Molecular Determinants of Voltage-dependent Slow Inactivation of the Ca$^{2+}$ Channel*

Chengzhang Shi and Nikolai M. Soldatov‡

From the NIA, National Institutes of Health, Baltimore, Maryland 21224

Received for publication, November 1, 2001, and in revised form, December 14, 2001
Published, JBC Papers in Press, December 18, 2001, DOI 10.1074/jbc.M110524200

Ba$^{2+}$ current through the L-type Ca$^{2+}$ channel inactivates essentially by voltage-dependent mechanisms with fast and slow kinetics. Here we found that slow inactivation is mediated by an annular determinant composed of hydrophobic amino acids located near the cytoplasmic ends of transmembrane segments S6 of each repeat of the $\alpha_{1C}$ subunit. We have determined the molecular requirements that completely obstruct slow inactivation. Critical interventions include simultaneous substitution of A752T in IIS6, V1165T in IIS6, and I1475T in IVS6, each preventing in additive manner a considerable fraction of Ba$^{2+}$ current from inactivation. In addition, it requires the S405I mutation in segment IS6. The fractional inhibition of slow inactivation in tested mutants caused an acceleration of fast inactivation, suggesting that fast and slow inactivation mechanisms are linked. The channel lacking slow inactivation showed $\sim$40% of the sustained Ba$^{2+}$ or Ca$^{2+}$ current with no indication of decay. The remaining fraction of the current was inactivated with a single-exponential decay ($\tau_C \sim 10$ ms), completely recovered from inactivation within 100 ms and did not exhibit Ca$^{2+}$-dependent inactivation properties. No voltage-dependent characteristics were significantly changed, consistent with the C-type inactivation model suggesting constriction of the pore as the main mechanism possibly targeted by Ca$^{2+}$ sensors of inactivation.

The voltage-gated inward current of Ca$^{2+}$ ions is a common mechanism of transient increase in the cytoplasmic free Ca$^{2+}$ concentration that stimulates a great variety of cellular responses. The rapid and complete inactivation of Ca$^{2+}$ current is the critical step terminating Ca$^{2+}$ influx and preventing Ca$^{2+}$ overloading of the cell. In the case of L-type ($\alpha_{1C}$) Ca$^{2+}$ channels, two different mechanisms are in control of the Ca$^{2+}$ current inactivation (1). One mechanism is driven by Ca$^{2+}$ ions on the cytoplasmic side of the membrane (2), whereas the other depends on transmembrane voltage. Replacement of Ca$^{2+}$ ions by Ba$^{2+}$ ions in the extracellular medium eliminates Ca$^{2+}$-dependent inactivation so that Ba$^{2+}$-conducting Ca$^{2+}$ channels are inactivated in a voltage-dependent manner. Two major mechanisms have been previously implicated in voltage-dependent inactivation (3). The ball and chain mechanism of an ion pore occlusion by a positively charged segment of the N-terminal tails was first described in the tetrameric Shaker K$^+$ channel where it supports fast N-type inactivation (4). Somewhat similarly, the hinged-lid mechanism in the Na$^+$ channel is mediated by the IFM motif of the cytoplasmic linker between repeats III and IV (5). In both Na$^+$ and K$^+$ channels, receptor sites for the different inactivation gates are located in S4–S5 intracellular loops (6, 7). The second, C-type mechanism of slower K$^+$ channel inactivation (8) was found to involve a constriction of the pore by the S6 segments lining the intracellular part of the pore and arranged as an inverted teepee structure (9, 10).

The voltage-dependent inactivation of $\alpha_{1C}$ Ca$^{2+}$ channels appears to be more complex (for review, see Refs. 11,12). Experimental trials of chimeras between $\alpha_{1C}$ and the faster inactivating Ca$^{2+}$ channels showed that multiple regions are involved in inactivation, including transmembrane segments IS6 (13), IIS6 (14, 15), IVS5 (16), IVS6 (17–19), repeats I–II linker (20–22), as well as the C-terminal determinants, E1537 of EF-hand motif (23) and the Ca$^{2+}$-sensing 80-amino acid domain 1572–1651 (24). In these regions, multiple amino acids were shown to be critical for the rate of inactivation of the Ba$^{2+}$ current (12). A systematic analysis of these multiple determinants has not been performed.

In this work we focused on determinants situated in all four transmembrane segments S6. The approach was based on our earlier observation (25) that Ca$^{2+}$ channel inactivation was impaired by the A752T mutation at a position–2 from the cytoplasmic end of IIS6 identified in the human fibroblast $\alpha_{1C}$ Ca$^{2+}$ channel transcript (26). Similarly, Val-1504 in IVS6 of the rabbit cardiac $\alpha_{1C}$ was shown to be critical for the channel inactivation (18). The goal of our work was to determine, by single and combined amino acid substitutions, the role and molecular requirements for the involvement of S6 segments in voltage-dependent inactivation. The results are consistent with the C-type inactivation model (4, 8, 12, 13) and suggest that the slow inactivation of Ca$^{2+}$ current is mediated by an annular determinant composed of amino acid residues situated in the cytoplasmic ends of transmembrane segments S6 in repeats I–IV. Complete removal of slow inactivation by a specific set of mutations in all four repeats gave us a unique opportunity to investigate the properties of Ca$^{2+}$ channels that retain only the fast component of inactivation.

MATERIALS AND METHODS

$\alpha_{1C}$ Channel Mutants—pHLCC94 coding for $\alpha_{1C}$ was constructed as described previously (25). $\alpha_{1C,IR}$, $\alpha_{1C,II}$, and its derivatives ($\alpha_{1C,IRF}$, $\alpha_{1C,IRH}$, $\alpha_{1C,II}$, $\alpha_{1C,IIH}$, and $\alpha_{1C,III}$) were generated by the “megaprimer” method (27). To prepare pHLCC250 coding for $\alpha_{1C,III}$, sense 5’-gtgtagccacctgta-3’ (3162–3177) and mutated antisense primer 5’-gaaagggcaagtGTAaagccagaaatg-3’ (3477–3508) were amplified in 25 cycles of PCR (40 s at 94 °C, 1 min at 42 °C, 40 s at 72 °C) with 400 ng of BamHI-linearized pHLC77 (GenBank™ Z34815) using the Ampli-Taq polymerase kit (PerkinElmer Life Sciences). The purified 347-bp megaprimer was subjected to 5 “conditioning” cycles (28), each composed of 1 min at 94 °C and 3 min at 68 °C. Then antisense primer 5’-tacctcgggtattgcta-
Molecular Determinant of Ca\(^{2+}\) Channel Inactivation

tatcaactgtcaccacaa-3\(^\prime\) (3870–3909) was added for 25 cycles of PCR (40 \(\pm\) 94 \(^\circ\)C, 1 min at 65 \(^\circ\)C, 2 min at 73 \(^\circ\)C). The S\(\text{bi1/\text{Nsi}}\) fragment (3342–3846) of the amplified DNA containing the mutation was incorporated at the respective restriction sites into pHCC77. Other II\(\text{S6}\) mutants were prepared in a similar way using the following antisense primers: 5'-gattaagcggagcggagaagatgttcatc-3' (3508–3474), V1165I (3508–3474, V1165F), 5'-cttcaataacgtgaaaggtacctgaggttc-3' (3511–3477), V1161K, 5'-cttcaataacgtgaaaggtacctgaggttc-3' (V1165D), 5'-cttcaataacgtgaaaggtacctgaggttc-3' (1227), or 5'-cttcaataacgtgaaaggtacctgaggttc-3' (3503–3486) Mun and Sph primers were selected to compare the kinetics (Table II and Fig. 1) with the stability of oocyte preparation. Currents were filtered at 1 kHz and the values of the double-exponential fitting are presented only for the faster inactivating component representing a 1.4- to 1.7-fold compared with the wild-type channel. None of the mutations have markedly altered the kinetics of inactivation. Indeed, the faster inactivating component of the current was further analyzed by double-exponential fitting as a sum of the fast and slow components. Increase of the pulse duration to 2 s did not greatly change the calculated parameters (see Table II, asterisk).

The results, summarized in Table II, show that the mutations to thrreonine introduced at the \(-2\) positions in any of the S6 segments in repeats II, III, or IV increased the size of the sustained \(\text{Ba}^{2+}\) current. The inactivating component of the current was further analyzed by double-exponential fitting as a sum of the fast and slow components. Increase of the pulse duration to 2 s did not greatly change the calculated parameters (see Table II, asterisk).

The results, summarized in Table II, show that the mutations to thrreonine introduced at the \(-2\) positions in any of the S6 segments in repeats II, III, or IV increased the size of the sustained \(\text{Ba}^{2+}\) current. The inactivating component of the current was further analyzed by double-exponential fitting as a sum of the fast and slow components. Increase of the pulse duration to 2 s did not greatly change the calculated parameters (see Table II, asterisk).

The results, summarized in Table II, show that the mutations to thrreonine introduced at the \(-2\) positions in any of the S6 segments in repeats II, III, or IV increased the size of the sustained \(\text{Ba}^{2+}\) current. The inactivating component of the current was further analyzed by double-exponential fitting as a sum of the fast and slow components. Increase of the pulse duration to 2 s did not greatly change the calculated parameters (see Table II, asterisk).

**Table I**

| Segment | Amino acid motif | Mutation | \(\alpha_c\) construct |
|---------|-----------------|----------|------------------------|
| S6      | NIFVGVIVF       | A732T    | \(\alpha_c\)II         |
|II\(\text{S6}\) | NIFVGVIVT      | V1165D   | \(\alpha_c\)III        |
|IV\(\text{S6}\) | NIFVGVIVT      | V1165K   | \(\alpha_c\)III        |
|          | NIFVGVIVT      | I1166S   | \(\alpha_c\)III        |
|          | NIFVGVIVT      | F1164S   | \(\alpha_c\)III        |
|          | NIFVGVIVT      | G1168S   | \(\alpha_c\)II,III     |
|          | NIFVGVIVT      | I1167V   | \(\alpha_c\)II,III     |
|          | NIFVGVIVT      | I1167V   | \(\alpha_c\)II,III     |

(See Table II) and normalized to the peak amplitude. This sustained component of the current (\(I_s\)) is a measure of the fraction of the \(\text{Ba}^{2+}\) current inaccessible to the \(-2\) position from 395 to 415 ms, \(i.e.\) in the range characteristic for the \(\alpha_c\) channel.

The results, summarized in Table II, show that the mutations to thrreonine introduced at the \(-2\) positions in any of the S6 segments in repeats II, III, or IV increased the size of the sustained \(\text{Ba}^{2+}\) current at the end of a 1-s depolarization pulse \(-1.4\) to \(-1.7\)-fold compared with the wild-type channel. None of the mutations have markedly altered the kinetics of inactivation. Indeed, the faster inactivating component representing \(60\%\)–70\% of the \(\text{Ba}^{2+}\) current decay has the time constant \(\tau_i\) ranging from 71 to 86 ms. The remaining slower component of the \(\text{Ba}^{2+}\) current continued with a \(\tau_i\) from 395 to 415 ms, \(i.e.\) in the range characteristic for the \(\alpha_c\) channel.

When single mutations were introduced, a 3- to 4-mV shift in both directions in steady-state inactivation curves was observed (Fig. 1C). At the end of the \(2\)-s conditioning pulse, 6.1 \(\pm\) 1.4\% of the maximum \(\text{Ba}^{2+}\) current through the wild-type channel remained inactivated. This fraction increased to 11.5–21.2\% by the threonine substitution in repeats II–IV, suggesting that inactivation of the mutated channels was obstructed. The maximum effect of a single mutation was seen with \(\alpha_c\) II, thus confirming the results of our earlier study of this mutant expressed in HEK293 cells (25). Thus, the hydrophobic residues of Ala-752 in II\(\text{S6}\), Val-1165 in III\(\text{S6}\), and Ile-1475 in IV\(\text{S6}\) each contribute to the voltage-dependent inactivation of the \(\alpha_c\) channel. Mutation of these amino acids to hydrophilic Thr appears to impair the transition from the open to inactivated state and/or stabilizes the inactivated state of the channel.

**Combined Mutations in Repeats II, III, and IV**—Given the similarity in the effects of the tested single mutations to Thr, our data may suggest that the hydrophobic amino acids in the \(-2\) positions of segments II\(\text{S6}\), III\(\text{S6}\), and IV\(\text{S6}\) are concurrently involved in voltage-dependent inactivation. To test this hypothesis, the S6 mutations in repeats II–IV were combined in the double and triple mutants listed in Table II. We observed that each additional homologous mutation inhibited a considerable fraction of the \(\text{Ba}^{2+}\) current inactivation (Fig. 1A and Table II) without significantly altering current-voltage relationships (Fig. 1B). The voltage dependences of the time con-
Molecular Determinant of Ca\textsuperscript{2+} Channel Inactivation

**Table II**

| Mutant          | Fraction of $I_s$ | Fraction of $I_I$ | $\gamma$ | $I_s$ Fraction | $\gamma^*$ | $\alpha$ |
|-----------------|-------------------|-------------------|----------|----------------|------------|---------|
| $\alpha_{1C,WT}$| 16.9 ± 1.0        | 44.4 ± 3.8        | 76.0 ± 4.4| 38.7 ± 3.8     | 425 ± 19   | 23      |

Repeats II, III, IV

$\alpha_{1C,II}$ | 25.2 ± 2.0        | 47.9 ± 3.0        | 71.4 ± 5.5| 26.9 ± 3.0     | 415 ± 26   | 22      |
$\alpha_{1C,III}$| 22.2 ± 1.8        | 46.7 ± 3.3        | 86.2 ± 9.1| 31.1 ± 3.3     | 404 ± 32   | 14      |
$\alpha_{1C,IV}$ | 28.9 ± 2.0        | 51.0 ± 1.4        | 78.4 ± 2.9| 20.1 ± 1.4     | 395 ± 16   | 19      |
$\alpha_{1C,III,II}$ | 52.1 ± 4.3        | 31.2 ± 5.3        | 69.9 ± 7.8| 16.7 ± 5.3     | 635 ± 83   | 9       |
$\alpha_{1C,III,IV}$ | 47.0 ± 3.1        | 29.4 ± 4.9        | 81.0 ± 9.9| 24.0 ± 4.9     | 671 ± 49   | 11      |
$\alpha_{1C,IV,III}$ | 50.7 ± 3.1        | 32.6 ± 2.8        | 63.2 ± 5.4| 16.7 ± 2.8     | 752 ± 79   | 17      |
$\alpha_{1C,IV,IV}$ | 69.7 ± 2.0        | 22.9 ± 2.0        | 53.8 ± 3.3| 7.4 ± 2.0      | 708 ± 64   | 29      |

Repeat I

$\alpha_{1C,II}$ | 18.0 ± 2.7        | 50.0 ± 4.7        | 77.2 ± 7.4| 30.0 ± 4.7     | 507 ± 49   | 17      |
$\alpha_{1C,III}$ | 29.7 ± 3.0        | 60.3 ± 1.8        | 46.9 ± 3.3| 10.0 ± 1.8     | 337 ± 36   | 9       |
$\alpha_{1C,IV}$ | 7.8 ± 2.1         | 66.8 ± 3.3        | 56.2 ± 2.2| 25.4 ± 3.3     | 354 ± 20   | 8       |

All four repeats

$\alpha_{1C,II,IV}$ | 29.9 ± 2.3        | 50.5 ± 4.8        | 37.7 ± 3.5| 19.6 ± 4.8     | 641 ± 91   | 12      |
$\alpha_{1C,III,IV}$ | 43.0 ± 4.9        | 56.1 ± 1.0        | 14.8 ± 1.8| 0.9 ± 1.0      | 471 ± 27   | 11      |
$\alpha_{1C,IV,IV}$ | 45.0 ± 4.8        | 55.0 ± 4.8        | 10.8 ± 0.4| –              | –         | –       |

In each of the double mutants tested ($\alpha_{1C,III,II}$, $\alpha_{1C,III,IV}$, and $\alpha_{1C,IV,III}$), the sustained component of Ba\textsuperscript{2+} current increased by 2-fold compared with $\alpha_{1C,WT}$ and other mutants. Steady-state inactivation curves showed an increase of the noninactivating component of Ba\textsuperscript{2+} current from 61 ± 1.4% in $\alpha_{1C,WT}$ to 48.5 ± 3.0% in $\alpha_{1C,III,IV}$ (Fig. 1C). For some of the mutants, these curves were shifted by 3–4 ms toward positive potentials, whereas their slopes in $\alpha_{1C,III}$ and $\alpha_{1C,III,III}$ were less steep than in $\alpha_{1C,WT}$ and other mutants. These data suggest that the combined double and triple mutations to threonine-589 may slightly change the voltage dependence and cooperativity of the voltage sensors for inactivation in these channels.

In the three double mutants tested ($\alpha_{1C,III,II}$, $\alpha_{1C,III,IV}$, and $\alpha_{1C,IV,III}$), the sustained component of Ba\textsuperscript{2+} current increased by 2-fold compared with the single mutants, and accounted for 47–52% of the total current (Table II). An augmentation of the sustained current was due to both the fast and slow components of the current, which decreased about proportionally in the analyzed single and double mutants ($I_I/\bar{I}$). In the case of $\alpha_{1C,III,IV}$, the fraction of the sustained current increased to 70% of the total current (Fig. 1A and Table II). It is important to note that the ratio of $I/I_{\bar{I}}$ – 3 determined for the $\alpha_{1C,III,IV}$ channel suggests a substantial additional decrease of the slower inactivating component.

The triple mutant did not appear to greatly affect the property of Ba\textsuperscript{2+}-dependent inactivation (Fig. 2). First, the dominating (87.8 ± 0.2%) fast component of the decay of the Ca\textsuperscript{2+} current at $V_{\text{app}} = +10$ mV ($\gamma = 16.0 ± 1.2$ ms, $n = 7$; Fig. 2A) exhibited a 4.6-fold acceleration as compared with the Ba\textsuperscript{2+} current (Table II) but had kinetics essentially similar to those through the wild-type channel ($\gamma = 15.3 ± 1.5$ ms; $I_{\text{ba}} = 95.1 ± 0.5%$; $n = 16$). Second, we observed a U-shape voltage dependence of $\gamma$ (Fig. 2B) reflecting the relationship between the inactivation rate and the size of Ca\textsuperscript{2+} current expected for Ca\textsuperscript{2+}-induced inactivation (32). However, with Ca\textsuperscript{2+} as a charge carrier, a sustained component of the current was also observed, but its amplitude (11.6 ± 1.8%, $n = 5$) was smaller than those measured with Ba\textsuperscript{2+} current (Table II) and appreciably larger than that in the case of $I_{\text{ca}}$ through $\alpha_{1C,WT}$ (2.8 ± 1.0%; $n = 16$). One of the possible explanations for the significant residual sustained component of the Ca\textsuperscript{2+} current in the triple mutant may be partial compensation of the impaired voltage-dependent inactivation in $\alpha_{1C,III,IV}$ by the Ca\textsuperscript{2+}-induced inactivation. Although the hydrophobic amino acid mutations were introduced in the presumed pore region of the channel (9, 10), the ratio of maximum Ba\textsuperscript{2+} to Ca\textsuperscript{2+} currents (–2.7; $n = 3$) through the $\alpha_{1C,III,IV}$ channel did not change substantially compared with the wild-type channel, indicating that the ion selectivity was not appreciably affected.

**Distinct Role of Repeat I Examined by Single and Combined Mutations**—Segment IS6 appears to contribute to the voltage-dependent inactivation differently as compared with other S6 segments. The inactivation properties of the L404T mutant $\alpha_{1C,II}$ are very similar to those of the wild-type channel (Table II). However, incorporation of the L404T mutation into the $\alpha_{1C,III,IS}$ channel signally reversed the effect caused by the combined mutations to threonines in repeats II–IV leading to $\alpha_{1C,III,IV}$ (Fig. 3, decays of Ba\textsuperscript{2+} current through the $\alpha_{1C,III,IV}$ channel in Fig. 1A and those through the $\alpha_{1C,IV,IV}$ channel in Fig. 3A). The sustained component of the Ba\textsuperscript{2+} current through the $\alpha_{1C,II,IV}$ channel was reduced 2.3-fold. Both fast and slow components of the decay increased in size, and the faster inactivating component of the Ba\textsuperscript{2+} current was accelerated (Table II). Steady-state inactivation curves for $\alpha_{1C,II}$ and $\alpha_{1C,IV}$ were shifted by 7 and 13 mV toward negative voltages, and their slopes were steeper compared with $\alpha_{1C,WT}$ and $\alpha_{1C,III,IV}$, respectively (Fig. 3B). In the case of the $\alpha_{1C,IV,IV}$ channel, the half-maximal activation was also shifted by 12 mV toward negative potentials (Fig. 3C), but the current-voltage relation
for α1C,IL was not appreciably different from those for the other isoforms (Fig. 1B).

Unlike transmembrane segments IIS6–IVS6, segment IS6 contains a hydroxyl amino acid Ser-405 at the adjacent position –1 (Table I). The simultaneous double conversion L404T,S405I was introduced into the α1C,WT channel to create a microenvironment at the cytoplasmic end of segment IS6 analogous to those in the mutated S6 segments of α1C,II, α1C,III, or α1C,IV. The resulting double mutant α1C,ILS, however, was inactivated significantly faster than the wild-type channel (Table II). Strong acceleration of the Ba2+ current decay was observed in the composed mutant α1C,ILS (Fig. 3A, Table II) that exhibited two characteristic features: (a) a robust sustained component of the current that is a hallmark of the mutations to Thr at positions –2 of S6 in repeats II–IV and (b) an impressive acceleration of the time course of fast inactivation, which is a characteristic result of the S405I mutation. Voltage dependences of activation and inactivation of the α1C,ILS channel were both shifted to negative potentials (Fig. 3, B and C). Despite acceleration of fast inactivation and impairment of slow inactivation, the voltage dependences of the time constants were not greatly changed (Fig. 3D).

The Combined Mutations Obstructing Slow Inactivation—The kinetic analysis revealed that the slow component of the Ba2+ current inactivation was substantially reduced in the α1C,ILS channel. It appears that Ser-405 is one of the major determinants of slow inactivation of Ca2+ channel. Indeed, the S405I mutation alone greatly reduced the slow inactivation of Ba2+ current through the α1C,IL channel and completely inhibited it in the α1C,IS,IV channel (Table II). In fact, ~45% of the maximum Ba2+ current through the α1C,IS,IV channel showed almost no decay during the 30-s test pulse (Fig. 4A) indicating that a conducting state is the major favorable stable state of this channel at lasting depolarizations. Up to 55% of the Ba2+ current through the α1C,IS,IV channel become inactivated with a single-exponential decay characterized by a time constant of 10.8 ± 0.4 ms, which is in fact somewhat faster than the Ca2+ current through the wild-type channel (τs = 18 ± 2 ms, n = 3).

Fig. 4B shows the traces of Ba2+ current through the α1C,IS,IV channel recorded at different voltages. Similar to other S6 mutants tested, the time constant of the faster inactivation did not change more than 1.6-fold with membrane potential (Fig. 4C, ■). The current-voltage relationships measured for the sustained and inactivating components of the Ba2+ current (Fig. 4C) were very similar to those for the wild-type channel (cf. Fig. 1B, ●). As expected, the voltage dependence of inactivation of the sustained component of the Ba2+ current was completely inhibited in the α1C,IS,IV mutant (Fig. 4D, ○). The analysis of the voltage dependence of inactivation of the rapidly inactivating Ba2+ current component showed that up to 60% of the α1C,IS,IV channels remained available at positive potentials (Fig. 4D, □). The steady-state inactivation curve for the inactivating component of the Ba2+ current through the α1C,IS,IV channel (VO.5 = 27.7 ± 1.5 mV, n = 7) showed a negative shift characteristic for the tested IS6 mutations incorporated into the α1C,ILS,IV channel.

Taken together, these data are consistent with the idea that
component of the Ba\(^{2+}\) current decay. This dependence be-

served in the \(\alpha_{1C,IV}\) channel that contribute to inactivation similar to the segments IIIS6 and IVS6. To examine the importance of the amino acid in

slow inactivation in \(\alpha_{1C,IV}\) is completely inhibited, and the respective long-lasting conducting state is preceded by the rapidly inactivating one. We would predict that the recovery of this fast transient fraction from inactivation should also be markedly accelerated. Recovery from inactivation was measured as a time dependence of the ratio of maximum Ba\(^{2+}\) currents elicited by two consecutive test pulses. We have found (Fig. 5) that recovery was substantially accelerated by the introduction of single- or double-threonine mutations in the S6 segments of repeats II–IV. This effect, however, did not progress with additional mutations leading to \(\alpha_{1C,III,IV}\), \(\alpha_{1C,II,IV}\), and \(\alpha_{1C,III,IV}\) channels as compared with the double mutants. In sharp contrast, the recovery of the \(\alpha_{1C,IV}\) channel from inactivation was complete after 100 ms. Thus, the inhibition of slow inactivation in \(\alpha_{1C,IV}\) correlated with the observed elimination of multiple slow phases of recovery that is characteristic for the \(\alpha_{1C,WT}\) channel (24).

Among the 14 mutants tested, the apparent values of the slower inactivation time constants, \(\tau_s\), measured at the peak of current-voltage relationships vary ~2.2-fold, whereas the faster inactivation time constants, \(\tau_f\), vary over 8-fold (Table II). In fact, the faster inactivation was accelerated with the reduction of the apparent fraction of the slower inactivating component of the Ba\(^{2+}\) current decay. This dependence becomes evident when \(\tau_f\) is plotted as a function of \(I_f/(I_f + I_s)\) and fitted by linear regression with \(r = 0.926\) (Fig. 6).

We have noted that this empirical relation is true also for the Ba\(^{2+}\) current through the \(\alpha_{1C,IS,IV}\) channel isomorph with disrupted Ca\(^{2+}\) sensors mediating Ca\(^{2+}\)-dependent inactivation (24, 29) (Fig. 6, inset). Inactivation of the Ba\(^{2+}\) current through \(\alpha_{1C,IS,IV}\) is strongly accelerated (\(\tau_f = 23.5 \pm 5.6\) ms), whereas the slow component of inactivation accounts for only a small fraction of the decay (\(I_p = 7.2 \pm 4.7\%\); \(I_s = 0\)) (Fig. 6). These data suggest that the slow and fast mechanisms of voltage-dependent inactivation of Ca\(^{2+}\) channels are not entirely independent. Both Ca\(^{2+}\) sensors of the C-terminal tail and the outlined annular determinant appear to be important, possibly in cooperative manner, for the voltage-dependent inactivation.

\(\text{Ca}^{2+}\)-induced Inactivation Property Is Missing in the \(\alpha_{1C,IS,IV}\) Channel—It is interesting that Ca\(^{2+}\) current through the \(\alpha_{1C,IS,IV}\) channel exhibited inactivation with a single time constant (\(\tau_f = 9.5 \pm 0.7\) ms; \(n = 3\)) very close to those found for the Ba\(^{2+}\) current (Fig. 7A). A characteristic 3-fold difference between the peak Ba\(^{2+}\) and Ca\(^{2+}\) currents (29, 33) was preserved in the \(\alpha_{1C,IS,IV}\) channel indicating that ion selectivity in this mutant was not altered. Fig. 7 (B and C) shows dependence of the Ca\(^{2+}\) currents through the \(\alpha_{1C,IS,IV}\) channel on membrane potential. The sustained component of Ca\(^{2+}\) current was observed over a wide range of membrane potentials and comprised up to 49% of the total current (Fig. 7B).

The Ca\(^{2+}\)-induced inactivation of the \(\alpha_{1C}\) channels is supported by two calmodulin-binding domains located in the C-terminal tail (34–36). These Ca\(^{2+}\) sensors remain intact in the \(\alpha_{1C,IS,IV}\) channel, but their function mediating Ca\(^{2+}\)-dependent inactivation appears to be lost. Indeed, simultaneous co-expression of either wild-type calmodulin (CaM\(_{WT}\))\(^{3}\) or its Ca\(^{2+}\)-insensitive mutant CaM\(_{1234}\) (35, 37) did not appreciably affect the properties of Ba\(^{2+}\) or Ca\(^{2+}\) currents through the \(\alpha_{1C,IS,IV}\) channel. Even when CaM\(_{WT}\) was overexpressed in oocytes, the voltage dependence of the time constant of inactivation of the Ca\(^{2+}\) current (Fig. 7C, D) did not show a U-shape characteristic for the Ca\(^{2+}\)-induced inactivation property (cf., Fig. 2B). Similar observations were made for the \(\alpha_{1C,IS,IV}\) channel co-expressed with CaM\(_{1234}\) (data not shown). Thus it appears that the \(\alpha_{1C,IS,IV}\) channel lacks the property of Ca\(^{2+}\)-dependent inactivation.

\(\text{Amino Acid Requirements for the Determinant of Slow Inactivation in S6 Segments of Repeats II–IV}\)—Initial selection of sites for the mutation analysis described in this report was based on the observation that the A752T mutation impaired inactivation in the fibroblast \(\alpha_{1C,94}\) channel isoform (25). Having established an annular nature of the determinant of voltage-dependent slow inactivation as well as the critical amino acids in the IS6 region, we also evaluated the role of the IIIS6 segment that contribute to inactivation similar to the segments IIIS6 and IVS6. To examine the importance of the amino acid in position 1165 of IIIS6, the hydrophilic Thr-1165 residue in the outlined sensors of the C-terminal tail and the outlined annular determinant was replaced by guest on July 25, 2018 http://www.jbc.org/ Downloaded from

3 The abbreviations used are: CaM\(_{WT}\), wild-type calmodulin; CaM\(_{1234}\), Ca\(^{2+}\)-insensitive calmodulin mutant.
transmembrane segment S6 affects voltage-dependent inactivation. To examine this, the conversion of the hydrophobic to a hydrophilic amino acid (Ser or Thr) was shifted from position 1165 in the \( \alpha \)-IIS6 channel to the neighbor amino acids of IIIS6 indicated in Table I. Each of the shifted upward mutations F1164S, G1163, or V1162T has partially restored the slow inactivation of the channel (Fig. 8) and slowed down the recovery from inactivation (not shown). These results are compatible with the view that amino acids in the tested positions are not particularly critical for the slow inactivation. However, the I(1166)S conversion in the position \( \alpha \)-IIIS6 was almost as effective as the mutations in the position \( \alpha \)-IIIS6, indicating that hydrophobic amino acids at this location of the putative cytoplasmic end of S6 are important for slow inactivation. These data support our initial conclusion on the annular nature of the determinant that may involve amino acid residues in positions \( \alpha \)-IIIS6 and \( \alpha \)-IIIS6.

**DISCUSSION**

In this study, we have dissected the fast and slow mechanisms of \( \alpha \)-I C-type \( \mathrm{Ca}^{2+} \) channel inactivation by introducing a number of mutations that selectively and completely inhibited slow inactivation. Guided by the inactivation-impairing A752T mutation in segment IIS6, we performed molecular interventions leading to complete inhibition of slow inactivation require simultaneous substitution to threonines of Ala-752 in IIS6, Val-1165 in IIIS6, and Ile-1475 in IVS6, each causing an additional increase in the fraction of non-inactivating \( \mathrm{Ba}^{2+} \) current. In addition, it requires a S405I mutation in segment IS6 creating a ring of hydrophobic amino acids in the \( \alpha \)-I positions of the S6 segments. The critical role of hydrophobic amino acids in position \( \alpha \)-IIIS6 was also revealed by the mutation I(1166)S in IIIS6 (Fig. 8). We have found that repeat I contributes to inactivation somewhat differently when compared with repeats II–IV. To explain these differences, one would need data on the molecular architecture of the pore region. However, the fact that all four repeats contribute to the outlined determinant conforms to their arrangement around the central pore and suggests that slow inactivation is supported by an “annular” structure involving critical amino acids in positions \( \alpha \)-IIIS6 and \( \alpha \)-IIIS6.

Properties of the S6 mutants studied in our work are consistent with the C-type inactivation model (3, 8). Similar to C-type inactivation, the rate of inactivation of the S6 mutants is essentially voltage-independent. Therefore, S6-mediated inactivation does not involve a large membrane charge movement, which suggests constriction of the pore as the main mechanism. We speculate that the residues of the annular determinant are oriented away from the ion-conducting pore.
and play a critical role in stabilizing hydrophobic interactions supporting slow inactivation (38). Mutations to hydrophilic Thr or Ser residues appear to destabilize these interactions so that the conducting state becomes favorable compared with the inactivated state.

Approximately 55% of the Ba$^{2+}$ current through the $\alpha_{1C,IS-IV}$ channel...
channel inactivates as a single-exponential decay, characterized by the time constant of $\tau_1$ 11 ms. The steady-state inactivation curve of this current is shifted to negative voltages indicating that the voltage sensors for fast inactivation operate at lower depolarization levels. However, neither the time course of activation nor its voltage dependence was significantly affected by inhibition of the slow inactivation mechanism. Single-channel studies are required to further clarify the microscopic properties and determine the relationship between rapidly inactivating and non-inactivating conducting states of the $\alpha_{1C,IS-IV}$ channel. Although a molecular determinant for the fast inactivation has not been identified yet, $\alpha_{1C,IS-IV}$ may be a valuable channel isofrom in such investigation.

It appears that the fast and slow inactivation mechanisms are linked. The fractional inhibition of slow inactivation component in the tested mutants caused an acceleration of the fast inactivation (Fig. 6). A similar effect (see asterisk in Fig. 6) was described earlier (29) for the $\alpha_{1C,IS-IV}$ isoform deprived of Ca$^{2+}$-induced inactivation by the mutation of Ca$^{2+}$ sensors in the cytoplasmic C-terminal tail. Although Ca$^{2+}$ sensors (34–36) remain intact in the $\alpha_{1C,IS-IV}$ channel, no acceleration of inactivation was observed when Ba$^{2+}$ was replaced by Ca$^{2+}$ as the charge carrier (Fig. 7). This result may suggest that Ca$^{2+}$-induced inactivation of L-type Ca$^{2+}$ channels predominantly targets the slow mechanism of inactivation mediated by S6 segments and obstructed in the $\alpha_{1C,IS-IV}$ channel. Several observations support this hypothesis. The pore region, which is thought to be formed on the cytoplasmic side by the S6 segments (9, 10), can in fact be reached by the distant C-terminal Ca$^{2+}$ sensors. Indeed, the segmental mutations disrupting the Ca$^{2+}$ sensors were found to accelerate voltage-dependent inactivation (24, 29) possibly via the loss of critical calmodulin interaction (39). These mutations affected the unitary conductance (40) quite likely via the annular determinant in a manner similar to those linking C-type inactivation to the Shaker K$^+$ channel ion selectivity (3). Taken together, our data are consistent with the idea that the voltage-dependent slow inactivation mechanism involves the targeting of the annular determinant characterized here by the Ca$^{2+}$ sensors of inactivation. Additional studies revealing the role of the individual Ca$^{2+}$ sensors as well as of the $\beta$ subunit in this mechanism are in progress.

Acknowledgments—We thank D. R. Abernethy and I. Josephson for critical discussions and comments on the manuscript; E. Kobrinskys for help in electrophysiology; A. Blatt, O. Carlson, and Z. Zhang for help in molecular biology; F. Hofmann and V. Flockerzi for a gift of clones of $\alpha_1$ subunit in this mechanism are in parentheses.
Molecular Determinant of Ca\textsuperscript{2+} Channel Inactivation

11. Hering, S., Berjukow, S., Aczel, S., and Timin, E. N. (1998) Trends Pharmacol. Sci. 19, 439–443
12. Hering, S., Berjukow, S., Sokolov, S., Marksteiner, R., Weiβ, R. G., Kraus, R., and Timin, E. N. (2000) J. Physiol. (Lond.) 528, 237–249
13. Zhang, J. F., Elliner, P. T., Aldrich, R. W., and Tsien, R. W. (1994) Nature 372, 97–100
14. Tang, S., Yatani, A., Bahinski, A., Mori, Y., and Schwartz, A. (1993) Neuron 11, 1013–1021
15. Hering, S., Aczel, S., Kraus, R. L., Berjukow, S., Striessnig, J., and Timin, E. N. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13323–13328
16. Motoike, H. K., Bodi, I., Nakayama, H., Schwartz, A., and Varadi, G. (1999) J. Biol. Chem. 274, 9409–9420
17. Doering, F., Degtiar, V. E., Grauber, M., Striessnig, J., Hering, S., and Glossmann, H. (1996) J. Biol. Chem. 271, 11745–11749
18. Berjukow, S., Gapp, F., Aczel, S., Sinneker, M. J., Mitterdorfer, J., Glossmann, H., and Hering, S. (1999) J. Biol. Chem. 274, 6154–6160
19. Berjukow, S., Marksteiner, R., Gapp, F., Sinneker, M. J., and Hering, S. (2000) J. Biol. Chem. 275, 22114–22120
20. Spastegens, R. L., and Zamponi, G. W. (1999) J. Biol. Chem. 274, 22428–22436
21. Stotz, S. C., Hamid, J., Spaetgens, R. L., and Zamponi, G. W. (2000) J. Biol. Chem. 275, 24575–24582
22. Restituito, S., Cens, T., Barrere, C., Geib, S., Galas, S., De Waard, M., and Charnet, P. (2000) J. Neurosci. 20, 9046–9052
23. Bernatchez, G., Talwar, B., and Parent, L. (1998) Biochem. J. 327, 1727–1739
24. Soldatov, N. M., Zühlke, R. D., Boren, A., and Reuter, H. (1997) J. Biol. Chem. 272, 3560–3566
25. Soldatov, N. M., Zholshin, S., AllBanna, B., Abernethy, D. R., and Morad, M. (2000) J. Membr. Biol. 177, 129–135
26. Soldatov, N. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4628–4632
27. Sarkar, G. (1990) BioTechniques 8, 404–407
28. Datta, A. K. (1995) Nucleic Acids Res. 23, 4530–4531
29. Soldatov, N. M., Oz, M., O'Brien, K. A., Abernethy, D. R., and Morad, M. (1998) J. Biol. Chem. 273, 957–963
30. Ruth, P., Rohrkasten, A., Biel, M., Bosse, E., Regula, S., Meyer, H. E., Flockerzi, V., and Hofmann, F. (1989) Science 245, 1115–1118
31. Singer, D., Biel, M., Letai, F., Flockerzi, V., Hofmann, F., and Dascal, N. (1991) Science 253, 1553–1557
32. Neely, A., Olcese, R., Wei, X. Y., Birnbaumer, L., and Stefani, E. (1994) Biophys. J. 66, 1895–1903
33. Guia, A., Stern, M. D., Lakatta, E. G., and Josephson, I. R. (2001) Biophys. J. 80, 2742–2750
34. Pate, P., Mochma-Morales, J., Wu, Y., Zhang, J.-Z., Rodney, G. G., Serysheva, I. I., Williams, B. Y., Anderson, M. E., and Hamilton, S. L. (2000) J. Biol. Chem. 275, 39796–39799
35. Romanin, C., Gamsjäger, R., Kahr, H., Schaufler, D., Carlson, O., Abernethy, D. R., and Soldatov, N. M. (2000) FEBS Lett. 487, 301–306
36. Pitt, G. S., Zühlke, R. D., Hudman, A., Schulman, H., Reuter, H., and Tsien, R. W. (2001) J. Biol. Chem. 276, 30784–30802
37. Xia, X.-M., Fakler, B., Rivard, A., Wayman, G., Johnson-Pais, T., Keen, J. E., Ishii, T., Hirashima, B., Bond, C. T., Lutsenko, S., Maylie, J., and Adelman, J. P. (1998) Nature 395, 503–507
38. Espinosa, F., Fleischhauer, R., McMahon, A., and John, R. H. (2001) J. Gen. Physiol. 118, 157–170
39. Mouton, J., Felz, A., and Maulet, Y. (2001) J. Biol. Chem. 276, 22359–22367
40. Keppinger, K. J. P., Kahr, H., Förster, G., Sonnleitner, M., Schneider, H., Schmidt, T., Groschner, K., Soldatov, N. M., and Romanin, C. (2000) FEBS Lett. 477, 161–169
Molecular Determinants of Voltage-dependent Slow Inactivation of the Ca\textsuperscript{2+} Channel
Chengzhang Shi and Nikolai M. Soldatov

J. Biol. Chem. 2002, 277:6813-6821.
doi: 10.1074/jbc.M110524200 originally published online December 18, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M110524200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 22 of which can be accessed free at
http://www.jbc.org/content/277/9/6813.full.html#ref-list-1