Structural Regions of the Cardiac Ca Channel $\alpha_{1C}$ Subunit Involved in Ca-dependent Inactivation

Brett Adams* and Tsutomu Tanabe‡

From the *Department of Physiology and Biophysics, University of Iowa, Iowa City, Iowa, 52242; and ‡Department of Cellular and Molecular Physiology, and Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06536

ABSTRACT We investigated the molecular basis for Ca-dependent inactivation of the cardiac L-type Ca channel. Transfection of HEK293 cells with the wild-type $\alpha_{1C}$ or its $3'\prime$ deletion mutant ($\alpha_{1C-3'SL}$) produced channels that exhibited prominent Ca-dependent inactivation. To identify structural regions of $\alpha_{1C}$ involved in this process, we analyzed chimeric $\alpha$ subunits in which one of the major intracellular domains of $\alpha_{1C}$ was replaced by the corresponding region from the skeletal muscle $\alpha_{1S}$ subunit (which lacks Ca-dependent inactivation). Replacing the NH$_2$ terminus or the III–IV loop of $\alpha_{1C}$ with its counterpart from $\alpha_{1S}$ had no appreciable effect on Ca channel inactivation. In contrast, replacing the I–II loop of $\alpha_{1C}$ with the corresponding region from $\alpha_{1S}$ dramatically slowed the inactivation of Ba currents while preserving Ca-dependent inactivation. A similar but less pronounced result was obtained with a II–III loop chimera. These results suggest that the I–II and II–III loops of $\alpha_{1C}$ may participate in the mechanism of Ca-dependent inactivation. Replacing the final 80% of the COOH terminus of $\alpha_{1C}$ with the corresponding region from $\alpha_{1S}$ completely eliminated Ca-dependent inactivation without affecting inactivation of Ba currents. Significantly, Ca-dependent inactivation was restored to this chimera by deleting a nonconserved, 211-amino acid segment from the end of the COOH terminus. These results suggest that the distal COOH terminus of $\alpha_{1S}$ can block Ca-dependent inactivation, possibly by interacting with other proteins or other regions of the Ca channel. Our findings suggest that structural determinants of Ca-dependent inactivation are distributed among several major cytoplasmic domains of $\alpha_{1C}$.

KEY WORDS: $\alpha_{1S}$ • skeletal muscle • L-type Ca channel • chimeric proteins • heart

INTRODUCTION

L-type Ca channels perform essential roles in the cardiovascular system, where they trigger excitation–contraction coupling and contribute to pacemaker and action potentials (Boyett et al., 1996). Inactivation of L-type channels is induced by membrane depolarization and elevations in intracellular [Ca], although these two types of Ca channel inactivation appear to proceed via distinct and independent mechanisms (Hadley and Lederer, 1991; Obejero-Paz et al., 1991; Shirokov et al., 1993). Ca-dependent inactivation is a prominent feature of cardiac L-type Ca channels that has important implications for the function of these channels in cardiovascular physiology.

Voltage-gated Ca channels are heteromultimeric complexes composed of pore-forming $\alpha_1$ and accessory $\alpha_2\delta$ and $\beta$ subunits (Hofmann et al., 1994). Transfection of mammalian cell lines or Xenopus oocytes with the cardiac $\alpha_1$ subunit ($\alpha_{1C}$) by itself produces voltage-gated Ca channels that exhibit Ca-dependent inactivation (Neely et al., 1994; Perez-Garcia et al., 1995; Zong and Hofmann, 1996), suggesting that this type of inactivation is an intrinsic property of the $\alpha_{1C}$ subunit. Because Ca-dependent inactivation is induced by a rise in intracellular Ca concentration (Haack and Rosenberg, 1994), it is reasonable to postulate that cytoplasmic domains of $\alpha_{1C}$ participate in its molecular mechanism. The five major putative cytoplasmic domains of $\alpha_{1C}$ include the NH$_2$ and COOH termini and three linkers (the I–II, II–III, and III–IV loops) that connect the four major transmembrane domains (Mikami et al., 1989).

Two previous studies have provided evidence that one or more of these cytoplasmic domains play important roles in Ca-dependent inactivation. Thus, Ca-dependent inactivation is abolished by simultaneous replacement of all five of the major cytoplasmic domains of $\alpha_{1C}$ with the corresponding regions from the skeletal muscle $\alpha_{1S}$ subunit (Zong et al., 1994). Ca-dependent inactivation is also eliminated by replacing all or a portion of the COOH terminus of $\alpha_{1C}$ with the corresponding region of the neuronal $\alpha_{1E}$ subunit (de Leon et al., 1995). Because $\alpha_{1S}$ and $\alpha_{1E}$ both appear to exhibit only voltage-dependent inactivation (Donaldson and Beam, 1983; Beam and Knudson, 1988; de Leon et al., 1995), these results imply that the structural determinants of
Ca-dependent inactivation are encoded within the cytoplasmic domains of α1C. The goal of the present study was to test this hypothesis. Toward this end, we have studied a series of chimeric α subunits in which the major cytoplasmic domains of α1C were individually replaced by their counterpart from α1S. Our results suggest that the cytoplasmic COOH terminus, and I–II and II–III loops are all involved in the molecular mechanism of Ca-dependent inactivation. In addition, our findings indicate that Ca-dependent inactivation can be prevented by the distal COOH terminus from the skeletal muscle α1S. Some of these results have appeared previously in abstract form (Adams and Tanabe, 1996).

Materials and Methods

Cell Culture and Transfection

Human embryonic kidney cells were obtained from the American Type Culture Collection (CRL 1573; Rockville, MD) and propagated using standard techniques. The culture medium contained 90% DMEM (11995-065; Gibco BRL, Gaithersburg, MD), 10% heat-inactivated horse serum (26050-13; Gibco BRL) and 50 μg/ml gentamicin (15701045; Gibco BRL). Every 2–3 d, these cells were briefly trypsinized and replated onto the maintenance culture at a fourfold lower density. At the time of replating, additional 35-mm culture dishes (3001; Becton Dickinson & Co., Franklin Lakes, NJ) were seeded with ~10^5 cells/dish. Approximately 16 h later, the CaPO_4 precipitation technique (Cell Phect kit; Pharmacia LKB Biotechnology Inc., Piscataway, NJ) was used to transfect the seeded cells with a combination (at 1 μg of each plasmid cDNA per dish) of expression plasmids encoding the rabbit cardiac α1C (or chimeras constructed between α1C and the rabbit α1A) and the rabbit skeletal muscle α1D and β1S subunits. The transfection mixture also included an expression plasmid (EBO-pCD-Leu2; 59565; American Type Culture Collection) encoding the human CD8 protein at a fivefold lower concentration (0.2 μg per dish). 1–3 d later, paramagnetic beads (4.5 μm diameter) coated with anti–CD8 antibody (Dynal, Inc., Great Neck, NY) were added to each dish. Cells expressing CD8 protein on the surface membrane were visually identified by virtue of being decorated with the beads (Jurman et al., 1994) and were selected for electrophysiological analysis.

Molecular Biology

The amino acid compositions and construction of the expression plasmids encoding the 3′ deletion mutant of α1C (α1C−3′del) and the chimeric α1 subunits CSk1, CSk2, CSk3, and CSk4 have been previously described (Tanabe et al., 1990b; Zong et al., 1994). The cDNAs encoding the chimeras CSk5 and CSk8 are composed of the following restriction fragments (the origin of the fragments is given in parentheses). pCSk5: 4.2-kb pair HindIII-AatII (pCARD1; see Mikami et al., 1989), 0.88-kb pair AatII-BglII (pCARD1), and 0.55-kb pair BglII-HindIII (pCDΔ1; see Beam et al., 1992). pCSk8: 3.8-kb pair HindIII-BspII (pCARD1), 0.24-kb pair BspII-BstXI (pCACE; see Tanabe et al., 1988), 0.19-kb pair BstXI-AatII (pCARD1), and 2.94-kb pair AatII-HindIII (pCARD1). The expression plasmids pCSk5 and pCSk8, carrying the cDNAs encoding the individual chimeric Ca channels, were constructed by inserting the corresponding cDNAs into the HindIII site of the plasmid pKRCH2 (Mishina et al., 1984).

The amino acid compositions of CSk5 and CSk8 are as follows (C and Sk, cardiac and skeletal muscle Ca channel, respectively; numbers in parentheses, amino-acid numbers [Tanabe et al., 1987; Mikami et al., 1989]; the junctional sequences common to the two Ca channels are represented by amino acid numbers of the cardiac Ca channel). CSk5: C (1–1063) and Sk (1510–1662). CSk8: C (1–1204), Sk (1074–1129), and C (1261–2171).

Electrophysiology

Patch pipettes were fabricated from 100-μl borosilicate micropipettes (53432921; VWR Scientific, West Chester, PA) and filled with a solution containing (mM): 155 CaCl, 10 CsEGTA, 4 MgATP, 0.38 Tris-GTP, and 10 HEPES, with pH adjusted to 7.4 using CsOH. Aliquots of this solution were stored at ~80°C and kept on ice after thawing. The internal solution was filtered (0.22 μm) immediately before use. Filled pipettes had DC resistances of 1–2 MΩ. Pipette tips were coated with paraffin to reduce capacitance, and then fire polished. Residual pipette capacitance was compensated in the cell-attached configuration, using the negative capacitance circuit of the Axopatch 200A amplifier (Axon Instruments, Inc., Foster City, CA). In earlier experiments, the external solution contained (mM): 145 tetraethylammonium (TEA) chloride, 40 CaCl, 10 BaCl, and 10 HEPES, with pH adjusted to 7.4 using TEAOH. In later experiments, an external solution containing (mM) 140 NaCl, 2 KCl, 40 CaCl or BaCl, and 10 HEPES (pH 7.4 with NaOH or HCl) was used because the cells appeared to remain healthy for longer periods in this solution. Because the voltage dependence of ionic currents recorded in the NaCl-based solution was shifted by ~10 mV relative to currents recorded in the TEACl-based solution, data obtained using the two different external solutions have been analyzed separately. Experiments were performed at room temperature (20–23°C). Voltages reported in this paper have not been corrected for liquid junction potentials.

Ca or Ba currents were recorded using the whole-cell patch-clamp technique (Hamill et al., 1981). After establishment of the whole-cell configuration, electronic compensation was used to minimize the access resistance and the time required to charge the cell capacitance. The DC resistance of the whole-cell configuration typically exceeded 1 Ω, and leakage currents were usually <50 pA at the steady holding potential of ~80 mV. Linear cell capacitance was monitored throughout each experiment and was calculated from the integral of charges required to clamp the membrane from ~80 to ~70 mV. The compensated series resistance (Rc) was calculated from the time constant for decay of the capacity transient and the linear cell capacitance. Depolarizing test pulses were delivered at 5-s intervals. Linear membrane capacitance and leakage currents were subtracted from test currents using the p-6/ method, and all analyzed and reported data were obtained from corrected currents. Currents were filtered at 0.5–10 kHz using the built-in Bessel filter (4-pole low pass) of the Axopatch 200A patch-clamp amplifier, and were sampled at 1–50 kHz using a Digidata 1200 analogue-to-digital board installed in a Gateway 486-66V personal computer. The pCLAMP software programs Clampex and Clampfit (version 6.0) were used for data acquisition and analysis, respectively. Figures were made using Origin (version 4.1).

Results

Fig. 1 shows whole-cell Ca or Ba currents recorded from a human embryonic kidney cell expressing the
wild-type α₁C subunits. As demonstrated by previous studies (Perez-Reyes et al., 1994; Zong et al., 1994; de Leon et al., 1995; Perez-Garcia et al., 1995; Ferreira et al., 1997), the heterologously expressed cardiac α₁C exhibits prominent Ca-dependent inactivation, as evidenced by the faster inactivation of Ca than Ba currents. The inactivation rate of currents mediated by the subunit combination expressed here (α₁C, α₂δ, and β₁a) closely approximates the inactivation rate of natively expressed cardiac channels (see Imredy and Yue, 1994), and is considerably faster than the inactivation rate of currents mediated by expression of α₁C and β₂a in the absence of α₂δ (Perez-Reyes et al., 1994; de Leon et al., 1995; Perez-Garcia et al., 1995; Ferreira et al., 1997).

To quantify the time course of inactivation, the decaying phase of Ca or Ba currents was fit with a single or double exponential function. Most Ca currents required two exponentials for a good fit, whereas Ba currents evoked by relatively short test pulses (250 ms) could usually be well fit by a single exponential. However, some Ba currents displayed two distinct components of inactivation. Fig. 1D plots the time constants for inactivation of α₁C currents as a function of test potential. For Ca currents, the time constants for inactivation had a U-shaped dependence on test potential, whereas time constants for inactivation of Ba currents decreased progressively with increasing test potential. These results are consistent with the expectation that α₁C undergoes Ca- but not Ba-dependent inactivation. However, in some cells the availability of Ba currents (measured using a double-pulse protocol) displayed a weak U-shaped dependence on test potential (data not shown), consistent with the idea that Ba can also trigger ion-dependent inactivation, although less effectively than Ca.

To investigate the structural basis for Ca-dependent inactivation, we expressed a series of chimeric α₁ subunits in which one of the major intracellular domains of α₁C was replaced by the corresponding region from the skeletal muscle α₁S subunit. The composition of these chimeras is represented diagrammatically in Fig. 2.

Previous studies have shown that deletion of the distal COOH terminus of α₁C or α₁S produces a fully functional Ca channel (Beam et al., 1992; Zong et al., 1994; de Leon et al., 1995). Fig. 3A confirms this result for the deletion mutant α₁C-3'del in which amino acids 1813–2166 have been removed from the COOH terminus of α₁C. As shown in Table I, inactivation of α₁C-3'del proceeded at the same rate as the wild-type α₁C, confirming that deletion of the distal COOH terminus of α₁C does not appreciably alter the process of Ca-dependent inactivation.

We next examined the potential role of the NH₂ terminus in Ca-dependent inactivation. The NH₂ terminus of α₁C is 103 amino acids longer than that of α₁S (Mikami et al., 1989) and contains four consensus sites for potential phosphorylation by PKC, whereas the NH₂ terminus of α₁S lacks predicted PKC sites. In chimera CSk1, the NH₂ terminus of α₁C has been replaced by its counterpart from α₁S (Fig. 2). Currents mediated by CSk1 closely resembled those produced by α₁C and α₁C-3'del (Fig. 3B). Thus, Ca currents inactivated faster than Ba currents, and time constants for inactivation of Ca currents were indistinguishable between CSk1 and α₁C (Table I). However, inactivation of Ba currents was significantly different.

**Figure 1.** Ca-dependent inactivation of wild-type α₁C Ca channels coexpressed in HEK293 cells with skeletal muscle α₂δ and β₁a subunits. (A) Representative whole-cell Ca currents mediated by α₁C. The illustrated currents were evoked by depolarizations from −10 to +90 mV. The compensated series resistance (Rₛ) was 4.2 MΩ. Linear cell capacitance (C) = 41 pF. File 96506016. (B) Representative Ba currents mediated by α₁C. Test pulses −20 to +90 mV. Same cell as in A. Rₛ = 2.1 MΩ. File 96506018. (C) Average I–V relations for Ca and Ba currents mediated by α₁C. Each plotted point represents the mean (±SEM) of 13 (Ca) and 6 (Ba) different cells. (D) Time constants for inactivation of Ca or Ba currents mediated by α₁C. Currents were evoked by 250-ms test pulses and were fit with either a single or double exponential function. For currents requiring two exponentials, only the fastest component was included in the analysis. Each plotted point represents the mean (±SEM) of 2–14 (Ca) and 3–7 (Ba) different cells. Experiments summarized in this figure were done using the TEACl-based external solution.
slightly faster for CSk1 than for $\alpha_{1C}$. Overall, these results suggest that Ca-dependent inactivation was not significantly altered by replacing the NH$_2$ terminus of $\alpha_{1C}$ with the corresponding region from $\alpha_{1S}$.

The Ca channel $\alpha$ subunit is highly homologous to the $\alpha$ subunit of voltage-gated Na channels, and the cytoplasmic linker between transmembrane domains III and IV (the III–IV loop) of Na channels is a critical structural determinant of fast inactivation (Vassilev et al., 1988; Stühmer et al., 1989). To examine the possibility that the homologous III–IV loop of Ca channels is involved in Ca-dependent inactivation, we constructed chimera CSk8 in which the III–IV loop and the first half of IVS1 of $\alpha_{1C}$ were replaced by the corresponding region from $\alpha_{1S}$ (Fig. 2). As shown in Fig. 3 C, Ca currents mediated by CSk8 inactivated faster than Ba currents and in general closely resembled currents produced by expression of $\alpha_{1C}$–3' del or CSk1. Furthermore, the time constants for inactivation of currents mediated by CSk8 were comparable to those obtained for $\alpha_{1C}$, $\alpha_{1C}$–3' del, and CSk1 (Table I), suggesting that Ca-dependent inactivation is not altered in CSk8.

In both $\alpha_{1C}$ and $\alpha_{1S}$, the I–II loop contains a number of negatively charged aspartate and glutamate residues that could potentially form a Ca-coordination site or sites. There are 25 negatively charged residues within the I–II loop of $\alpha_{1C}$ and 19 such residues within the I–II loop of $\alpha_{1S}$. To examine the possibility that the extra acidic residues within the I–II loop of $\alpha_{1C}$ might play a functional role in Ca-dependent inactivation, we expressed chimera CSk2 in which the I–II loop and most of the IIS1 segment of $\alpha_{1C}$ were replaced by the corresponding region from $\alpha_{1S}$ (Fig. 2).

Fig. 4 A presents Ca and Ba currents mediated by CSk2. For comparison, currents recorded under identical conditions from cells expressing $\alpha_{1C}$ are shown in Fig. 4 B. Ca currents produced by CSk2 inactivated significantly faster than Ba currents, indicating the presence of Ca-dependent inactivation. Furthermore, when relatively long test pulses (1.25 s) were used, both Ca and Ba currents exhibited two distinct components of inactivation. As recently shown by Ferreira et al. (1997) for $\alpha_{1C}$, the fast component of Ba current inactivation likely represents an ion-dependent process because it parallels Ba influx, whereas the slow component of inactivation likely represents a voltage-dependent process because it parallels the immobilization of gating charge. The presence of two components for inactivation of currents mediated by CSk2 is consistent with the thesis of Ferreira et al. (1997) that Ba as well as Ca can trigger inactivation. In the present work, we have analyzed only the faster time constants for inactivation of Ca and Ba currents.

The fast time constants for inactivation of Ca currents mediated by CSk2 exhibited a U-shaped dependence on test potential, whereas those for Ba currents progressively decreased with increasing test potential (Fig. 4 D). The time constants for inactivation of Ca currents were similar for CSk2 and $\alpha_{1C}$ currents of comparable densities (Table I). These results demonstrate that chimera CSk2 undergoes Ca-dependent inactivation. In marked contrast, the inactivation of Ba currents mediated by CSk2 was dramatically slowed (Fig. 4 A), with the fast time constants for inactivation being
approximately threefold larger than for αIC (Table I). If the fast phase of Ba current inactivation primarily reflects an ion-dependent process as proposed by Ferreira et al. (1997), then the slower inactivation of CSK2 may indicate that Ba is less effective in triggering ion-dependent inactivation when the I–II loop has skeletal muscle as opposed to cardiac sequence. Such an interpretation would imply that the I–II loop of αIC has a functional role in Ca-dependent inactivation if Ba- and Ca-dependent inactivation are equivalent. It is also possible that the process of voltage-dependent inactivation is affected somewhat by replacement of the I–II loop region.

We have previously demonstrated that the II–III loop of αIS performs a critical function in skeletal muscle-type excitation–contraction coupling (Tanabe et al., 1990b), perhaps by interacting directly with the ryanodine receptor. The II–III loops of αIC and αIS also contain numerous (35 and 27, respectively) negatively charged aspartate or glutamate residues that could potentially be involved in Ca coordination (Fujita et al., 1993). In addition, the II–III loop of αIS contains a consensus site for phosphorylation by PKA (Tanabe et al., 1987), whereas the II–III loop of αIC lacks predicted PKA sites (Mikami et al., 1989). To test whether the II–III loop of αIC performs a unique function in Ca-dependent inactivation, currents mediated by test pulses of 250 or 500 ms, or 1.25 or 5 s, as dictated by the inactivation rate. Mean ± SEM, with the number of cells (n) in parentheses.

**Figure 3.** Ca-dependent inactivation is normal in αIC–3del, CSK1, and CSK8. (A) Representative Ca (top) and Ba (bottom) currents mediated by αIC–3del, a deletion mutant of αIC lacking amino acid residues 1813–2166 from the COOH terminus. Test pulses from 0 to +40 mV (Ca) and −10 to +60 mV (Ba). Files 95929002, 95929006. C = 42 pF, R = 3.8 MΩ. (B) Representative Ca and Ba currents mediated by CSK1, a chimera in which the NH2 terminus of αIC was replaced by the corresponding region from αIS. Test pulses from 0 to +40 mV (Ca) and 0 to +60 mV (Ba). Files 95D01016, 95D01018. C = 21 pF, R = 6.3 MΩ. (C) Representative Ca and Ba currents mediated by chimera CSK8, in which the III–IV loop of αIC was replaced by the corresponding region from αIS. Test pulses from +30 to +50 mV (Ca) and +20 to +40 mV (Ba). Files 96118045, 96118053. C = 15 pF, R = 4.9 MΩ. Experiments summarized in this figure were done using the TEACl-based external solution.

**Table I**

*Data from HEK293 Cells Cotransfected with Expression Plasmids Encoding α1a, α1S, and β1a, Ca Channel Subunits*

| α1 construct | Ca current density | Fast tau Ica | Fast tau Icb | External solution |
|--------------|--------------------|--------------|--------------|------------------|
| αIC          | −56 ± 9 (17)       | 17 ± 2 (14)  | 88 ± 8 (7)   | TEACl            |
| αIC          | −17 ± 3 (7)        | 38 ± 6 (7)   | —            | TEACl            |
| αIC–3del     | −68 ± 21 (7)       | 20 ± 4 (8)   | 93 ± 21 (6)  | TEACl            |
| CSK1         | −28 ± 5 (12)       | 20 ± 1 (11)  | 57 ± 11 (6)  | TEACl            |
| CSK2         | −28 ± 11 (8)       | 35 ± 4 (9)   | 295 ± 38 (8) | TEACl            |
| CSK2         | −20 ± 7 (5)        | 51 ± 8 (5)   | 467 ± 107 (4)| NaCl             |
| CSK3         | −23 ± 6 (9)        | 31 ± 5 (9)   | 129 ± 15 (4) | TEACl            |
| CSK3         | −45 ± 9 (6)        | 38 ± 5 (6)   | 106 ± 22 (3) | NaCl             |
| CSK4         | −16 ± 3 (10)       | 70 ± 4 (5)   | 75 ± 5 (5)   | TEACl            |
| CSK4         | −13 ± 5 (4)        | 82 ± 15 (4)  | 80 ± 10 (4)  | NaCl             |
| CSK5         | −51 ± 12 (8)       | 32 ± 4 (10)  | 97 ± 9 (8)   | TEACl            |
| CSK5         | −17 ± 6 (5)        | 76 ± 7 (5)   | 111 ± 6 (7)  | NaCl             |
| CSK8         | −51 ± 12 (8)       | 23 ± 4 (8)   | 110 ± 10 (7) | TEACl            |

The external solution was based upon TEACl or NaCl (see materials and methods for compositions). Time constants for inactivation were derived by fitting maximal Ca or Ba currents (i.e., at the peak of the I–V relationship) with one or two exponential functions. Only the fast time constants (fast tau) were analyzed. Currents were evoked by test pulses of 250 or 500 ms, or 1.25 or 5 s, as dictated by the inactivation rate. Mean ± SEM, with the number of cells (n) in parentheses.
dent inactivation, we expressed chimera CSk3 in which the II–III loop of \( \alpha_{1C} \) was replaced by its counterpart from \( \alpha_{1S} \) (Fig. 2). CSk3 undergoes Ca-dependent inactivation because Ca currents inactivated much faster than Ba currents (Fig. 5). Furthermore, both Ca and Ba currents exhibited two distinct components of inactivation. The fast time constants for inactivation of Ca currents exhibited a U-shaped dependence on test potential; in contrast, such a relationship was not apparent for Ba currents (Fig. 5 D). Similar to the results obtained for CSk2, the fast component of Ba current inactivation was slightly slower for CSk3 than for \( \alpha_{1C} \) (Table I), raising the possibility that the II–III loop may perform a functional role in Ca- or voltage-dependent inactivation.

Several previous studies have identified the COOH terminus of \( \alpha_{1C} \) as an important structural determinant of Ca-dependent inactivation (de Leon et al., 1995; Soldatov et al., 1997; Zhou et al., 1997). To obtain further information regarding this issue, we expressed chimera CSk4 in which the distal 80% of the COOH terminus of \( \alpha_{1C} \) was replaced by the corresponding region from \( \alpha_{1S} \) (Fig. 2). In contrast to the other chimeras examined, Ca and Ba currents mediated by CSk4 inactivated at equivalent rates (Fig. 6, A and B). Both Ca and Ba currents exhibited two distinct components of inactivation. However, there was no difference between the fast time constants for inactivation of Ca currents and those for Ba currents over a wide range of test potentials and time constants for inactivation of Ca currents did not have a U-shaped dependence upon test potential (Fig. 6 D). These results demonstrate that CSk4 lacks Ca-dependent inactivation. As shown in Table I, the fast time constants for inactivation of Ca currents were three- to fourfold larger for CSk4 than for \( \alpha_{1C} \). This slow inactivation of Ca currents cannot be explained by low expression of CSk4, because inactivation was also slow compared with low density currents mediated by \( \alpha_{1C} \) (Table I). Interestingly, the fast time constants for inactivation of Ba currents were not different between CSk4 and \( \alpha_{1C} \), suggesting that the fast component of Ba current inactivation is unaltered in CSk4.

The results obtained with \( \alpha_{1C} \text{C}^{3,9}\text{del} \) (Fig. 3) confirm that a large segment of the distal COOH terminus is not required for Ca-dependent inactivation (Zong et al., 1994; de Leon et al., 1995). In contrast, the results obtained with CSk4 (Fig. 6) suggest that the COOH terminus of \( \alpha_{1S} \) can somehow prevent Ca-dependent inactivation. A comparison of the COOH termini of \( \alpha_{1C} \) and \( \alpha_{1S} \) (Fig. 7) reveals substantial conservation of their sequences for \( \sim 200 \) amino acids after the end of the last predicted transmembrane segment (IVS6). Beyond

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**Figure 4.** CSk2 retains Ca-dependent inactivation, but Ba current inactivation is dramatically slowed. (A) Representative Ca and Ba currents mediated by CSk2. Test pulses from \(-10 \) to \(+60 \) mV (Ca) and \(-10 \) to \(+50 \) mV (Ba). Files 97428010, 97428014. \( C = 17 \) pF, \( R_s = 2.2 \) MΩ. (B) Representative Ca and Ba currents mediated by \( \alpha_{1C} \). Test pulses from \(-10 \) to \(+40 \) mV (Ca) and \(-10 \) to \(+50 \) mV (Ba). Files 97502005, 97502012. \( C = 31 \) pF, \( R_s = 2.3 \) MΩ. (C) I–V relations for CSk2 currents. Plotted points represent the mean (±SEM) of 4–10 different cells. (D) Voltage dependence of fast time constants for inactivation of CSk2. Plotted points represent mean (±SEM) of four to five different cells. Experiments summarized in this figure were done using the NaCl-based external solution.
this point, however, the COOH termini of α_{1C} and α_{1S} diverge significantly. To test whether the distal, non-conserved portion of the COOH terminus from α_{1S} could be responsible for the inability of CSk4 to undergo Ca-dependent inactivation, we constructed chimera CSk5 (Fig. 2). This construct is a truncation mutant of CSk4 in which the final 211 amino acids of the COOH terminus have been deleted, effectively removing the majority of the sequence that is not conserved between α_{1C} and α_{1S} (Fig. 7).

CSk5 undergoes Ca-dependent inactivation, as evidenced by the significantly faster inactivation of Ca than of Ba currents (Fig. 8, A and B; Table I). Two distinct components of inactivation were present in both Ca and Ba currents mediated by CSk5. The fast time constants for inactivation of Ca currents exhibited a U-shaped voltage dependence, whereas those for Ba currents did not (Fig. 8 D). At test potentials of +10, +20, and +30 mV, the fast time constants for inactivation of Ca currents were significantly smaller than for Ba currents (Fig. 8 D). The fast component of Ca current inactivation was slower for CSk5 than for α_{1C}, suggesting that Ca-dependent inactivation proceeds at a slower rate than in α_{1C}. As was found for CSk4, the fast
time constant for inactivation of Ba currents was not different between CSk5 and αIC (Table I).

**Discussion**

The goal of the present study was to gain new insights into the molecular mechanism of Ca-dependent inactivation by identifying structural regions of αIC involved in this phenomenon. Toward this end, we compared inactivation of Ca and Ba currents mediated by chimeras constructed between the cardiac αIC, which exhibits prominent Ca-dependent inactivation, and the skeletal muscle αIS, which lacks this property (Donaldson and Beam, 1983; Beam and Knudson, 1988).

The protein sequence homology between αIC and αIS is ~66%, with the majority of the amino acid differences occurring within the major cytoplasmic domains (Mikami et al., 1989). For example, the NH2 terminus of αIC contains 154 amino acids, whereas that of αIS contains only 50 amino acids. At the other extreme, the III–IV loops of αIC and αIS are highly conserved, both being 53 amino acids in length with only 7 amino acid differences. Our results with chimeras CSk1 and CSk8 suggest that neither the NH2 terminus nor the III–IV loop of αIC performs an essential function in Ca-dependent inactivation, because Ca and Ba currents mediated by these constructs are nearly identical to those mediated by αIC (Fig. 3). However, because the III–IV loop is so highly conserved between αIC and αIS, this region may function interchangeably in Ca-dependent inactivation.

We have also found that inactivation of Ba current is dramatically slowed for chimera CSk2, in which the I–II loop of αIC is replaced by the corresponding region of αIS.
from $\alpha_1S$ (Fig. 4; Table I). This result is consistent with the relative inactivation rates of $\alpha_1C$ and $\alpha_1S$, when these two different L-type Ca channels are expressed in dysgenic myotubes (Tanabe et al., 1990a). Our results with CSk2 are also consistent with the finding of Page et al. (1997) that inactivation was slowed by replacing the entire I–II loop of the relatively fast inactivating $\alpha_1E$ with the I–II loop from the more slowly inactivating $\alpha_1B$. It is also interesting to compare our results for CSk2 with those of Zhang et al. (1994), who identified transmembrane segment IS6 and its immediately flanking regions as important determinants of voltage-dependent inactivation. A comparison of the I–II loops of $\alpha_1C$ and $\alpha_1S$ reveals that most amino acid differences occur within the COOH-terminal half, whereas the NH$_2$-terminal half of the I–II loop is comparatively well conserved (Mikami et al., 1989). The slowed inactivation of CSk2 may thus indicate an important role for the COOH-terminal portion of the I–II loop in Ca channel inactivation. However, because the NH$_2$-terminal portion of the I–II loop contains an interaction site for the Ca channel $\beta$ subunit (Pragnell et al., 1994), and different $\beta$ subunit isoforms can modulate the rate of Ca channel inactivation (Hullin et al., 1992), it is also possible that altered interactions between CSk2 and the $\beta$ subunit are partially responsible for its slower inactivation. No consensus sites for phosphorylation by PKA or PKC are present within the I–II loop of either $\alpha_1C$ or $\alpha_1S$; thus, it seems unlikely that differential phosphorylation could account for the slower inactivation of CSk2.

Ca-dependent inactivation is usually defined as the faster inactivation of Ca than Ba currents and by a U-shaped voltage dependence of the time constants for Ca current inactivation. Inactivation of Ba currents is usually assumed to proceed through a voltage-dependent process. However, Ferreira et al. (1997) have recently demonstrated that Ba can trigger the ion-dependent inactivation of $\alpha_{1C}$. They found that Ba currents inactivate with two distinct components, and that the rate and extent of the fast component parallels Ba influx, whereas the rate and extent of the slow component parallels immobilization of gating charge (Ferreira et al., 1997). If the fast component of Ba current inactivation measured in our experiments reflects an ion-dependent process, then this process is significantly slowed in chimera CSk2, and to a lesser extent in chimera CSk3. In this view, our results with CSk2 suggest that the I–II loop of $\alpha_{1C}$ may be an important structural determinant of ion- rather than voltage-dependent inactivation. Such inactivation could be triggered (physiologically) by Ca or (experimentally) by Ba binding to the I–II and II–III loops of $\alpha_{1C}$ but not to the homologous regions of $\alpha_{1S}$. If this interpretation is correct, then the I–II and II–III loops of $\alpha_{1C}$ are structural determinants of Ca-dependent inactivation.

We have demonstrated that CSk4, a chimera in which the COOH terminus of $\alpha_{1C}$ has been replaced by the corresponding region from $\alpha_{1S}$, lacks Ca-dependent inactivation. This result is not an artifact stemming from low channel expression because the current density in cells expressing CSk4 was not significantly different from that in cells expressing CSk2 or CSk5 (Table I), which both displayed prominent Ca-dependent inactivation. Furthermore, Ca-dependent inactivation was...
absent even from relatively high density CSk4 currents (not shown), whereas it was present in relatively low density α_{1C} CSk1, CSk2, or CSk3 currents (e.g., Figs. 3 and 4). The lack of Ca-dependent inactivation by CSk4 may explain why this property is not exhibited by the skeletal muscle L-type Ca channel. In this regard, it would be interesting to know whether the property of Ca-dependent inactivation was gained or retained by α_{1C} during the course of Ca channel evolution. A recent report that the neuronal α_{1D} (an L-type Ca channel) also exhibits Ca-dependent inactivation (Hans et al., 1997) suggests that this property has been retained by α_{1C} and α_{1D} and lost by α_{1S}.

The mechanism of Ca-dependent inactivation is not known, but it has been proposed that a putative EF hand motif located within the proximal COOH terminus of α_{1C} functions as the essential Ca-binding site responsible for triggering Ca-dependent inactivation (de Leon et al., 1995). However, recent evidence from other laboratories suggests that the putative EF hand motif is not important in the mechanism of Ca-dependent inactivation. Thus, transfer of the putative EF hand from α_{1C} into α_{1E} fails to confer Ca-dependent inactivation and, conversely, transfer of the EF hand from α_{1E} into α_{1C} fails to disrupt it (Zhou et al., 1997). Additionally, Ca-dependent inactivation is not abolished by point mutations within α_{1C} that eliminate the Ca-coordination site from the putative EF hand motif but leave the remainder of the COOH terminus intact (Zhou et al., 1997). These results strongly suggest that the exact site or sites of Ca binding remain to be identified.

Because CSk5 undergoes Ca-dependent inactivation (Fig. 8), it is reasonable to suppose that it contains one or more Ca binding sites. It follows that CSk4 contains the same site or sites, because it encompasses the entire sequence of CSk5 (Fig. 7). However, CSk4 lacks Ca-dependent inactivation (Fig. 6), which leads to the conclusion that Ca binding to the α_{1} subunit is only a prerequisite for Ca-dependent inactivation and is by itself insufficient. Presumably, Ca-dependent inactivation requires both Ca binding and a subsequent conformational shift of the channel protein(s).

Perhaps the most significant result of the present study is that Ca-dependent inactivation was restored in chimera CSk5 by deletion of the nonconserved, distal region of the COOH terminus present in CSk4 (Figs. 2 and 8). The COOH terminus of CSk5 is similar in length and composition to that of α_{1C-3’del} (Fig. 7). The behavior of α_{1C-3’del} clearly demonstrates that the most distal ~350 amino acids of the COOH terminus of α_{1C} are not required for Ca channel inactivation (Fig. 3 A; Zong et al., 1994; de Leon et al., 1995). In contrast, Ca-dependent inactivation is conferred upon the α_{1E} backbone by replacing a 134-amino acid segment immediately downstream from the putative EF hand region with the homologous 142-amino acid segment from α_{1C} (Zhou et al., 1997). Furthermore, Ca-dependent inactivation is profoundly influenced by splice variations within the proximal COOH terminus of α_{1C} immediately downstream from the putative EF hand region (Soldatov et al., 1997). Our results with CSk5 suggests that the proximal COOH termini of α_{1C} and α_{1S} (which are mostly conserved) can function interchangeably in Ca-dependent inactivation. When considered altogether, our results and those of other studies indicate that the proximal COOH terminus downstream from the putative EF hand region is an important structural determinant of Ca-dependent inactivation. However, de Leon et al. (1995) showed that Ca-dependent inactivation was only partially conferred upon the neuronal α_{1E} subunit by replacing its entire COOH terminus with 217 amino acids from the corresponding region of α_{1C}. Thus, while the proximal COOH terminus appears to be important for Ca-dependent inactivation, the participation of additional channel regions may also be required.

Our findings that CSk4 lacks Ca-dependent inactivation, whereas this property is restored in CSk5, suggests that the distal COOH terminus of α_{1S} (which is not well conserved between α_{1C} and α_{1S}) can somehow block Ca-dependent inactivation. The mechanism by which this block occurs is, at present, purely speculative. However, because ion channels appear to associate with many other proteins in vivo (Sheng and Kim, 1996), it seems plausible that CSk4 might be tethered through its distal COOH terminus to other proteins (such as ryphanidine receptors, the cytoskeleton, kinases, phosphatases, or other ion channels), and that such interactions might prevent the conformational shift underlying Ca-dependent inactivation.

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