 channel complex cleaved at position 1,800 or 1,821 have nearly identical functional properties when studied side-by-side (unpublished data). To determine the role of the proximal C-terminal EF-hand in the enhancement of VDI by the distal C terminus in CaV1.2\( _{1800} \), we studied the mutation D1546R, which reduces the effects of Mg on current amplitude and VDI of CaV1.2 (Brunet et al., 2005a,b). With EF-hand mutant CaV1.2\( _{1800(D1546R)} \) plus distal\( _{1801–2171} \), the distal C terminus did not enhance VDI of CaV1.2 (Fig. 9 B) in contrast to CaV1.2\( _{1800} \) with data. These effects of distal\( _{1822–2171} \) on VDI of CaV1.2\( _{1821} \) are consistent with previous results showing that distal\( _{1822–2171} \) reduces the current amplitude of CaV1.2\( _{1821} \) through interaction with the EED motif in the DCRD (Hulme et al., 2006). Overall, these results suggest that the increased VDI of L-type Ca\( ^{2+} \) current in cardiac myocytes results at least in part from the interaction with distal C terminus of CaV1.2, which enhances VDI of CaV1.2 channels through electrostatic interactions with the PCRD.

**Cooperative modulation of VDI by the distal C terminus and Mg**

Because both the distal C terminus and Mg enhance inactivation of CaV1.2 channels, we examined the impact of the distal C terminus on the Mg modulation of VDI of truncated CaV1.2 channels. As in previous work, Mg enhanced the inactivation of the full-length CaV1.2 channel (Fig. 8 A) (Brunet et al., 2005a), with \( r_{1000} = 0.54 \pm 0.03 \) for 0.26 mM versus 0.15 \pm 0.03 for 7.2 mM Mg (\( P < 0.001 \)). Similar to the Mg effect on full-length channels, Mg also enhanced VDI of CaV1.2\( _{1821} \) (Fig. 8 B). The \( r_{1000} \) value was 0.54 \pm 0.03 at 0.26 mM Mg versus 0.10 \pm 0.05 (\( P < 0.001 \)) for 7.2 mM Mg. In contrast, when distal\( _{1822–2171} \) was coexpressed with CaV1.2\( _{1821} \) channels, 7.2 mM Mg did not significantly enhance VDI of CaV1.2 (Fig. 8 C; 0.26 mM, \( r_{1000} = 0.28 \pm 0.03 \); 7.2 mM, \( r_{1000} = 0.25 \pm 0.03 \); \( P = 0.52 \)). To determine whether reduced Mg regulates this autoinhibitory complex, a lower Mg concentration (0.1 mM) was tested.

**Figure 9.** Requirement for a functional proximal C-terminal EF-hand for the effects of the distal C terminus on CaV1.2 current. Effect of coexpression of distal\( _{1801–2171} \) on mean normalized Na\(^+\) currents resulting from expression of (A) CaV1.2\( _{1800} \) or (B) CaV1.2\( _{1800(D1546R)} \) containing mutation D1546R in the EF-hand (CaV1.2\( _{1800(D1546R)} \)). \( n = 15, \ 17, \ 10, \) and 15 for CaV1.2\( _{1800(D1546R)} \), CaV1.2\( _{1800(D1546R)} \) plus distal\( _{1801–2171} \), CaV1.2\( _{1800} \), and CaV1.2\( _{1800 \text{ plus distal}_{1801–2171}} \), respectively. Mg was 0.26 mM. (C) Mean coupling ratios measured as the ratio of peak tail current at \( -40 \text{ mV} \) (nA) after a depolarization to the reversal potential and gating charge movement measured at the reversal potential (pC). Gating charge movement was measured by applying a series of test pulses from the HP of \( -80 \text{ mV} \) to potentials between +60 and 80 mV in 2-mV increments and integrating the gating charge movement at the reversal potential for the ionic current (Hulme et al., 2006). \( n = 10, \ 9, \ 11, \) and 8. (D) Normalized conductance–voltage relationships. Peak tail currents were measured upon repolarization to \( -40 \text{ mV} \) after 20-ms depolarization to potentials between \( -40 \text{ and } 200 \text{ mV} \). Ba\(^{2+}\) was used as charge carrier, and Mg was 0.8 mM for the experiments in C and D.
WT distal1801-2171 (Fig. 9 A). These results indicate that interaction of Mg, with the EF-hand in the proximal C terminal is required for regulation of VDI by the distal C-terminal domain.

The distal C-terminal domain inhibits CaV1.2 channel activity by positively shifting the voltage dependence of activation and reducing the coupling ratio of gating charge movement to channel opening (Hulme et al., 2006). Using Ba2+ as a charge carrier to allow comparison of our results with the previous work, we found that the D1546R mutation in the EF-hand in the proximal C-terminal domain prevented both the reduction of the coupling ratio (Fig. 9 C) and the positive shift in the voltage dependence of activation of CaV1.2Δ1800 (Fig. 9 D). These results demonstrate that a functional EF-hand in the proximal C-terminal domain is required to mediate all of the effects of the noncovalently associated distal C terminus on CaV1.2 channels, including increased VDI, positively shifted activation, and reduced coupling ratio.

DISCUSSION

Cation-dependent inactivation and Ba2+ as charge carrier

We found that use of Ba2+ as a charge carrier resulted in a significant level of cation-dependent inactivation of CaV1.2 channels. In our transfected cell system, we observed a correlation between Ba2+ current amplitude and r1000 as an index of inactivation. This result is in agreement with previous work (Zhang et al., 1994; Ferreira et al., 1997; Sun et al., 2000), including previous studies of cation-dependent inactivation of CaV1.2 channels expressed in tsA-201 cells by Ba2+ (Ferreira et al., 1997). It was proposed that Ba2+ could bind weakly to the same sensor as Ca2+, which is now known to be calmodulin, and could activate the same inactivation mechanism when high local concentrations are achieved (Sun et al., 2000). Based on our results and this previous work, it is evident that unambiguous measurement of VDI requires use of Na+ or another monovalent cation as charge carrier, taking advantage of the ability of Ca2+ channels to conduct monovalent cations with high efficiency in the presence of low extracellular concentrations of Ca2+. Because Ca2+-dependent inactivation is much faster than VDI for CaV1.2 channels, a small contamination by this cation-dependent inactivation mechanism can have a major impact on measurements of VDI. In addition, in transfected cell systems that yield variable expression of CaV1.2 channels from different transfections and from different mutants, elimination of cation-dependent inactivation that varies with channel density is especially important. For these reasons, we used Na+ as permeant ion for most of our experiments on VDI reported here.

Mg, reduces peak amplitude and increases VDI of native cardiac L-type Ca2+ currents at physiological concentrations

Previous reports have suggested that Mg, could reduce peak L-type Ca2+ currents and enhance the VDI in ventricular myocytes, as measured with Ba2+ as the permeant ion, (Hartzell and White, 1989; Yamaoka and Seyama, 1996b; Wang et al., 2004). However, it was uncertain whether the Ba2+-dependent inactivation observed in our experiments in transfected cells might contribute substantially to those effects in ventricular myocytes. Our present results with Na+ as permeant ion clearly demonstrate that, in the absence of permeant divalent ions, increases in Mg concentration both reduce peak Ca2+ current and enhance VDI in native ventricular myocytes.

Mg, is ~0.8 mM in intact cardiac myocytes under physiological conditions (Murphy et al., 1989; Headrick and Willis, 1991; Haigney et al., 1998). There is a two- to threefold increase in Mg in ventricular myocytes as ATP levels decrease during transient ischemia (Murphy et al., 1989; Headrick and Willis, 1991). Similar two- to threefold increases in Mg are observed in cerebral ischemia (Brooks and Bachelard, 1989; Helpern et al., 1993; Williams and Smith, 1995) and in traumatic brain injury (decrease Mg) (Vink et al., 1988; Heath and Vink, 1996). In contrast, a decrease of two- to threefold in Mg is observed in heart failure (Haigney et al., 1998). Our results show that both the peak amplitude and VDI of L-type Ca2+ currents in ventricular myocytes would be substantially regulated by these changes in Mg. In heart failure, reduction of Mg would increase Ca2+ currents and slow their inactivation, which may contribute to dysregulation of Ca2+ signaling, hypertrophy, and cytotoxicity. In ischemia, the increase in Mg would reduce L-type Ca2+ current by reducing peak current and by enhancing VDI. These effects would reduce cytotoxicity caused by Ca2+ overload under ischemic conditions. Moreover, this regulatory mechanism would be cell autonomous, reducing Ca2+ currents only in those individual cardiac myocytes that are ischemic, while leaving neighboring cells with normal ATP levels uninhibited. This unique cell-autonomous protective mechanism may contribute significantly to preventing Ca2+ overload in ischemia.

Upon maintained depolarization, the cardiac L-type calcium current inactivates by dual mechanisms dependent on Ca2+ and voltage (Lee et al., 1985; Nilius and Benndorf, 1986). Calcium-dependent inactivation accelerates the decay of the calcium current as calcium accumulates inside the cell during the action potential, and these changes in inactivation kinetics play an important role in the control of action potential duration and excitation–contraction coupling (Kleiman and Houser, 1988; Keung, 1989). However, Ca2+-dependent inactivation does not reduce the peak Ca2+ current until after
Ca\textsuperscript{2+} overload has occurred and is not able by itself to reduce the Ca\textsuperscript{2+} current to zero, even at high Ca\textsuperscript{2+} levels. In contrast, VDI can reduce the peak Ca\textsuperscript{2+} current in response to sustained depolarization, even before Ca\textsuperscript{2+} overload has occurred, and it is powerful enough to reduce the Ca\textsuperscript{2+} current to zero as shown in our records. Thus, we propose that in ischemic conditions with sustained membrane depolarization, Mg\textsubscript{i} can reduce Ca\textsuperscript{2+} entry via L-type Ca\textsubscript{V1.2} channels before Ca\textsuperscript{2+} overload occurs and sustain inhibition as Ca\textsuperscript{2+} is sequestered and pumped out of the cytosol.

**Effects of PKA stimulation on Mg\textsubscript{i} regulation of L-type Ca\textsuperscript{2+} channels**

Previous studies have shown that Mg\textsubscript{i} modulation of the cardiac L-type Ca\textsuperscript{2+} channel depends on the state of activation of channels by cAMP-dependent phosphorylation (White and Hartzell, 1988; Wang et al., 2004; Wang and Berlin, 2006). These investigators concluded that the actions of Mg\textsubscript{i} to reduce peak currents and enhance VDI are mediated by a separate mechanism from protein phosphorylation, but the extent of modulation by Mg\textsubscript{i} is dependent on the phosphorylation state of the Ca\textsubscript{V1.2} channel. These conclusions are consistent with the work we present here, in which we find that the effects of Mg\textsubscript{i} on peak current and on VDI are caused by the binding of Mg to the C-terminal EF-hand. In other experiments (unpublished data), we found that PKA inhibitors reduced Ca\textsubscript{V1.2} current \(~50\%\) under our basal physiological conditions; therefore, our results presented here reflect the effects of Mg on Ca\textsubscript{V1.2} channels whose activity is partially up-regulated by basal cAMP-dependent phosphorylation.

**Mg\textsubscript{i} enhances VDI of Ca\textsubscript{V1.2} channels**

Our results show that altering Mg\textsubscript{i} has striking effects on VDI of Ca\textsubscript{V1.2} channels expressed in tsA-201 cells in the absence of other cardiac-specific proteins. The rate and extent of VDI increase dramatically with increased Mg\textsubscript{i}. This enhancement of inactivation occurs with an apparent K\textsubscript{d} of 0.9 mM. Ca\textsubscript{V1.2} is the predominant Ca\textsuperscript{2+} channel in cardiac cells, and this value falls in the range of normal physiological levels of Mg\textsubscript{i} in cardiac cells (0.6–1.0 mM) (Murphy et al., 1989; Headrick and Willis, 1991; Haigney et al., 1998). Thus, the dependence of peak current amplitude and VDI of Ca\textsubscript{V1.2} channel on Mg\textsubscript{i} that we have defined in the tsA-201 cell expression system likely reflects the Mg\textsubscript{i} dependence of peak current amplitude and VDI of the Ca\textsubscript{V1.2} channel in vivo.

The proximal C-terminal EF-hand mediates the effects of Mg\textsubscript{i} on VDI

Our previous studies of the effects of Mg\textsubscript{i} on peak Ca\textsuperscript{2+} currents showed that EF-hand mutations at the \(\_z\) position (D1546A/NS/K/R) reduce the inhibitory effects of Mg\textsubscript{i} because of a decrease in apparent affinity for Mg\textsubscript{i} (Brunet et al., 2005b). Our present results extend those findings to VDI and lead to the conclusion that both the

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**Figure 10.** Schematic illustration of the Ca\textsubscript{V1.2} \(\alpha\) subunit emphasizing the spatial relationship of key regulatory sites located at the proximal C terminus. Ca\textsubscript{V1.2} \(\alpha\) subunit composed of the N terminus, the four homologous domains with six transmembrane segments, and a reentrant pore loop in each (I-IV), and the C-terminus. The arrows point to the conformational interactions between the distal C terminus (DCT; red arrow) and the IQ motif (Ca\textsuperscript{2+}/CaM) (orange arrow) independently converging on the EF-hand motif (Mg\textsuperscript{2+}), which subsequently may modify the conformation of domain IV S6 segment of the Ca\textsubscript{V1.2} \(\alpha\) subunit to alter channel-gating properties. The scissors illustrate the site of proteolytic cleavage. CaM, calmodulin.
Noncovalent association of the distal C-terminal domain increases the rate of VDI in transfected cells

Although our results describing the modulation of Mg, on L-type Ca\(^{2+}\) current in myocytes are qualitatively similar to our results on Mg, modulation of CaV1.2 channels in tsA-201 cells, there are some important quantitative differences. First, Mg, was more effective at enhancing the inactivation of CaV1.2 in tsA-201 cells compared with the inactivation of the L-type Ca\(^{2+}\) current in ventricular myocytes. Second, VDI was greater for CaV1.2 channels in cardiac myocytes than for CaV1.2 expressed in tsA-201 cells, as reported previously using Ba\(^{2+}\) as permeant ion (Colecraft et al., 2002). Subunit structure and composition of CaV1.2 channels in ventricular myocytes and CaV1.2 channels in tsA-201 cells may contribute to these differences.

Our results show that Ca\(_{\alpha}\)\(\beta\)s have small but significant effects on the VDI of CaV1.2 measured with Na\(^+\) as the permeant ion in the presence of low Mg,. These effects of Ca\(_{\alpha}\)\(\beta\)s on VDI are smaller than in previous work, and Ca\(_{\alpha}\)\(\beta\)s did not fully restore VDI of CaV1.2 as reported previously (Colecraft et al., 2002). Our results indicate that Ca\(_{\alpha}\)\(\beta\)s cannot fully account for the difference in VDI between CaV1.2. channels in ventricular myocytes and CaV1.2 channels in transfected cells in the absence of permeant divalent cations.

In myocytes, the distal C terminus of CaV1.2 is proteolytically processed in situ, whereas it is not cleaved in tsA-201 cells (De Jongh et al., 1991, 1994, 1996; Hulme et al., 2005). Our results demonstrate that expression of the distal C terminus of CaV1.2 together with the truncated CaV1.2Δ1821 channel markedly enhanced VDI under our recording conditions. This enhancement of VDI was blocked by the distal C terminus mutation EED-QQQ, supporting a role for an electrostatic interaction between the PCRD and DCDR in mediating the effect of the distal C terminus on VDI. This effect of the distal C terminus on VDI was greater than the effects of Ca\(_{\alpha}\)\(\beta\)s tested, but it was comparable to the effects of Mg,. These findings support an important role of the distal C-terminal domain in regulating Ca\(^{2+}\) currents via control of coupling ratio, voltage-dependent activation, and VDI. These results are consistent with the autoinhibitory function demonstrated for the distal C terminus of CaV1.2 in previous studies (Hulme et al., 2006).

Mg, and the proximal C-terminal EF-hand are required for the functional effects of the noncovalently associated distal C-terminal domain

Because the effect of the distal C terminus on VDI was comparable in amplitude to that of Mg,, we investigated the possible functional interaction between Mg, and the distal C terminus. To our surprise, when the distal C terminus was expressed together with the cleaved CaV1.2 channel, increasing Mg, concentration from 0.26 to 7.2 mM had little effect. On the other hand, decreasing Mg, to lower levels reduced VDI, indicating that the apparent affinity for Mg, was increased by noncovalent association of the distal C terminus. These results demonstrate a cooperative interaction between the distal C terminus and Mg, binding to the proximal C-terminal EF-hand motif in enhancement of VDI. Consistent with this idea, the mutation D1546R reduces affinity for the binding of Mg, to the proximal C-terminal EF-hand and reduces the functional effects of the distal C terminus. Overall, these results place the EF-hand as a downstream structural element through which the distal C terminus regulates VDI of CaV1.2 channels. Evidently, Mg, enhancement of VDI is cooperatively coupled to enhancement of VDI by the distal C terminus.

A C-terminal signaling complex controlling inactivation of CaV1.2 channels

The inactivation of CaV1.2 channels is both Ca\(^{2+}\) and voltage dependent. Our results show that VDI of CaV1.2 channels is regulated by the distal C terminus, Mg, and the proximal C-terminal EF-hand. At the same time, Ca\(^{2+}\)-dependent inactivation of CaV1.2 channels is regulated by the binding of Ca\(^{2+}\) and calmodulin to an IQ motif just downstream from the EF-hand motif in the proximal C-terminal domain (Peterson et al., 1999; Zuhlke et al., 2000; Pitt et al., 2001; Ohrtman et al., 2008), and the effects of Ca\(^{2+}\)/calmodulin binding to the IQ motif on CDI are blocked by mutations on the external, non-cation–binding face of the EF-hand (Peterson et al., 2000). The spatial relationship of these regulatory sites is illustrated in Fig. 10, which emphasizes that regulatory influences on both Ca\(^{2+}\)-dependent inactivation and VDI from downstream in the C-terminal domain are mediated via the proximal C-terminal EF-hand and are potentially influenced by the binding of Mg,. We propose that conformational changes induced by noncovalent association of the distal C-terminal domain and Ca\(^{2+}\)/calmodulin with the proximal C-terminal domain are propagated from the regulatory regions of the C terminus to the pore-lining IVS6 segment via the EF-hand motif. These results further emphasize the interactive nature of the multiple regulatory elements in the C terminus of CaV1.2 channels, and place Mg, binding to the proximal C-terminal EF-hand motif in position to serve as a key integrator of multiple regulatory signals that control Ca\(^{2+}\) channel function.
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