Identification of Inactivation Determinants in the Domain IIS6 Region of High Voltage-activated Calcium Channels*

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We have recently reported that transfer of the domain IIS6 region from rapidly inactivating R-type (α₁R) calcium channels to slowly inactivating L-type (α₁L) calcium channel confers rapid inactivation (Stotz, S. C., Hamid, J., Spaetgens, R. L., Jarvis, S. E., and Zamponi, G. W. (2000) J. Biol. Chem. 275, 24575–24582). Here we have identified individual amino acid residues in the IIS6 regions that are responsible for these effects. In this region, α₁L and α₁E channels differ in seven residues, and exchanging five of those residues individually or in combination did not significantly affect inactivation kinetics. By contrast, replacement of residues Phe-823 or Ile-829 of α₁C with the corresponding α₁E residues significantly accelerated inactivation rates and, when substituted concomitantly, approached the rapid inactivation kinetics of R-type channels. A systematic substitution of these residues with a series of other amino acids revealed that decreasing side chain size at position 823 accelerates inactivation, whereas a dependence of the inactivation kinetics on the degree of hydrophobicity could be observed at position 829. Although these point mutations facilitated rapid entry into the inactivated state of the channel, they had little to no effect on the rate of recovery from inactivation. This suggests that the development of and recovery from inactivation are governed by separate structural determinants. Finally, the effects of mutations that accelerated α₁C inactivation could still be antagonized following co-expression of the rat β₂L subunit or by domain I–II linker substitutions that produce ultra slow inactivation of wild type channels, indicating that the inactivation kinetics seen with the mutants remain subject to regulation by the domain I–II linker. Overall, our results provide novel insights into a complex process underlying calcium channel inactivation.

The voltage-dependent inactivation of ionic channels is a fundamental biological process that prevents the breakdown of ionic gradients and determines action potential duration and the refractory period of excitable tissues (2). In calcium channels, this process serves several unique purposes. First, it is a key mechanism by which calcium channels are able to achieve a tight regulation of internal calcium levels and thus the temporal precision of calcium signals. This is of particular significance in view of the pivotal role calcium plays as a cytoplasmic messenger (3, 4). Along these lines, calcium channel inactivation helps to prevent the build up of excessive and cytotoxic levels of intracellular calcium (5, 6). Furthermore, the inactivation of calcium currents in nerve terminals appears to contribute to the short term depression of neurosecretion (7, 8). Finally, a number of pharmacological agents, including many clinically used calcium channel therapeutics, preferentially interact with inactivated channels (9, 10). Thus, understanding the molecular determinants that govern this process is of fundamental importance.

Whereas the basic principles underlying fast inactivation of voltage-gated sodium and potassium channels have been elucidated (for review see Ref. 11), the detailed mechanisms underlying the voltage-dependent inactivation of calcium channels have remained somewhat enigmatic. In potassium channels, an ∼20-amino acid cluster of residues in the N-terminal region acts to physically occlude the channel pore (12–14), thus preventing potassium influx (termed ball and chain mechanism). In sodium channels, the cytoplasmic loop between domains III and IV of the α subunit serves as a pore-occluding inactivation particle (termed hinged-lid mechanism) (15–17). In voltage-gated calcium channels, it has been known for some time that the β subunit that interacts with the α subunit domain I–II linker region exerts a pronounced modulatory effect on inactivation kinetics (18–22), and there is now direct evidence that this region is indeed an important determinant of the inactivation process (1, 23–25). These results suggested that the domain I–II linker structure may perhaps serve as a physical inactivation gate (1, 26, 27). However, point mutations in a number of locations throughout the calcium channel sequence can exert dramatic effects on inactivation rates (28–32), indicating that inactivation may be more complex than just a simple pore plug mechanism. We have recently reported that multiple transmembrane domains contribute to the inactivation process (33). Furthermore, our work has revealed that insertion of either the domain IIS6 or the domain IIIS6 region of rapidly inactivating R-type (α₁E) channels into normally slowly inactivating L-type (α₁C) calcium channels is sufficient to induce rapid inactivation of L-type channels (1). Here we have identified a pair of amino acid residues in the domain IIIS6 region that are key inactivation determinants of α₁C-Phe-823 and Ile-829. We show that amino acid substitutions in these two positions to the corresponding α₁E residues are capable of inducing rapid, α₁E-like inactivation rates. A systematic replacement of these residues with a series of amino

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acids reveals amino acid side chain size in position 823 as a major inactivation determinant, with the \( \alpha_{1C} \) F823A mutant producing inactivation kinetics that approach those of T-type calcium channels. Our data, however, also show that this effect can be greatly attenuated by either coexpression of the \( \beta_{2a} \) subunit or by manipulation of the domain I–II linker structure. Our data are consistent with a mechanism in which the domain I–II linker may serve as a physical inactivation gate structure that functionally interacts with the pore lining S6 segments to induce inactivation.

**MATERIALS AND METHODS**

**Molecular Biology**—We have previously engineered a series of silent restriction enzyme sites into the wild type rat brain \( \alpha_{1C} \) subunit in the pMT2 expression vector (1, 33). This construct was modified further as follows. An SpeI site in the 3′-untranslated region of the channel was eliminated using the QuickChange Site-directed Mutagenesis Kit (Stratagene) using the complete expression construct as the template. An ~2.2-kilobase pair SbfI-NcoI fragment containing the mutation was then excised and reintroduced into the original full-length pMT2/\( \alpha_{1C} \) cDNA. The mutated SbfI-NcoI fragment was completely sequenced to confirm the removal of the SpeI site and the absence of errors. By using a similar strategy, a silent MutI site was then introduced into this modified construct 129 bp upstream of the domain IIS6 region. Subsequently, an ~1-kilobase pair BseGI-SpeI fragment containing the silent MutI site was isolated and then reintroduced into the construct lacking the SpeI site in the 3′-untranslated region. The BseGI-SpeI fragment was sequenced in its entirety to confirm the presence of the MutI site and the absence of other errors. The resulting pMT2/\( \alpha_{1C} \) cDNA construct encoding a wild type \( \alpha_{1C} \) channel thus carried a unique MutI site 129 bp upstream of the IIS6 region, and an endogenous unique SpeI site 570 bp 3′ to the end of the segment, and was used as the template for all mutagenesis of residues of the IIS6 region. When possible, each further change incorporated the silent addition or elimination of a diagnostic restriction site such that mutated sequence could be readily identified via restriction enzyme digestion. SpeI sites were used in the creation of V815S and C816A, FokI for I821V, AflIII for I829T, BglII for F823Q, F823E, and F823K, and NdeI for F823T, F823S, F823C, F823Y, F823S, F823D, F823R, F823A, F823I, and F823V. If a diagnostic restriction site could not be easily designed, the mutations were confirmed by immediate sequencing (ABI Prism BigDye Terminating Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems); University of Calgary Sequencing). This was necessary in the creation of I824L and C816A, 

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\begin{align*}
\text{\textit{Table I}}\text{ Summary of half-activation and half-inactivation potentials for domain IIS6 mutants}\\
\begin{array}{cccc}
\text{Construct} & V_{1/2,activation} & n & V_{1/2, inactivation} & n \\
\hline
\alpha_I & -0.43 & \pm 1.72 & 12 & -21.23 & \pm 1.70 & 10 \\
\alpha_{1C}(IIS6) & -9.43 & \pm 2.24^a & 8 & -32.67 & \pm 0.89^a & 7 \\
\alpha_{1E} & -19.70 & \pm 2.77^a & 8 & -79.80 & \pm 0.90^a & 8 \\
V815S & -3.01 & \pm 1.18 & 10 & -20.42 & \pm 1.73 & 9 \\
C816A & -3.81 & \pm 1.39 & 12 & -24.69 & \pm 1.59 & 8 \\
I821V & -0.65 & \pm 1.85 & 8 & -12.51 & \pm 0.15^a & 4 \\
F823T & -4.18 & \pm 1.11 & 20 & -27.57 & \pm 1.31 & 17 \\
I824L & -6.12 & \pm 1.43^a & 16 & -24.51 & \pm 3.18 & 6 \\
C825F & -0.04 & \pm 1.52 & 16 & -26.37 & \pm 1.92 & 10 \\
I829T & -20 & \pm 0.97 & 14 & -29.99 & \pm 1.39^a & 14 \\
F823T/F823T & -2.40 & \pm 1.33 & 9 & -29.49 & \pm 2.21 & 6 \\
\text{Quintuple mutant} & -2.69 & \pm 1.56 & 10 & -26.88 & \pm 1.67 & 7 \\
\end{array}
\end{align*}
\]

\( ^a \) Statistical significance \( p < 0.05 \) relative to \( \alpha_{1C} \) control is shown. Values are significantly different from \( \alpha_{1C} \), \( p < 0.05 \) (Student's t test).

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for 24–72 h, and then individual coverslips were transferred to a 3-cm culture dish for electrophysiological recordings.

**Patch Clamp Recordings**—Cells were bathed in a recording solution consisting of 20 mM BaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, 40 mM tetraethylammonium chloride, 10 mM glucose, 65 mM CsCl (pH 7.2 with tetraethylammonium-OH). Whole cell patch clamp recordings were performed with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) connected to a personal computer equipped with pCLAMP version 6.0. Patch pipettes (Sutter borosilicate glass, BF150-86-15) were pulled using a Sutter P-87 puller and fire-polished using a drive of the computer. Series resistance and capacitance were compensated by the use of barium as the carrier ion allows us to isolate voltage-dependent inactivation without contamination from calcium-sensitive inactivation (33). Data were filtered at 1 kHz and recorded directly onto the hard drive of the computer. Series resistance and capacitance were compensated, but no on-line leak subtraction was used. Unless stated otherwise, currents were evoked by stepping from −100 mV to various test potentials. Steady state inactivation curves were obtained by maintaining the cells at various holding potentials for 5 s prior to stepping to a test depolarization of +10 mV. Recovery from inactivation was determined by providing a conditioning pulse from a holding potential of −100 mV to +10 mV for 1 s followed by a test pulse of 10 mV for 40 ms. The time interval between the conditioning pulse and the test pulse was increased in 10-ms increments until the 100-ms interval point. Hereafter, the duration between conditioning pulse and the test pulse was increased in 100-ms intervals. In the period between the conditioning pulse and the test pulse, the holding potential was returned to −100 mV.

The raw data were analyzed using Clampfit software. The time course of inactivation was fitted monoexponentially, and as a second measure of inactivation rate, the percentage of inactivation that had developed during a 125-ms test depolarization was used. Steady state inactivation curves were fitted with standard Boltzmann relations using Sigmaplot software (Jandel Scientific). Half-activation potentials were estimated from the whole cell current voltage relations by means of Boltzmann fits. The time course of recovery from inactivation was obtained by biexponential fits to the amplitude normalized data or, in the case of $\alpha_{1E}$, with a monoexponential fit. All figures were generated in Sigmaplot. Numbers in parentheses reflect individual experiments that typically were obtained in multiple transfections. Error bars given reflect standard errors. Student’s $t$ tests were carried out between wild type data and individual mutations within the IIS6 segment to ascertain the statistical significance of the results ($p < 0.05$). To determine the significance of differences in data obtained in the characterization of IIS6 positions Phe-823 and Ile-829 one-way ANOVAs were performed and followed by the appropriate post-ANOVA test (Student-Newman-Keuls method, $p < 0.05$).

**RESULTS**

Phe-823 and Ile-829 in the $\alpha_{1C}$ Domain IIS6 Region Are Key Inactivation Determinants—We have recently reported that replacement of the domain IIS6 region of the (L-type) $\alpha_{1C}$ subunit with the corresponding region in $\alpha_{1E}$ was sufficient to confer the rapid inactivation rates seen with $\alpha_{1E}$ (1). As illustrated in Fig. 1, $A$ and $B$, the $\alpha_{1C}$(IIS6E) chimera displays rapid inactivation kinetics that qualitatively approach those of wild type $\alpha_{1E}$ channels. The domain IIS6 regions of these two chan-
nel isoforms contain 25 amino acid residues, of which 18 are completely conserved (Fig. 1C), indicating that one, or a combination of the remaining seven residues, must be responsible for the effects observed in Fig. 1, A and B. To determine the role of each of these seven amino acids, we individually replaced $\alpha_{1C}$ residues of the IIS6 regions with the corresponding residues found in $\alpha_{1E}$, and we then assessed their effects on inactivation. As in our previous work (1), the rate of inactivation was determined either from monoexponential fits to the decaying phase of the raw current traces or from the fraction of current that has inactivated during a 125-ms test depolarization. In addition, for each mutant, the half-activation potential was estimated from whole cell current-voltage relations, and the half-inactivation potential was determined from steady state inactivation curves (see Table I).

Figs. 2 and 3 illustrate the effects of individual point mutations in the domain IIS6 region of $\alpha_{1C}$. Substitutions of residues Val-815, Cys-816, and Cys-825 did not significantly affect inactivation of $\alpha_{1C}^{1E} + \beta_{1A} + \alpha_{2E}$ calcium channels, whereas mutagenesis of residue Ile-821 to the corresponding $\alpha_{1E}$ Residue (valine) appeared to produce a significant slowing of inactivation rather than acceleration toward $\alpha_{1E}$-like kinetics. Mutagenesis of residue 824 to leucine statistically and significantly increased the net percentage of inactivation that occurred over 125 ms (see Fig. 3A for detail); however, this was due to a slightly smaller non-inactivating fraction of the $\alpha_{1C}$ current. The time constant of inactivation of this mutant did not differ significantly from that observed with wild type channels (Fig. 3B), suggesting that this position contributes only in a minor fashion to the observed differences between $\alpha_{1C}$ and $\alpha_{1E}$ calcium channels. By contrast, replacement of residues Phe-823 or Ile-829 with the threonines found in the corresponding positions in $\alpha_{1E}$ mediated a statistically significant ($p < 0.05$) acceleration of inactivation kinetics, which was maintained over a wide range of test potentials but which did not completely approach those seen with wild type $\alpha_{1C}$ calcium channels or the $\alpha_{1C}(\text{IIS6E})$ chimera. Hence, no single residue was able to account for the observations obtained with the $\alpha_{1C}(\text{IIS6E})$ chimera.

To determine if the effects of the F823T and I829T substitutions were additive, we generated a double $\alpha_{1C}(\text{F823T/I829T})$ mutant and examined its inactivation profile. As seen from Fig. 4A, the double mutant displayed rapid inactivation kinetics that did not differ significantly from the $\alpha_{1C}(\text{IIS6E})$ chimera or wild type $\alpha_{1E}$ calcium channels over a wide range of test potentials, indicating that the combination of these two residues is sufficient to confer rapid $\alpha_{1E}$-like inactivation kinetics onto $\alpha_{1C}$. To corroborate our data further, we produced a quintuple mutant in which all of the domain IIS6 residues of $\alpha_{1C}$ except for Phe-823 and Ile-829 were mutated to the corresponding $\alpha_{1E}$ residues. As shown in Fig. 4, B and C, this mutant produced inactivation rates that did not differ significantly from those of wild type $\alpha_{1C}$ channels, thereby further supporting the notion that only residues 823 and 829 are responsible for the effects of the $\alpha_{1E}$ domain IIS6 region on L-type calcium channel inactivation.

As shown in Table I, the individual substitutions mediated only minor effects on the voltage dependence of activation, indicating that the observed effects did not secondarily arise from a change in activation gating. Furthermore, with the exception of the I829T construct, the half-inactivation potentials observed with the mutant channels did not differ significantly from those of the wild type $\alpha_{1C}$ channels. The observation that the F823T/I829T double mutant inactivated with a time course comparable to $\alpha_{1E}$ channels, but displayed ~50 mV more positive half-inactivation potential, indicates that the rate and voltage dependence of inactivation can be governed by separate structural determinants (but see below).

The Inactivation Rate of $\alpha_{1C}$ Is Dependent on the Size of Residue 823—To gain a more detailed understanding of the roles of residues 823 and 829 in the inactivation process, we replaced these residues with a number of different amino acids of varying size and polarity, and we examined their effects on the functional properties of the channel. Fig. 5 shows the effects of substitutions of Phe-823 with a number of non-polar amino acid residues. As seen from the figure, replacement of the phenylalanine with either glycine or alanine results in very rapid inactivation kinetics that approach the rates seen with T-type calcium channels (34–36). Hence, a single amino acid substitution in a slowly inactivating channel is sufficient to accelerate inactivation by almost an order of magnitude. More importantly, there appears to be a clear dependence of the inactivation rates on the size of the amino acid side chain, with smaller residues resulting in faster kinetics than bulkier side chains.

Fig. 6 examines the effects of polar substitutions on the rate of inactivation. As with the non-polar residues, increasing the size of polar groups resulted in a slowing of inactivation. As evident from the summary of the data in Fig. 7, residues with similar size, but differing polarities, yielded similar inactivation rates, indicating that polarity per se is not a major factor in setting the rates of inactivation. However, the presence of a hydrophilic residue in this position appeared to result in rapid inactivation kinetics irrespective of charge or size (Fig. 7), possibly indicating that side chain size, while clearly impor-
tant, is not the sole inactivation determinant in position 823 and that charge per se is not a factor. This result could be consistent with a mechanism in which residue 823 might sterically interfere with gate closure or, alternatively, that this residue could be involved in a conformational change of the channel during the inactivation process.

We carried out a similar analysis of position 829 (Fig. 8). Although increasing side chain size among polar residues appeared to decrease the rate of inactivation, a similar trend could not be observed among the non-polar substitutions, and residues of similar size (i.e. valine and threonine) differentially affected inactivation rate (Fig. 8A). In contrast, the rate of inactivation appeared to show a better correlation with the degree of hydrophobicity of the substituent in position 829 (Fig. 8B). Thus, despite the apparent size dependence seen with the polar residues, hydrophobicity may be the true determining

**Fig. 4.** Effect of concomitant residue substitutions on $\alpha_{1c}$ inactivation. A, current records and rates of inactivation obtained with the F823T/I829T double mutant. Note that this mutant approaches the inactivation rates observed with the $\alpha_{1c}$(IIIS6E) chimera over a wide range of test potentials. B, concomitant substitution of the remaining five residues that differ in the IIIS6 regions of the wild type channels does not significantly accelerate inactivation of $\alpha_{1c}$. The conditions in A and B were analogous to those in Fig. 2, and data obtained for $\alpha_{1c}$ and the $\alpha_{1c}$(IIIS6E) chimera are shown in the form of solid circles and gray squares, respectively. C, comparison of the effects of the double and quintuple mutants relative to the wild type $\alpha_{1c}$ channels and the $\alpha_{1c}$(IIIS6) mutant at a test potential of +10 mV. Note that the inactivation rates obtained with the quintuple and double mutants closely resemble those of wild type $\alpha_{1c}$ and $\alpha_{1c}$(IIIS6E) channels, respectively. Error bars denote S.E., numbers in parentheses reflect numbers of experiments, and asterisks denote statistical significance ($p < 0.05$) relative to the $\alpha_{1c}$ control.

**Fig. 5.** Effect of substitution of $\alpha_{1c}$ residue 823 for a series of non-polar residues, arranged in rows according to increasing side chain size. The outline of the figure parallels that of Fig. 3. Note that with increasing side chain size, the rate of inactivation becomes slowed. Also note the particularly rapid inactivation kinetics seen with the F823A mutant that occurs over a wide range of test potentials. Records were obtained as outlined in Figs. 1 and 2, and error bars denote S.E.
factor underlying the importance of this residue in determining the inactivation rate.

Several of the mutations resulted in small (5–10 mV) hyperpolarizing shifts in half-activation potentials (Table II). It is unlikely that these shifts were secondarily responsible for the observed changes in inactivation rates since the effects on inactivation were observed over a wide range of test potentials. In addition, a number of mutants with fast inactivation kinetics did not display a change in the voltage dependence of activation (e.g. F823S). Interestingly, a number of mutations that speeded inactivation also induced small hyperpolarizing shifts in half-inactivation potential (Table II), although they did not approach the negative inactivation range seen with wild type $\alpha_{1C}$ channels. This suggests that there could be some overlap in the determinants that underlie the rate and voltage dependence of inactivation.

The Effects of IIS6 Mutations Are Modulated by the Domain I–II Linker Region—We have previously suggested that the domain I–II linker region mediates a key role in the inactivation process (1, 27). If so, then manipulations of the domain I–II linker region that slow the kinetics of wild type calcium channels should be able to override the effects of domain IIS6 mutations that speed inactivation. It has been well documented that the rat $\beta_{2\alpha}$ subunit mediates a dramatic slowing of the inactivation kinetics of high voltage-activated calcium channels (21–37). The $\beta_{2\alpha}$ subunit effect is dependent on palmitoylation and membrane insertion of the $\beta_{2\alpha}$ N terminus (22, 26, 38, 39) which may result in restricted mobility of the domain I–II linker region of the channel (27). To determine whether the effects of the domain IIS6 mutations could occur independently of the domain I–II linker region, we therefore determined the inactivation kinetics of the $\alpha_{1C}(F823A)$ mutant in the presence of the rat $\beta_{2\alpha}$ subunit. As shown in Fig. 9, A and C, the presence of this subunit mediated a dramatic slowing of inactivation kinetics compared with channels coexpressed with $\beta_{1\alpha}$, indicating that the inactivation kinetics of $\alpha_{1C}(F823A)$ remain subject to $\beta$ subunit regulation.

During our previous work, we had created a chimeric calcium channel in which the first 80% of the domain I–II linker of $\alpha_{1C}$ was replaced by $\alpha_{1B}$ sequence (CecCCC), and we observed that this channel displayed ultra slow inactivation kinetics. Compared with wild type $\alpha_{1C}$ channels, the CecCCC chimera displayed a negative half-activation potential ($V_{1/2,act} = -15.6 \pm 0.4$, $n = 6$) indicating that the I–II linker region may perhaps contribute to the voltage dependence of L-type calcium channel activation gating, whereas the channel displayed a "normal" half-inactivation potential ($V_{1/2,inact} = -22.8 \pm 1.8$, $n = 5$). To support further the idea that the overall effects of the IIS6 linker mutations are dependent on the domain I–II linker region, we introduced the F823A mutation into the CecCCC construct. As shown in Fig. 9, B and C, the CecCCC environment remained capable of slowing the overall inactivation kinetics of the channel despite the presence of the F823A mutation. Together with the results shown in Fig. 9A, this supports the notion that the total degree of inactivation seen with the mutation in the domain IIS6 regions is critically dependent on the nature of the domain I–II linker and that the domain I–II linker region is a key structural element involved in fast calcium channel inactivation. The data, however, also show that the F823A substitution remains capable of overriding in part the slowing of inactivation introduced by coexpression of $\beta_{2\alpha}$ and/or manipulation of the domain I–II linker, indicating that the effects mediated by the I–II linker and position 823 occur independently of each other.

Speeding of Inactivation Is Not Paralleled by Faster Recovery from Inactivation—To gain additional mechanistic insights into the role of residue 823 in the inactivation process, we

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Footnote:

$^a$ S. C. Stotz, J. Hamid, and G. W. Zamponi, unpublished observations.
examined the recovery from inactivation for both wild type channels, as well as a selected set of mutants with varying effects on inactivation. Recovery from inactivation of wild type \( \alpha_{1C} \) channels was best described by a double exponential fit, yielding a rapid recovery phase with a time constant of 23.2 ± 4.6 ms, followed by a slower component with a time constant on the order of 450 ms. In contrast, wild type \( \alpha_{1E} \) calcium channels appeared to display only a single time constant for recovery from inactivation of 235.5 ± 42.5 ms (Fig. 10). Perhaps not unexpectedly, the quintuple IIS6 mutant that displayed \( \alpha_{1C} \)-like inactivation kinetics displayed recovery kinetics that required a double exponential fit with time constants that did not differ significantly from those of the wild type channels. Interestingly, however, as shown in Fig. 10, examination of a subset of mutations that speeded the inactivation of \( \alpha_{1C} \) channels (such as F823A or the F823T/I829T double mutant) reveals a requirement of a double exponential fit with time constants that did not differ significantly from those of the wild type channels. Interestingly, however, as shown in Fig. 10, examination of a subset of mutations that speeded the inactivation of \( \alpha_{1C} \) channels (such as F823A or the F823T/I829T double mutant) reveals a requirement of a double exponential fit with time constants that did not differ significantly from those of the wild type channels. Interestingly, however, as shown in Fig. 10, examination of a subset of mutations that speeded the inactivation of \( \alpha_{1C} \) channels (such as F823A or the F823T/I829T double mutant) reveals a requirement of a double exponential fit with time constants that did not differ significantly from those of the wild type channels. Interestingly, however, as shown in Fig. 10, examination of a subset of mutations that speeded the inactivation of \( \alpha_{1C} \) channels (such as F823A or the F823T/I829T double mutant) reveals a requirement of a double exponential fit with time constants that did not differ significantly from those of the wild type channels. Interestingly, however, as shown in Fig. 10, examination of a subset of mutations that speeded the inactivation of \( \alpha_{1C} \) channels (such as F823A or the F823T/I829T double mutant) reveals a requirement of a double exponential fit with time constants that did not differ significantly from those of the wild type channels.
Our data show that the effects of substituting the entire domain IIS6 region of α_{1C} with α_{1K} is exclusively due to residues 823 and 829, whereas even concomitant substitution of the remaining five residues did not accelerate α_{1C} inactivation kinetics. In high voltage-activated non-L-type channels (including marine ray doe I channels), these two residues are highly conserved threonines, and thus, the chimeric approach employed by Zhang et al. (28) would not have detected this region as a key inactivation determinant. Although substitution of these two residues could dramatically accelerate the time course of inactivation, there was no significant effect on the rate of recovery from inactivation, and for some of the mutants (e.g. F823T/I829T) there was no change in the voltage dependence of inactivation. Indeed, even for the mutants with the fastest inactivation behavior (F823A and I829S), the rates of recovery from inactivation were not affected, further supporting the notion that these two residues do not participate in the stabilization of the inactivated state. Thus, there appears to be a selective role of residues 823 and 829 in controlling the access to the inactivated conformation that can (under certain circumstances) be functionally uncoupled from the voltage dependence of steady state inactivation.

Although the domain IIS6 region is an important determinant of inactivation, it is clearly not the only one. Based on our previous work (1) and the work of others (28, 31, 32), it is likely that the S6 segments in domains I, III, and IV also play important roles in the inactivation process. For example, it was recently shown that replacement of α_{1C} domain IVS6 residues with corresponding α_{1K} residues can result in accelerated inactivation kinetics (32), and as stated above, the presence of the entire domain IIS6 segment of α_{1K} is sufficient to induce rapid inactivation in α_{1C} (1). It will therefore be important to investigate systematically the contributions of individual residues in these regions to the overall inactivation process. Moreover, we note that a chimeric approach does not permit the identification of contributions from conserved residues. Since all high voltage-activated calcium channels contain highly conserved residues near the cytoplasmic end of the domain IIS6 region (see Fig. 1) as well as in the other S6 segments (40), it is possible that they may also mediate important roles in inactivation (see below). However, future site-directed mutagenesis studies will be required to test this possibility.

### Table II

| Construct | \( V_{1/2, \text{activation}} \) | \( V_{1/2, \text{inactivation}} \) |
|-----------|------------------|------------------|
| F823G     | -8.04 ± 1.35 \( ^a \) | -10.28 ± 2.48 \( ^b \) |
| F823A     | -7.78 ± 1.22 \( ^a \) | -9.43 ± 2.32 \( ^b \) |
| F823V     | -6.94 ± 0.35 \( ^a \) | -7.22 ± 3.03 \( ^b \) |
| F823I     | -5.83 ± 0.55 \( ^a \) | -6.85 ± 4.67 \( ^b \) |
| F823S     | -5.91 ± 3.40 \( ^a \) | -3.97 ± 2.01 \( ^b \) |
| F823C     | -5.08 ± 1.42 \( ^a \) | -10.35 ± 2.01 \( ^b \) |
| F823Q     | -3.67 ± 1.43 \( ^a \) | -29.29 ± 0.97 \( ^b \) |
| F823Y     | -3.00 ± 1.86 \( ^a \) | -23.15 ± 2.44 \( ^b \) |
| F823D     | -4.33 ± 0.43 \( ^a \) | -31.11 ± 1.04 \( ^b \) |
| F823E     | -2.01 ± 1.68 \( ^a \) | -30.30 ± 1.13 \( ^b \) |
| F823K     | -3.20 ± 1.10 \( ^a \) | -29.77 ± 1.63 \( ^b \) |
| F823R     | -9.66 ± 2.11 \( ^a \) | -33.58 ± 2.77 \( ^b \) |
| I829G     | -4.41 ± 1.77 \( ^a \) | -28.13 ± 2.62 \( ^b \) |
| I829A     | -9.96 ± 0.80 \( ^a \) | -28.12 ± 7.77 \( ^b \) |
| I829V     | -2.41 ± 1.48 \( ^a \) | -24.14 ± 2.77 \( ^b \) |
| I829F     | -5.86 ± 1.34 \( ^a \) | -30.96 ± 3.46 \( ^b \) |
| I829S     | -11.36 ± 1.06 \( ^a \) | -38.58 ± 1.72 \( ^b \) |
| I829Y     | -2.33 ± 1.94 \( ^a \) | -25.18 ± 2.56 \( ^b \) |

\( ^a \) Statistical significance relative to α_{1C} controls is shown. Values are significantly different from α_{1C}, \( p < 0.05 \) (ANOVA).

\( ^b \) Values are significantly different from α_{1C}, \( IIS6E \), \( p < 0.05 \) (ANOVA).

**Possible Models for Calcium Channel Inactivation**—We have recently reported (1) that replacement of the α_{1C} domain I–II linker region with the α_{1K} sequence is sufficient to produce a significant speeding of inactivation. Based on this observation and on the data of Cens et al. (41), we have suggested that the domain I–II linker may serve as a physical inactivation gate that functionally interacts with the domain II and IIS6 regions (27). Our observations with the CecCCC chimera provides further support for a key involvement of the domain I–II linker region in the inactivation process. It is interesting to note that mutations described previously (29–31) in the I–II linker regions that affected inactivation rates occur much closer to the N-terminal end of the linker. The rapidly inactivating CecCCC chimera differs from the CecCCC construct described here only in the last 18 residues of the domain I–II linker, and yet, inactivation of the latter construct is dramatically slowed compared with CecCCC (1). This hints at the possibility that the domain I–II linker may contain multiple functionally important regions that are involved in inactivation, including both its rate and its voltage dependence.

At the same time, our data show that individual amino acid residues in the domain IIS6 regions are critical modulators of inactivation rates. This raises the question as to what might be the functional relation between the domain I–II linker and the S6 segments. We envision two possible models. First, the do-

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** FIG. 9. Effect of the domain I–II linker structure on inactivation of the F823A mutant.** A, current records illustrating the effects of coexpression of the F823A mutation. In both cases, the I829Y mutant results in a channel with ultra slow inactivation, which can be accelerated by introducing the F823A mutation. In both cases, the I829Y and α_{1A} subunits were coexpressed. Current records in A and B were obtained at a test potential of +10 mV. C, summary of the effects shown in A and B in bar graph form for a number of experiments (\( n \) values are indicated in parentheses). The test potential was +10 mV, and error bars denote S.E., and asterisks denote significance (\( p < 0.05 \)).
main I–II linker region could form a physical pore plug that enters the internal vestibule of the channel and directly interacts with residues Phe-823, Ile-829, and possibly other residues in the domain II S6 region, as well as with residues contained within the remaining S6 segments. In such a scenario, the importance of the size dependence on inactivation rate seen with substitutions of residue 823 could arise from steric interference with the docking of the linker to its receptor site. On the other hand, residue 829 may influence inactivation rate by participating in hydrophilic interactions with the I–II linker structure. However, in potassium channels whose crystal structure has been resolved, residues in the S6 segment corresponding to the position of α1C residue 823 are located immediately adjacent to the selectivity filter of the channel (42, 43). Provided that the geometry of the inner vestibule of calcium channels is not radically different from that of potassium channels, then it would seem unlikely that the domain I–II linker would be able to penetrate rapidly the inner vestibule of the pore all the way to the level of the selectivity filter to interact with these specific residues, given that this region associates with the large cytoplasmic β subunit (44) and, in some cases, G protein βγ subunits (45, 46).

A second and in our view more likely possibility could be that the domain I–II linker might dock to one or perhaps all of the four S6 segments at their cytoplasmic end. This docking process could then result in a conformational change in the S6 segments that may ultimately result in pore closure. Alternatively, membrane depolarization could induce a conformational change in the S6 segments that generate a binding/blocking site for the domain I–II linker at the inner mouth of the channel (see Fig. 11). In this scenario, the decrease in inactivation rate that occurs upon increasing the size of residue 823 or increasing the hydrophobicity of residue 829 could affect the rate by which these conformational changes in the S6 segments may occur, but these residues would not necessarily have to be involved in the physical interaction with the gate structure. Our observation (Fig. 10) that recovery from inactivation was not affected in mutants that displayed accelerated rates of inactivation would also be consistent with such a mechanism, as the mutations could promote the availability of the docking site for the I–II linker in response to membrane depolarization, without affecting the stability of the binding interaction. Furthermore, the scenario described in Fig. 11 could account for the observation that, although the F823A mutation accelerated inactivation kinetics, a concomitant manipulation of the domain I–II linker region could still slow the inactivation of this mutant. Within the framework of such a model, the high degree of conservation of residues near the cytoplasmic ends of the four S6 segments also raises the intriguing possibility that the four S6 segments could provide the inactivation gate with a choice of as many as four similar receptor sites. This would fit nicely with the observation that insertion of either the domain II S6 or III S6 region of α1E into α1C is sufficient to confer rapid inactivation kinetics in a non-additive manner (1). However, more detailed structural information about the inner vestibule of the calcium channel pore will be required to substantiate further this possibility.

Nonetheless, our data provide several novel insights into the molecular basis of calcium channel inactivation and further support the notion that calcium channel inactivation is a highly complex process that involves the concerted interaction of different mechanisms. Besides the identification of novel channel structural determinants underlying the inactivation process, our work also indicates that individual features of inactivation such as its development, voltage dependence, and reversibility can be functionally uncoupled. The notion that a single amino acid substitution can dramatically accelerate inactivation kinetics in slowly inactivating channels suggests that the basic inactivation machinery is highly conserved across different

![Fig. 10. Time constants for recovery from inactivation, $\tau$ recovery, obtained with wild type and mutant channels (coexpressed with β1b and α1E). With exception of wild type α1E calcium channels, the time course of recovery from inactivation was fitted with two exponentials yielding a fast and a slow component. None of the mutants differed significantly from wild type α1E calcium channels in their recovery kinetics (p < 0.05). Error bars denote S.E., and numbers in parentheses indicate numbers of experiments.](http://www.jbc.org/)

![Fig. 11. Possible model of voltage-dependent inactivation of calcium channels. In response to membrane depolarization, the S6 segments might undergo a conformational change which unmask one or more binding sites (indicated as stars) for the inactivation gate (formed by the domain I–II linker). Certain mutations in the S6 regions (indicated by hexagons) could aid the conformational changes in these segments, thus promoting the availability of the gate receptor site and speeding inactivation. Manipulation of the domain I–II linker region might interfere with its ability to bind effectively to the docking site. The rate-limiting step for recovery from inactivation would be the dissociation of the I–II linker from the docking site and would not be affected by the mutations in the S6 segment.)](http://www.jbc.org/)
types of calcium channels, thus underlining the significance of the inactivation process in cellular function.

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