Multiple Structural Domains Contribute to Voltage-dependent Inactivation of Rat Brain α₁E Calcium Channels*

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We have investigated the molecular determinants that mediate the differences in voltage-dependent inactivation properties between rapidly inactivating (R-type) α₁E and noninactivating (L-type) α₁C calcium channels. When coexpressed in human embryonic kidney cells with ancillary β₁b and α₁EΔδ subunits, the wild type channels exhibit dramatically different inactivation properties; the half-inactivation potential of α₁E is 45 mV more negative than that observed with α₁C, and during a 150-ms test depolarization, α₁E undergoes 65% inactivation compared with only about 15% for α₁C. To define the structural determinants that govern these intrinsic differences, we have created a series of chimeric calcium channel α₁ subunits that combine the major structural domains of the two wild type channels, and we investigated their voltage-dependent inactivation properties. Each of the four transmembrane domains significantly affected the half-inactivation potential, with domains II and III being most critical. In particular, substitution of α₁C sequence in domains II or III with that of α₁E resulted in 25-mV negative shifts in half-inactivation potential. Similarly, the differences in inactivation rate were predominantly governed by transmembrane domains II and III and to some extent by domain IV. Thus, voltage-dependent inactivation of α₁E channels is a complex process that involves multiple structural domains and possibly a global conformational change in the channel protein.

The influx of calcium through neuronal voltage-gated calcium channels regulates a wide range of cellular processes, including neurotransmitter release, activation of Ca²⁺-dependent enzymes and second messenger cascades, gene regulation, and proliferation. To date, the primary structures of at least nine different neuronal Ca²⁺ channel α₁ subunits have been identified. α₁C, α₁D, and α₁F encode L-type channels (1–3); α₁B defines N-type channels (4–6); α₁A encodes both P- and Q-type channels (7–11); α₁G, α₁H, and α₁I form T-type channels (12, 13); and α₁E probably encodes a component of the “resistant” current identified in several neuronal preparations (14–16).

A key mechanism by which these channels achieve the tight regulation of internal calcium levels is a fast, voltage-dependent inactivation process. Calcium channel inactivation is a critical determinant of the temporal precision of calcium signals and serves to prevent long term increases in intracellular calcium levels, which are cytotoxic to neurons (17–19). The inactivation of calcium channels at presynaptic terminals may also contribute to short term synaptic plasticity (20). Additionally, cumulative inactivation of neuronal calcium channels during a train of action potentials can lead to variable depression of calcium entry depending on the subunit composition of calcium channels present (21). Unlike the well characterized “ball and chain” (22–24) and “hinged lid” (25–28) inactivation mechanisms of voltage-dependent potassium and sodium channels, the molecular mechanisms for voltage-dependent inactivation in calcium channel proteins are incompletely understood. A study by Zhang et al. (29) has revealed that the domain I S6 region is a critical determinant of the differences in voltage-dependent inactivation properties observed with marine ray (doo-I) α₁E and rabbit brain α₁A calcium channels. More recently, several individual amino acid substitutions throughout the calcium channel α₁ subunit, including the domain I-H linker region, the proximal carboxyl-terminal region, and the S6 regions in domains III and IV (7, 11, 30–36) have been shown to reduce or abolish voltage-dependent inactivation. These observations suggest that voltage-dependent inactivation of calcium channels may perhaps involve multiple structural elements.

In order to more systematically examine the molecular determinants governing calcium channel inactivation, we have created a series of chimerical calcium channel α₁ subunits, which combine the structural features of rapidly inactivating α₁E and noninactivating α₁C calcium channels, expressed them transiently in HEK cells, and assessed their inactivation properties via patch clamp. Our data indicate that each transmembrane domain contributes, to varying degrees, to voltage-dependent inactivation of rat brain α₁E calcium channels and that inactivation probably involves a complex global conformational change throughout the channel protein. We hypothesize that the molecular mechanisms underlying fast voltage-dependent inactivation in neuronal calcium channels may be analogous to the slower C-type inactivation process common to many types of potassium channels (23, 37, 38).

EXPERIMENTAL PROCEDURES

Materials
cDNAs coding for wild type rat brain α₁E (rbE-II; GenBank™ accession no. L15453), α₁E (rbC-II; GenBank™ accession no. M67515), and α₁EΔδ subunits were kindly donated by Dr. T. P. Snutch. The β₁b subunit was provided by Dr. Perez-Reyes. The pEGFP-C1 was donated by Dr. Robert Dunn. Unless stated otherwise, chemical reagents were purchased from Sigma. Restriction enzymes were obtained from Life Technologies, Inc. and from New England Biolabs.

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Determinants of Calcium Channel Inactivation

Site-directed Mutagenesis

Unique restriction enzyme sites were inserted into the α1C and α1S cDNA constructs via site-directed mutagenesis at exactly complementary positions at the beginning of the domain I-II (AvrII) and the domain II-III (SalI) linker regions and about 20 amino acid residues into the domain III-IV linker region (MluI) as outlined below.

Site-directed Mutagenesis of α1C—The rbC-II construct in Bluescript(SK−) was first cut with SalI, blunted and then religated in order to eliminate two undesired SalI sites in the polylinker and the 5′-untranslated region. Subsequently, the construct was cut with NheI and recircularized to temporarily eliminate 2.5 kilobase pairs of coding region at the 3′-end. This construct was used as the template for all subsequent rounds of mutagenesis. Using the QuikChange site-directed mutagenesis kit (Stratagene), the following mutations were created: silent mutation at bp 1199 to create an amino acid residue found in an undesired endogenous 2256 to generate a silent mutation at bp 2256 to create an undesired endogenous 2256 to generate a silent mutation at bp 2256 to create an undesired endogenous 2256 to generate a silent mutation at bp 2256 to create an undesired endogenous AvrII site. In order to create chimeras that involved domain switches at the junction between domains II and IV, it was necessary to engineer an MluI site into the cDNA sequence. In rbC-II, this required a nonsilent mutation at bp 3606 to the corresponding amino acid residue found in α1S (arginine to threonine substitution). Thus, a fourth round of mutagenesis to create the MluI site was carried out; however, this particular construct was used only for chimeras involving transitions between domains III and IV. Because the amino acid was switched to the complementary α1S residue, the mutation became silent once the domain IV switch had occurred. For all other chimeras, the construct lacking the MluI site was used as the parent channel construct. The successful addition of restriction sites was first confirmed via restriction digests, and the complete coding region was sequenced to confirm the absence of errors. For both constructs, the excised 2.5-kilobase pair NheI fragment was reintroduced to yield two full-length clones in Bluescript containing the unique restriction sites (CCCC, CCCC+(MluI)). CCCC was subcloned into PMT2 (XS) using the KpnI and NotI sites flanking the 5′- and 3′-ends of the clone, respectively.

Site-directed Mutagenesis of α1S—Prior to mutagenesis, rbE-II in Bluescript(SK−) was cut with NotI and recircularized to eliminate a 200-bp fragment in the 5′-untranslated region, thus leaving a single NotI site at the 5′-end. The construct was then cut with XhoI and recircularized to temporarily reduce its size by 1.7 kilobase pairs at the 3′-end. This construct was used as the template for all subsequent rounds of mutagenesis. Using the QuikChange kit, the following silent mutations were created: the addition of an AvrII site at bp 890; the addition of a SalI site at bp 1259; and the addition of a MluI site at bp 4224. Because the NotI and KpnI flanking sites in rbE-II were in opposite orientation compared with those flanking the rbC-II insert, it was necessary to replace the NotI and KpnI sites flanking rbE-II with KpnI and NotI, respectively, again using the QuikChange kit. The successful completion of the five rounds of mutagenesis was confirmed via restriction digests, and the excised 1.7-kilobase pair XhoI fragment was reintroduced to yield the full-length clone EEEE. This construct was completely sequenced from the 5′-end to the first XhoI (bp 5044) site to ensure that no PCR errors had occurred. The insert was then subcloned into PMT2 (XS) using KpnI and NotI.

Creation of α1S/α1C Chimeras

CCCC/EEEC—These chimeras were assembled in Bluescript using CCCC (+MluI) and EEEE by switching the MluI-NotI fragments among the two parent channels. Both chimeras were subsequently subcloned into PMT2 (XS) using KpnI and NotI. Again, note that in these two chimeras the nonsilent MluI mutation in CCCC (+MluI) becomes silent.

CCEC—CCEC was created by replacement of a SalI fragment between the II-III linker and the 3′ pMT2 polylinker of CCCC with the corresponding fragment of EEEC in pMT2.

CCEC—CCEC was created by replacement of an AvrII fragment between the 5′ pMT2 sequence (900 bp 5′ to the pMT2 polylinker) and the I-III linker region of EEEC with the corresponding fragment of CCCC in pMT2.

EECC—EECC was created by replacement of an AvrII fragment from CCCE (900 bp 5′ to the pMT2 polylinker, I-II linker) with the corresponding fragment from EEEC.

CCAECCEC—Domains I of EEEC and CCCCE were swapped via excision of the AvrII (pMT2, I-II linker) fragments.

ECCECCE—Domains III and IV of EEEC and CCCCE were swapped via excision of the SaII (I-II linker, 3′ pMT2 polylinker) fragments.

ECCE—Domains III and IV of CCCCE were replaced via excision of the SaII (I-II linker, 3′ pMT2 polylinker) fragments of EEEC.

ECCE—Domains III and IV of CCCCE were replaced via excision of the SaII (I-II linker, 3′ pMT2 polylinker) fragments from CCCCE.

ECCE—EECC was created by replacement of the domain III and IV fragment from CCCCE (via SaII digest) with the corresponding fragment from EEEC.

EECE—EECE was created by replacement of domain III and IV fragment from CCEC (via SaII digest) with the corresponding fragment from CCCE.

Electrophysiology

Immediately prior to recording, individual coverslips were transferred to a 3-cm culture dish containing external recording solution composed of 20 mM BaCl2, 1 mM MgCl2, 10 mM HEPES, 40 mM TEACl, 200 mM NaCl, 10 mM Hepes, 40 mM TEACl, 10 mM glucose, and 65 mM CsCl (pH 7.2). Whole cell patch clamp recordings were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) linked to a personal computer equipped with pCLAMP version 6.0. Patch pipettes (Sutter borosilicate glass, BF150-56-15) were pulled using a Sutter P-87 microelectrode puller and then coated using a Narishige microforge, and filled with 0.5 M KCl. The typical resistance of about 3–4 megahms. The series resistance was typically around 8 megohms. Because most currents were smaller than 1 nA at the peak of the current voltage relation, voltage errors were calculated to be less than 10 mV in the worst case. For steady state inactivation curves, this has little effect on the half-inactivation potential for two reasons. First, the conditioning potential is not affected by voltage errors, because with the exception of one chimera (ECEE) there was little current activation during the conditioning pulses. Second, the test potential used during the recording of steady state inactivation curves (+20 mV) was typically 10–30 mV more positive than the peak of the I-V relation, resulting in smaller currents and, thus, smaller voltage errors. Any remaining errors may cause a slight skewing of the shape of the steady state inactivation curve at the initial falling phase but very little effect on the measured half-inactivation potential. Consistent with this notion, for any given channel construct we found no correlation between half-inactivation potential and current size. The internal pipette solution contained 105 mM CsCl, 25 mM tetraethylammonium chloride, 11 mM EGTA, and 10 mM Hepes (pH 7.2). Recordings were made from cells expressing the green fluorescent protein gene as visualized by a fluorescence signal. The bath was connected to ground via a 3 M CsCl AGAR bridge. Seals were formed directly in the external control solution. After gigaseal formation, cells were allowed to dialyze for 5–10 min before recordings were performed. Unless stated otherwise, currents were typically elicited from a holding potential of −100 mV to various test potentials using Clampex software (Axon Instruments). We observed only negligible leak currents. At potentials more positive than +50 mV, because the reversal potential, outward currents carried presumably by cesium ions could be observed; however, these did not significantly affect our determination of the reversal potential, because data points close to reversal were not considered for fitting of macroscopic current-voltage relations.

Data were filtered at 1 kHz and recorded directly onto personal computer. Data were analyzed using Clampfit (Axon Instruments). All

1 The abbreviation used is: bp, base pair(s).
curve fitting was carried out in Sigmaplot 4.0 (Jandel Scientific). Steady state inactivation curves were fitted to the Boltzman equation,
\[ I_{peak} = \frac{C}{1 + e^{z(V-V_h)/S}} \]
where \( I_{peak} \) is the peak current, \( V \) is the voltage, \( V_h \) is the half-inactivation potential, \( z \) is a slope factor, and \( C \) is the non-inactivating fraction.

RESULTS

Wild type \( \alpha_{1C} \) and \( \alpha_{1E} \) Calcium Channels Exhibit Distinct Voltage-dependent Inactivation Properties—It is well established that neuronal (L-type) \( \alpha_{1C} \) calcium channels undergo little voltage-dependent inactivation in response to membrane depolarization. In contrast, \( \alpha_{1E} \) channels are among the most rapidly inactivating high voltage-activated calcium channel isoforms. Fig. 1 illustrates these intrinsic differences between \( \alpha_{1C} \) and \( \alpha_{1E} \) (both coexpressed in HEK cells with ancillary \( \delta \) and \( \beta_3 \) subunits). As seen in Fig. 1A, \( \alpha_{1E} \) channels inactivate much more rapidly than the \( \alpha_{1C} \) isoform, and this difference is maintained over a large range of test potentials (Fig. 1C). Generally, we observed some variability in the number of time constants required for fitting the time course of inactivation of \( \alpha_{1E} \) (i.e. while in the majority of the cases, a single exponential yielded a satisfactory fit, in some cases two exponentials were required). Hence, in order to facilitate comparison among the different channels, the rate of inactivation is reflected, in Fig. 1C and throughout, as the percentage of peak current that has inactivated over a time course of 150 ms. Note that, because barium was used as the external charge carrier and due to effective buffering of intracellular calcium with EGTA, voltage-dependent inactivation processes are not contaminated by the calcium-sensitive inactivation process intrinsic to L-type calcium channels (39).

In addition to their rates of inactivation, the wild type channels also exhibited pronounced differences in their half-inactivation potentials, with \( \alpha_{1E} \) inactivating at potentials about 40 mV more negative than \( \alpha_{1C} \) (Fig. 1D). In contrast, at least with 20 mM barium as the charge carrier, the half-activation potentials (estimated from Boltzman fits to current voltage relations) of the two wild type channels differed by only about 10 mV (Fig. 1B; see also Table I). Overall, the differences between the inactivation properties of the two wild type channels are sufficiently large to permit a chimerical approach toward the molecular identification of the underlying structural determinants.

All Four Transmembrane Domains Contribute to Steady State Inactivation—To investigate the molecular mechanism underlying these differences in voltage-dependent inactivation properties, we constructed a series of 14 chimeras between wild type \( \alpha_{1C} \) and \( \alpha_{1E} \) calcium channels. Each chimeric construct is formed via combination of the four major transmembrane domains of the respective parental channels (see Fig. 2). As described in more detail under “Experimental Procedures,” the chimeras were designed such that switches occurred immediately after the end of the S6 segments in domains I and II and about 20 amino acids past that of domain III, and thus each domain remains associated with the preceding cytoplasmic linker region. Of the 14 chimeras, nine constructs were found to form
Functional calcium channels when expressed in HEK cells. 

Fig. 2 depicts representative current traces and ensemble steady state inactivation curves for each of the nine functional chimeras. In each case, the voltage dependence of steady state inactivation could be nicely described with a Boltzman relation. Several of the chimeras, as well as the wild type α1C channels, did not inactivate completely during the 5-s conditioning pulse, and hence the Boltzman fit was modified to incorporate this noninactivating fraction (see “Experimental Procedures”).

The half-inactivation potentials and slope factors obtained from these fits (see also Table I) and the shapes of the current waveforms (Fig. 2) were consistent with what one might have expected from our observations with the two wild type channels. Chimera ECCC exhibited somewhat shallow voltage dependences of both inactivation and activation (Table I, Fig. 2).

Fig. 3A compares the half-inactivation potentials of the nine chimeras to the wild type channels in form of a bar graph. Upon examination of Fig. 3A, two observations can be made. First, no single domain switch appears to be able to confer the entire steadystate inactivation properties from one parent channel to another. Instead, a continuous spectrum of half-inactivation properties spanning the range between the two wild type channels was evident. Second, two of the chimeras (CEEC, CCCE) exhibited half-inactivation potentials outside of that range, and hence the Boltzman fit was modified to incorporate this noninactivating fraction (see “Experimental Procedures”).

The half-inactivation potentials and slope factors obtained from these fits (see also Table I) and the shapes of the current waveforms (Fig. 2) were consistent with what one might have expected from our observations with the two wild type channels. Chimera ECCC exhibited somewhat shallow voltage dependences of both inactivation and activation (Table I, Fig. 2).

From the graph in Fig. 3A, it is difficult to assess the effects of individual transmembrane domain switches on half-inactivation potential. Hence, in order to isolate the individual contributions of each of the four transmembrane domains to the overall voltage dependence of inactivation, the data of Fig. 3A were divided into individual pairs of chimeras in which only a single domain was exchanged. Fig. 3B examines the effect of replacement of the α1C sequence in domain I with the corresponding sequence from α1E. As evident from the figure, three out of the four chimera pairs examined exhibited a negative shift in half-inactivation potential when domain I contained the α1E sequence. Upon replacement of the α1C sequence in either domain II or III with that of α1E, a large hyperpolarizing shift of about −20 mV was observed in every case. Thus, structures residing within each of the first three domains contribute to the more negative half-inactivation potential seen with wild type α1C channels. Surprisingly, when the same type of analysis was carried out for substitutions in domain IV, the opposite effect was observed, with α1E domain IV mediating a depolarizing shift in half-inactivation potential when replacing α1C sequence. These data indicate that all four transmembrane domains contribute to steady state inactivation properties of voltage-dependent calcium channels, and furthermore, that the absolute value of the half-inactivation potential of α1E channels is determined through an equilibrium formed by hyperpolarizing and depolarizing structural elements.

Half-inactivation Potential Shifts Are Not Correlated with Activation Effects—It is known for sodium and potassium channels that inactivation can be tightly coupled to activation. Thus, the above conclusions are somewhat complicated by the notion that not all of the chimeras exhibited identical half-inactivation potentials (see Table I). The wild type channel differed by less than 10 mV in their half-activation potentials, and yet they exhibited a greater than 40 mV difference in their half-inactivation potentials. Furthermore, when examining the data presented in Table I, one can identify two clusters of constructs with half-activation potentials of about −21 mV (EEEE, EECC, CCEC, CCCE, CEEE) and about −12 mV (CCCC, CEEC, CECC, ECCC), respectively, and yet, within each of these clusters, the half-inactivation potentials varied by as much as 35 and 50 mV, respectively. Overall, these considerations suggest that the distinct activation potentials of the channels are not correlated with the observed differences in inactivation properties. Nonetheless, it is possible that in some cases the activation effects might skew the absolute inactivation potential changes induced by domain swapping. To assess the extent of any putative contamination by activation effects, we calculated the ratio of the change in half-activation potential to the change in half-inactivation potential for each pair of chimeras (see numbers in parentheses, Fig. 3B). A value of 1 indicates that the change in half-inactivation potential parallels the changes in half-activation potential in magnitude, a value near 0 indicates that there is only little if any contamination by activation effects, and a negative value reflects a scenario in which a domain switch resulted in opposite shifts in half-activation and half-inactivation potentials. As seen from Fig. 3, in only two out of 15 cases did the index approach 1, and only four additional chimera pairs displayed ratios greater than 0.1. It is also noteworthy that chimera CEEC differed from the wild type α1C channels by only 4 mV in half-activation potential, while exhibiting a half-inactivation potential that was 55 mV more negative, thus further supporting the notion that domains II and III carry the bulk of the voltage dependence of inactivation. Overall, these considerations suggest that for the majority of chimera pairs, differences in voltage-dependent activation properties could not account for the observed changes in half-inactivation potentials.

We did not systematically examine the effects of individual domains on half-activation potential, because the differences between the two wild type channels was relatively small (<10 mV mV pA/picofarads

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**Table I**

Activation and inactivation properties of wild type and chimeric calcium channels

Values were obtained via Boltzman fits of steady state inactivation curves (Fig. 2) and macroscopic current-voltage relations. Note that there is no correlation between half-activation and half-inactivation potentials. Current-density measurements obtained at the peak of the current voltage relation reveal variable levels of expression among the chimeras.

| Construct | Half-inactivation potential | z | Half-activation potential | S | Current-density |
|-----------|-----------------------------|---|----------------------------|---|-----------------|
| mV        | mV                          |   | Current-density            |
| CCCC      | −16.4 ± 1.9 (n = 9)         | 3.0| −11.2 ± 2.4 (n = 9)        | 5.2| 14.6 pa/picofarads |
| EEEE      | −57.5 ± 1.6 (n = 14)        | 3.0| −20.6 ± 3.2 (n = 12)       | 3.3| 29.3 pa/picofarads |
| CEEC      | −66.6 ± 1.4 (n = 9)         | 2.6| −15.0 ± 1.7 (n = 8)        | 3.8| 5.1 pa/picofarads |
| ECCC      | −58.5 ± 1.5 (n = 9)         | 2.7| −21.4 ± 2.3 (n = 8)        | 5.7| 15.3 pa/picofarads |
| CEEE      | −47.7 ± 2.5 (n = 8)         | 2.9| −27.9 ± 3.8 (n = 7)        | 2.6| 51.8 pa/picofarads |
| CECC      | −44.4 ± 2.5 (n = 10)        | 2.2| −13.6 ± 2.5 (n = 14)       | 4.0| 29.9 pa/picofarads |
| CCEC      | −43.7 ± 2.6 (n = 10)        | 2.9| −22.6 ± 2.0 (n = 8)        | 2.9| 37.4 pa/picofarads |
| CCEE      | −32.4 ± 2.7 (n = 8)         | 3.8| −21.7 ± 2.4 (n = 12)       | 4.7| 29.0 pa/picofarads |
| ECCCE     | −29.8 ± 3.4 (n = 6)         | 1.4| −12.5 ± 3.8 (n = 6)        | 11.6| 8.3 pa/picofarads |
| CCCE      | −19.6 ± 2.8 (n = 9)         | 3.1| −21.6 ± 2.2 (n = 5)        | 6.4| 5.8 pa/picofarads |
| CCEE      | 0.4 ± 1.8 (n = 4)           | 2.9| 5.4 ± 3.3 (n = 3)          | 8.6| 15.9 pa/picofarads |
mV), and perhaps with the exception of α1E domain III, which tended to shift the half-activation potential into the negative direction (see Table I), none of the individual transmembrane domains appeared to have a clear cut effect on activation range. We suspect that even with more accurate tail current protocols (rather than relying on fits to macroscopic current-voltage relations), we would be unable to attribute the distinct activation ranges of the two wild type channels to individual transmembrane domains.

Transmembrane Domains II, III, and IV Determine Inactivation Rates—To determine the effects of domain swapping on the rate of inactivation, we compared inactivation of wild type and chimeric calcium channels by using the ratio of peak current to the current observed at the end of a 150-ms test depolarization as a single measure of all voltage-dependent inactivation processes. Fig. 4 depicts the percentage of inactivation that occurred over 150 ms for three different test pulses (0, +10, and +20 mV). Similar to what was observed with the half-inactivation potentials, the inactivation rates of the individual chimeras formed a continuum within the range spanned by the two wild type channels. Overall, this indicates that the rate of inactivation may also be determined by multiple structural domains.

Fig. 5 examines the role of each individual transmembrane domain in determining the rate of inactivation. As seen from Fig. 5A, in only one out of four cases did exchanges of domain I exert a significant effect, suggesting that domain I does not contribute in a substantial manner to the differences in inactivation rate between rat brain α1C and α1E channels. In contrast, in seven out of eight cases, replacing α1C sequences in domains II or III with those corresponding to α1E mediated increases in inactivation rate by 2.5- and 4-fold, respectively.
Consistent with what we had observed with steady state inactivation, domain IV of α1E actually slowed the rate of inactivation (by 2.5-fold). This behavior is further illustrated in Fig. 5 with the current records and voltage dependences of the inactivation rates of selected chimera pairs. Insertion of domain I of α1E into the wild type α1C channel had little effect on current waveform or on the magnitude and voltage dependence of the rate of inactivation. Insertion of domain II or III of the wild type α1E channel into α1C mediated a significant speeding of inactivation at all test potentials (Fig. 5, B and C, insets). In fact, there was no significant difference in inactivation rate between chimeras CCEC and the wild type α1E channel at any of the test potentials used indicating that domain III might be perhaps be the most critical determinant of inactivation rate. Finally, in further support of the idea that domain IV of α1E slows inactivation, the CCE1 chimera inactivated significantly more slowly than CCEC at all potentials tested.

Overall, our data implicate multiple transmembrane domains in the overall voltage-dependent inactivation process of neuronal α1E calcium channels, with domains II and III accounting for the bulk of the effect.

**DISCUSSION**

**Ca**^2+** Channel Inactivation Is Fundamentally Different from That of Na**^+** and K** Channels—**The molecular mechanisms of fast voltage-dependent inactivation of voltage-dependent sodium and potassium channels are well understood. In Shaker K channels, fast inactivation occurs via physical occlusion of the pore by a cluster of about 20 amino acid residues at the amino terminus of the α1 subunit (22, 40). Due to the tetrameric structure of these channels, the presence of four identical inactivation particles has been proposed (41). In voltage-dependent sodium channels, fast inactivation appears to be caused by occlusion of the pore by three hydrophobic residues (Ile, Phe, and Met) located in the domain III-IV linker region of the sodium channel α1 subunit (26). Voltage-dependent calcium channels do not contain analogous structural elements, and no evidence for a pore blocking mechanism has been presented. Zhang et al. (29) have provided compelling evidence that the differences between the inactivation rates of marine ray α1E and rabbit brain α1A channels can be exclusively located to the domain I S6 region. However, more recently, individual point mutations in the domain I-III linker region (7, 11, 31, 35) and the S6 regions of domains III and IV (32–34) have been shown to attenuate or abolish voltage-dependent inactivation of α1A calcium channels, perhaps suggesting the possibility that inactivation of neuronal calcium channels might involve a more diffusely located effect.

Here, we have systematically investigated the roles of each of the four transmembrane domains in voltage-dependent inactivation of α1E channels. Our chimERIC approach was designed to be constructive; i.e. our goal was to confer the inactivation properties from a rapidly inactivating channel onto a relatively noninactivating one rather than simple destruction of inactivation. However, while we could confer certain aspects of voltage-dependent inactivation of α1E onto α1C channels through insertion of individual domains, we were unable to attribute the
mechanism underlying voltage-dependent inactivation to a single transmembrane domain. Instead, our data indicate that all four transmembrane domains contribute to varying degrees to fast inactivation. The overall half-inactivation potential appears to be determined through an equilibrium between hyperpolarizing (∆a1E domains I, II, and III) and depolarizing (∆a1E domain IV) elements. Similarly, the differences in inactivation rate between the two wild type channels appeared to involve predominantly domains II and III, with some contribution from domain IV. Thus, consistent with previous suggestions, the molecular mechanisms that mediate fast inactivation of voltage-dependent calcium channels appear to differ fundamentally from those observed with other types of voltage-gated cation channels.

Putative Effects of Differential Activation Properties—In principle, it is possible that some of the differences in half-inactivation potentials observed with the chimeric calcium channels might be secondarily due to intrinsic differences in activation properties. Therefore, we explored the role of each transmembrane domain in the inactivation process by constructing chimeric channels in which individual domains were exchanged between the wild type and chimeric sequences. Figure 5 illustrates the results of these experiments.

In Figure 4, inactivation rates for wild type and chimeric calcium channels are shown. Inactivation rates were measured at 0, 10, and 20 mV, and error bars denote S.E.; numbers in parentheses denote the numbers of experiments. Inactivation rates observed with the chimeras are widely distributed between those seen with the wild type channels.

Figure 5 shows the contribution of individual transmembrane domains to inactivation rate. A, exchanging domain I has little effect on inactivation rate. B and C, replacement of a1C sequence in domain II or III with the corresponding a1E sequence mediates a substantial increase in the rate of inactivation. D, replacement of a1C sequence in domain IV with that of a1E slows the rate of inactivation. Inactivation rates for each construct in Fig. 5 were measured at +20 mV and were taken from Fig. 4. The asterisks denote statistically significant changes in inactivation rates (p < 0.05). Insets, representative whole cell current records illustrating the effects of single domain switches on inactivation rates (step depolarizations to +10 mV). Current traces are scaled to the same peak amplitude to allow for comparison of inactivation rates. The voltage dependences of the inactivation rates for a number of experiments are depicted below the current records.
their activation properties. In 20 mM barium, we observed an approximate 10-mV difference in the half-activation potentials of wild type \( \alpha_{1E} \) and \( \alpha_{1C} \) channels. In contrast, their difference in half-inactivation potential is more than 4-fold larger. Furthermore, with the exception of two out of 15 chimera pairs, we found no correlation between shifts in half-activation and half-inactivation potentials resulting from the switching of individual or multiple domains, suggesting that changes in half-activation potential cannot account for our observations and that the effects observed with our chimeras are indeed due to structural changes in the voltage-dependent inactivation machinery.

The effects of individual domain exchanges on the inactivation rate were examined at the same test potential (+100 mV). Because the rate of inactivation may be coupled to channel activation, any variability in half-activation potential among the chimeras/wild type channels might affect the comparison of inactivation rates measured at a single arbitrary test potential. However, this variability in half-activation potential was fairly small (nine of the 11 chimeras activated within a 10-mV window), and thus, given the shallow voltage dependences of the inactivation rates, our interpretations are not likely to be affected.

Comparison with Previous Work—At first glance, our data appear to contradict those of Zhang et al. (29). However, two issues must be taken into consideration. First, wild type \( \alpha_{1E} \) and \( \alpha_{1C} \) channels exhibit much more pronounced differences in inactivation rate and in half-inactivation potential compared with the channels used by Zhang et al. (29) to create their chimeras (i.e. \( \alpha_{1E} \) and \( \alpha_{1A} \), which are phylogenetically quite closely related). Thus, it is possible that both parent channels may carry similar “inactivation” motifs in domains II, III, and IV but differ predominantly in domain I. In our case, due to the lower degree of overall homology shared by \( \alpha_{1E} \) and \( \alpha_{1C} \) channels, the regions critical for inactivation might perhaps be more divergent, thus revealing the contributions of additional domains to the overall inactivation process. A second fundamental difference between the present study and that of Zhang et al. lies in the type of transient expression system used, with Zhang et al. (29) using Xenopus oocytes as compared with the HEK cells in our experiments. It is well established that the type of host system frequently affects the functional and pharmacological properties of transiently expressed ion channels (e.g. Ref. 42), and it will be interesting to examine the properties of our chimeras in Xenopus oocytes. Finally, our data do support some contribution of domain I in the overall inactivation properties, since substitution of \( \alpha_{1C} \) sequence in domain I with that corresponding to \( \alpha_{1E} \) mediated a 10–15-mV negative shift in half-inactivation potential.

Two groups have pinpointed individual amino acid residues in the domain I–II linker as critical determinants of voltage-dependent inactivation. An alternative splice variant of the rat brain \( \alpha_{1L} \) channel that carries a single valine insertion in the domain I–II linker completely lacks voltage-dependent inactivation (7, 11, 31). Herlitze et al. (35) identified a single amino acid residue in the domain I–II linker of \( \alpha_{1A} \) that can confer positive inactivation properties onto L-type calcium channels. In the present study, the domain I–II linker was always associated with domain II. Thus, although Zhang et al. (29) showed that exchanging the I–II linker region between doe-I and \( \alpha_{1A} \) did not affect inactivation rate, we cannot rule out that the effects that we attribute to “domain II” may be contained in part, in the domain I–II linker rather than the actual domain II region per se. The same consideration applies in principle to domain III and the carboxyl-terminal region. Ultimately, swapping of individual cytoplasmic linker regions will be required to elucidate any putative contributions of the cytoplasmic linkers to voltage-dependent inactivation properties.

What Might Be the Molecular Mechanism of Fast \( \text{Ca}^{2+} \) Channel Inactivation?—Our observation that each of the four transmembrane domains appeared to affect voltage-dependent inactivation might suggest that calcium channel inactivation involves a complex global conformational change in the channel protein. Most of the structures or amino acid residues that have been linked to changes in voltage-dependent inactivation of various types of calcium channels have been located to the S6 regions in domains I, III, and IV (29, 32–34, 43), or to cytoplasmic regions directly linked to these S6 segments such as the domain I–II linker or the carboxyl-terminal region (36, 44). In addition, cytoplasmic proteins such as ancillary \( \beta \) subunits and syntaxin that physically bind to the domain I–II linker (45) and II–III linker regions, respectively, have been shown to affect voltage-dependent inactivation properties. In view of our current understanding of the slower C-type inactivation process in certain types of voltage-dependent potassium channels (46–51), we hypothesize that voltage-dependent inactivation of calcium channels could perhaps involve a physical constriction of the pore. Similar to what has been proposed to occur during C-type inactivation of potassium channels, the cytoplasmic ends of the S6 segments might come together to form an inverted teepee structure during inactivation, thereby preventing the passage of permeant ions through the pore. Such a mechanism could account for the previously reported mutagenesis data in both the S6 regions and the associated linker regions as well as for the effects of protein interactions with the linker regions. For example, the structural changes in the linker regions might affect the mobility or flexibility of the associated S6 regions and thus the overall inactivation properties. Furthermore, in such a model, one would expect to observe some contribution from each transmembrane domain as reported here. Hence, while we cannot rule out the possibility that the four transmembrane domains might cooperatively form a docking site for a yet to be identified inactivation gate particle, a mechanism similar to that proposed to underlie C-type inactivation of potassium channels is an attractive possibility. Construction of additional chimeras and/or site-directed mutagenesis will be required, however, to further support such a hypothesis. Overall, irrespective of the detailed molecular mechanisms involved, our data are consistent with the notion that voltage-dependent inactivation of \( \alpha_{1C} \) calcium channels is complex process that involves multiple structural domains and thus differs fundamentally from fast inactivation of sodium and potassium channels.

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