Molecular Determinants of L-type Ca\(^{2+}\) Channel Inactivation

SEGMENT EXCHANGE ANALYSIS OF THE CARBOXYL-TERMINAL CYTOPLASMIC MOTIF ENCODED BY EXONS 40–42 OF THE HUMAN \(\alpha_{1C}\) SUBUNIT GENE

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Recently we have described a splice variant of the L-type Ca\(^{2+}\) channel (\(\alpha_{1C,86}\)) in which 80 amino acids (1572–1651) of the conventional \(\alpha_{1C,77}\) were substituted by another 81 amino acids due to alternative splicing of exons 40–42. Ba\(^{2+}\) current \((I_{Ba})\) through \(\alpha_{1C,86}\) exhibited faster inactivation kinetics, was strongly voltage-dependent, and had no Ca\(^{2+}\)-dependent inactivation. An oligonucleotide-directed segment substitution and expression of the mutated channels in \(Xenopus\) oocytes were used to study the molecular determinants for gating of the channel within the 80-amino acid domain. Replacement of segments 1572–1598 or 1595–1652 of the \(\alpha_{1C,77}\) channel with the respective segments of the \(\alpha_{1C,86}\) gave rise to rapidly inactivating \(\alpha_{1C,86}\)-like channel isomers. We found that replacement of either motifs 1572–1576 or 1600–1604 of \(\alpha_{1C,86}\) caused strong but partial acceleration of \(I_{Ba}\) inactivation. Replacement of both sequences produced an \(\alpha_{1C,86}\)-like fast channel which had no Ca\(^{2+}\)-dependent inactivation. These results support the hypothesis that motifs 1572–1576 and 1600–1604 of \(\alpha_{1C,77}\) contribute cooperatively to inactivation kinetics of \(\alpha_{1C}\), and are critical for Ca\(^{2+}\)-dependent inactivation of the channel.

The voltage-gated class C ("cardiac") L-type Ca\(^{2+}\) channel is composed of the pore-forming \(\alpha_{1C}\) subunit, containing the high affinity binding sites for dihydropyridines and other organic Ca\(^{2+}\) channel blockers, and the auxiliary \(\beta\) and \(\delta\) subunits (1, 2). Unlike dihydropyridine-insensitive Ca\(^{2+}\) channels, which exhibit voltage-dependent inactivation of Ca\(^{2+}\) current \((I_{Ca})\), L-type Ca\(^{2+}\)-channels are in addition inactivated by Ca\(^{2+}\), but not Ba\(^{2+}\) ions permeating through the channel (3). The Ca\(^{2+}\)-binding site responsible for the Ca\(^{2+}\)-induced inactivation of the class C Ca\(^{2+}\) channel is thought to be located near the inner mouth, but outside the electric field of the pore (4). Substituting a small 142-amino acid segment of the Ca\(^{2+}\)-insensitive \(\alpha_{1E}\) channel with a homologous segment of the cytoplasmic tail of \(\alpha_{1C}\) confers Ca\(^{2+}\) sensitivity on the \(\alpha_{1E}\) channel (5).

Alternative splicing of the human \(\alpha_{1C}\) subunit generates multiple isoforms of the channel (6), including those with structurally altered carboxyl-terminal tail (7). Recently two splice variants of the principal 2138-amino acids pore-forming \(\alpha_{1C}\) subunit, \(\alpha_{1C,56}\) and \(\alpha_{1C,77}\), exhibiting strong differences in their gating properties were described (8). The \(\alpha_{1C,86}\) channel has 80 amino acid residues (1572–1651) in the second quarter of the 662-amino acid cytoplasmic tail of the conventional channel (\(\alpha_{1C,77}\)), replaced with 81 non-identical amino acids (Fig. 1) due to alternative splicing of exons 40–42 (7). Both splice variants retained high sensitivity toward dihydropyridine blockers, but \(I_{Ba}\) through \(\alpha_{1C,86}\) inactivated 10 times faster than \(\alpha_{1C,77}\) at +20 mV. The inactivation rate of \(\alpha_{1C,86}\) was strongly voltage-dependent but essentially Ca\(^{2+}\)-independent suggesting that the segment 1572–1651 of the carboxyl-terminal tail of \(\alpha_{1C}\) is critical for the kinetics as well as for voltage and Ca\(^{2+}\)-dependence of inactivation of \(\alpha_{1C}\) channel. Extended segment-substitution studies, reported here, show that amino acid residues 1572–1576 and 1600–1604 of \(\alpha_{1C,77}\) contribute in a cooperative manner to the Ca\(^{2+}\)-binding motif(s) responsible for the feedback inhibition of \(\alpha_{1C}\) channel by Ca\(^{2+}\) and the kinetics of decay of \(I_{Ba}\) in the absence of Ca\(^{2+}\)-

**MATERIALS AND METHODS**

Preparation of \(\alpha_{1C,77}\) Mutants—All mutations were incorporated into the nt\(^1\) sequence of pHLLC77 cDNA encoding human Ca\(^{2+}\) channel \(\alpha_{1C,77}\) subunit (9) subcloned into pAlter-1 vector (Promega) or Blue-script SK(–) vector (Stratagene) and composed of exons 1–20, 22–30, 32–44, and 46–50 (EMBL Data Bank accession number Z34815). To improve expression in \(Xenopus\) oocytes, pHLLC77 was supplemented at the 5′-end with HindIII/BgII and at the 3′-end with BgIII/BamHI fragments of untranslated region sequences of the \(Xenopus\) \(\beta\)-globin gene (10), respectively. Mutations were introduced through segment exchange using specifically designed mutagen primers and the Altered Sites\(^®\) II in vitro Mutagenesis System (Promega) according to the manufacturer’s manual. Table I shows mutation primers that have been synthesized (Genosys Biotechnologies, Inc.) and used for segment exchange with pHLLC77 in pAlter-1 vector as a template. Double mutant \(\alpha_{1C,77M1,3}\)-encoding construct was prepared using the M1 primer and the mutated recombinant plasmid pHLCC77M3 as a template. Mutated plasmid pHLLC77L, encoding \(\alpha_{1C,77L}\), was constructed by co-ligating the SfuI (3342)/SacI (4787) 1.4-kilobase fragment of the recombinant plasmid pHLLC86, encoding \(\alpha_{1C,56}\) (8), and 0.7-kilobase SacI (4787)/AatII (5485) fragment of the mutated plasmid pHLLC77M2 with the AatII (5498)/SfuI (3342) 6.7-kilobase fragment of pHLLC86 containing Blue-script vector. Similarly, the mutated plasmid pHLLC77K encoding \(\alpha_{1C,77K}\) was prepared by co-ligating the AatII (5498)/SacI (3342) fragment of pHLLC86 with the SfuI (3342)/SacI (4787) fragment of the mutated plasmid pHLLC77M2 and SacI (4787)/AatII (5498) fragment of the recombinant plasmid pHLLC86. Nucleotide sequences of the obtained cDNAs were verified using the ABI PRISM\(^®\) Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase (Perkin-Elmer). All template DNAs were linearized by digestion with BamHI. Capped transcripts were synthesized in vitro with T7 (pHLCC77L and pHLLC77K) or SP6 (all other mutants) RNA polymerase (Perkin-Elmer).
merases using the mRNA cap kit (Stratagene) and stored as suspensions in ethyl alcohol at −70 °C. Before injection, mRNA samples were dried and dissolved in water (0.5 μg/μl).

Functional Expression of Ca2+ Channels in Xenopus Oocytes—Xenopus laevis oocytes were defolliculated 6–12 h prior to injection with 50–100 nl of a mixture containing mRNAs for α1C, β1, and δ subunits (12) in equimolar ratio. Injected oocytes were incubated at 18 °C in sterile Barth’s medium supplemented with 10,000 units/liter penicillin, 6 mg/liter streptomycin, 50 mg/liter gentamicin, and 90.1 mg/liter theophylline. Membrane currents were recorded at room temperature (20–22 °C) by a two-electrode voltage clamp method using a GeneClamp 500 amplifier (Axon Instruments). Electrodes were filled with 3 M CsCl and had resistance between 2 and 1 meq/hm. The Ba2+ and Ca2+ extracellular (bath) solutions contained 50 mM NaOH, 1 mM KOH, 10 mM HEPES, and 40 mM Ba(OH)2 or 40 mM Ca(NO3)2, respectively (pH adjusted to 7.4 with methanesulfonic acid). In some experiments, about 1 h prior to recording, Ca2+ current (ICa)-expressing oocytes were injected with 50 nl of 94 mM Cs3-BAPTA (pH 7.4). Currents were filtered at 1 kHz. Data were acquired using pClamp 5.5 software (Axon Instruments), corrected for leakage using an on-line P/4 subtraction paradigm and analyzed with KaleidaGraph software. Results are shown as mean ± S.E. "Endogenous" current, determined in the presence of 5 μM XX-PFN200–110 to block the L-type current, did not exceed 3% of the total current.

RESULTS

Strategy of Segment Exchange Analysis—Since it was unclear whether the altered properties of the α1C,δ6 channel were due to the lost determinants normally present in α1C,77 and/or due to those imposed by the new 81-amino acid motif, we chose a well defined slow L-type Ca2+ channel isoform (α1C,77 channel (9)) as a primary target for the mutation studies. A series of segment exchange experiments were performed on pHLCC77 plasmid encoding α1C,77 to map the molecular determinants for the α1C,77 inactivation kinetics as well as its voltage and Ca2+ dependence within the experimentally targeted 80-amino acid (1572–1651) sequence of the carboxyl-terminal tail. To narrow the search, we substituted initially two large fragments of this sequence in α1C,77 with respective sequences from α1C,δ6 (Fig. 1, mutants 77L and 77K) by introducing an

| Table I |
|-------------------|-------------------|-------------------|
| Primer | Mutant | Nt sequence (5' → 3') | Nt positions |
| α1C | R | Q | Amino acids |
| 86 | ETEHSSGQVQAYEKSSLERKSSKPSKSTKPHLLSLGSSTGNGVEDARALKQVLARGCNWLSSLERERQPHPPGLF | (1572–1652) |
| 77K | SSSPSKSTKPHLLSLGSSTGNGVEDARALKQVLARGCNWLSSLERERQPHPPGLF | (1595–1652) |
| 77L | ETEHSSGQVQAYEKSSLERKSSKPSKSTKPHLLSLGSSTGNGVEDARALKQVLARGCNWLSSLERERQPHPPGLF | (1572–1598) |
| 77M1 | ETELS | LLERR | (1572–1595) |
| 77M2 | SSHP | (1595–1598) |
| 77M1,3 | ETELS | SSSSP | (1572–1576;1600–1604) |
| 77M3 | SSSSP | (1600–1604) |
| 77M5 | FVSSTK | (1590–1604) |
| 77M7 | KREBQNLLOQSKKLRA11111KWRNTSHMKIDGEQVFPAGDEVTGKFLYATYL-1QYEYFREKKKKEEQQVLGFSQRNHSL | (1572–1561) |

Fig. 1. Scheme illustrating segment exchange analysis of the specific carboxyl-terminal motif (1572–1651) of α1C,77. Corresponding sequence of α1C,δ6 (1572–1652) is shown on the top. Indicated amino acids of α1C,δ6 replace the respective residues in the amino acid sequence of α1C,77 thus forming mutants in which all other amino acids remain identical to α1C,77. Positions of amino acids are shown on the right. Amino acids sharing identical positions in both α1C,77 and α1C,δ6 are shown in bold caps. Vertical arrow marks a position of 19-amino acid insertion in α1C,77 (8).

Introduction of HLCC86-specific SacI Restriction Site Within the Segment (1595–1598)-coding Sequence Does Not Change Properties of Mutated α1C,77M2—The nt sequence of the α1C,77M2-encoding plasmid contains a convenient SacI restriction site which is absent from pHLCC77. This site, when introduced into the α1C,77M2-coding DNA sequence, allows the transfer of large segments of the 81-amino acid motif of the “fast” α1C,δ6 channel (e.g. 77L and 77K, Fig. 1) into α1C,77.

We introduced this SacI restriction site into pHLCC77 by replacing the segment (nt 4783–4794), which encodes the amino acid residues 1585–1598 of the “slow” α1C,77 channel, with the respective segment of pHLCC86 coding for SSSH (mutant 77M2, Fig. 1). The resulting α1C,77M2 channel showed no significant electrophysiological differences compared with α1C,77 (Table II, III, and IV; Fig. 2, A-C). Similar to α1C,77, the 77M2 mutant showed marked acceleration of inactivation rate when Ca2+ was the charge carrier through the channel (Fig. 2A). Consistent with previous observations (13), ICa was 4.0 ± 0.3 (n = 6) times smaller than IBa, τ1 had a U-shaped voltage dependence (Fig. 2C) for the Ca2+-transporting channel consistent with previous observations (4).

Large Segments Exchange Between α1C,δ6 and α1C,77—Using the pHLCC86-specific SacI restriction site in α1C,77M2-coding

All primers were designed as antisense oligonucleotides. Here and throughout the paper, nt positions are given relative to the first coding triplet. Mismatches are presented in lower case, while nt shown in upper case are identical to pHLCC77.
nt sequence, α1C,77L and α1C,77K mutants were constructed by substituting segments 1572–1598 and 1595–1651 of α1C,77M2 with the corresponding 27- and 58-amino acid segments of the fast α1C,86 channel (Fig. 1). Electrophysiological analysis of these mutants indicated that both α1C,77L and α1C,77K have properties very similar to those of the fast α1C,86 channel. Indeed, superimposed normalized traces of \( I_{Ba} \) for 

\[
\frac{I(t)}{I_0} = 1 + \exp(-t/t_\text{f}) + \exp(-t/t_\text{s}),
\]

where \( I_0 \) is the steady state amplitude of the current, \( t \) is the amplitude of the initial current, \( f \) and \( s \) stand for fast and slow components, respectively.

### Table II

| \( \alpha_{1C} \)          | \( \tau_{f(20)} \) | Fraction of \( I_f \) | \( \tau_{s(20)} \) | Fraction of \( I_s \) | \( n^* \) |
|-----------------|-----------------|-----------------|-----------------|-----------------|--------|
| 77              | 84.6 ± 8.9      | 34.2 ± 4.5      | 462.4 ± 28.4    | 65.8 ± 4.5      | 14     |
| 77M2            | 95.8 ± 12.7     | 37.3 ± 3.8      | 400.4 ± 36.7    | 62.7 ± 3.8      | 12     |
| 77M5            | 98.1 ± 10.5     | 41.7 ± 2.7      | 424.0 ± 40.1    | 58.3 ± 2.7      | 4      |
| 77M1            | 86.9 ± 7.5      | 67.4 ± 2.3      | 329.0 ± 23.1    | 32.6 ± 2.3      | 27     |
| 77M3m           | 71.1 ± 9.0      | 64.2 ± 3.1      | 245.4 ± 20.2    | 35.8 ± 3.1      | 4      |
| 77M3            | 44.5 ± 33.3     | 62.6 ± 2.3      | 276.8 ± 8.5     | 37.4 ± 2.3      | 13     |
| 77M1,3          | 42.9 ± 3.4      | 89.1 ± 3.3      | 222.8 ± 25.0    | 10.9 ± 3.3      | 5      |
| 77K             | 46.4 ± 2.3      | 88.2 ± 1.4      | 302.9 ± 4.5     | 11.8 ± 1.4      | 4      |
| 77L             | 37.9 ± 3.4      | 86.9 ± 1.6      | 191.1 ± 15.7    | 13.1 ± 1.6      | 10     |
| 86              | 44.0 ± 3.9      | 90.0 ± 1.2      | 252.0 ± 27.9    | 10.0 ± 1.2      | 11     |

* \( n \), number of tested oocytes.

The voltage dependence of \( \tau_f \) for \( I_{Ba} \) through α1C,77L and α1C,77K channels was similar to that of α1C,86 (Fig. 3C). There was approximately a 2.5–3-fold decrease of \( \tau_f \) at +40 mV compared with \( \tau_f \) at 0 mV. In both channels the fraction of \( I_{Ba} \) following the fast decay accounted for approximately 95% of the total current at +40 mV. However, a larger fraction of the current through 77L and 77K mutants continued to inactivate rapidly at 0 mV. Thus, voltage dependence of inactivation of \( I_{Ba} \) through α1C,77L was stronger than in α1C,86.

Similar to α1C,86 (8), α1C,77L and α1C,77K channels did not exhibit Ca\(^{2+}\)-dependent inactivation of \( I_{Ca} \) (Fig. 3D) as indicated by the absence of characteristic U-shaped voltage dependence of \( \tau_f \) (e.g. see for example, α1C,77M2; Fig. 2C). Therefore, α1C,77L and α1C,77K channels appear to have electrophysiological properties quite similar to those of α1C,86 channel. This data suggests that there may be at least two molecular determinants for the gating kinetics and Ca\(^{2+}\) dependence of inactivation of the channel, one located possibly within the 22-amino acid segment 1572–1593 (see Fig. 1), and the other within the 54-amino acid motif (1599–1652).

**Motif M1 Determines the Fractional Ratio of Fast to Slow Inactivation**—To further narrow the 22-amino acid motif (1572–1593) that confers the inactivation properties of α1C,86 to α1C,77 channel, a number of α1C,77 mutants containing shorter segments of this motif were prepared. The carboxy-terminal part (1588–1592; mutant M5 in Fig. 1) of the 77L motif did not cause appreciable changes in the properties of the mutated α1C,77M5 channel as compared with α1C,77 (Fig. 4A, Tables II and III). Within the remaining 16-amino acid sequence (1572–1587), we identified a 5-amino acid segment (1572–1576, mutant 77M1 in Fig. 1) that was critical for the faster inactivation.

Electrophysiological properties of the α1C,77M1 channel are presented in Fig. 4. The inactivation kinetics of \( I_{Ba} \) through
Double Mutant M1 + M3 Shows the Best Fit to the \( \alpha_{1C,77M3} \) Channel—When M1 mutation was combined with the M3 mutation, the resulting \( \alpha_{1C,77M1,3} \) channel exhibited all the prop-
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Table III
Parameters of current-voltage relationships for segmental mutants of \(\alpha_{1C,77}\)

| \(\alpha_{1C}\) | \(I_{\text{Ba max}}\) | \(E_{\text{rev}}\) | \(V_{0.5}\) | \(k_{I-V}\) | \(n^a\) |
|---|---|---|---|---|---|
| 77 | 1.68 ± 0.22 | 63.6 ± 0.9 | -1.3 ± 1.9 | -4.8 ± 0.3 | 6 |
| 77M2 | -1.05 ± 0.14 | 64.7 ± 1.0 | 1.4 ± 1.7 | -5.1 ± 0.2 | 10 |
| 77M5 | -0.82 ± 0.09 | 65.7 ± 3.7 | -1.3 ± 7.7 | -5.3 ± 0.5 | 4 |
| 77M1 | -0.88 ± 0.14 | 62.3 ± 1.3 | 2.5 ± 1.8 | -6.9 ± 0.3 | 19 |
| 77M3m | -1.04 ± 0.20 | 65.6 ± 1.5 | 4.0 ± 3.0 | -5.4 ± 0.6 | 4 |
| 77M3 | -2.38 ± 0.47 | 63.9 ± 1.1 | -2.9 ± 1.6 | -5.1 ± 0.7 | 8 |
| 77M1,3 | 0.31 ± 0.05 | 64.9 ± 0.5 | 13.2 ± 1.0 | -7.6 ± 0.4 | 5 |
| 77K | -0.23 ± 0.04 | 61.8 ± 1.2 | 12.3 ± 2.9 | -8.4 ± 0.5 | 5 |
| 77L | -0.77 ± 0.24 | 67.9 ± 2.2 | 9.7 ± 3.4 | -8.0 ± 0.6 | 7 |
| 86 | 0.47 ± 0.07 | 68.3 ± 1.2 | 11.3 ± 1.7 | -7.2 ± 0.3 | 7 |

\(^a\) n, number of tested oocytes.

Table IV
Influence of segmental mutations of \(\alpha_{1C,77}\) on steady-state inactivation

Steady-state inactivation curves were measured using a 2-step voltage clamp protocol. A 2-s conditioning pulses were applied at 30-s intervals with 10-mV increments up to +20 mV from \(V_h = -90\) mV followed by a 250-ms test pulse to +20 mV. Peak current amplitudes were normalized to maximum value. The curves were fitted by a Boltzmann function: \(I_{\text{Ba}} = I_{\text{Ba max}} \exp[-(V - V_{0.5})/k_{I-V}]\), where \(V_{0.5}\) is the voltage at half-maximum of inactivation, and \(k_{I-V}\) is a slope factor.

| \(\alpha_{1C}\) | \(V_{0.5}\) | Slope | \(n^a\) |
|---|---|---|---|
| 77 | -19.6 ± 9.2 | 10.0 ± 1.8 | 3 |
| 77M2 | -24.9 ± 1.2 | 9.2 ± 0.5 | 4 |
| 77M1 | -37.6 ± 4.8 | 13.0 ± 0.8 | 11 |
| 77M5m | -43.8 ± 3.9 | 12.6 ± 0.9 | 4 |
| 77M3 | -21.5 ± 3.9 | 10.8 ± 0.5 | 6 |
| 77M1,3 | -43.5 ± 2.6 | 14.8 ± 0.5 | 7 |
| 77K | -35.5 ± 8.6 | 13.3 ± 0.8 | 3 |
| 77L | -47.0 ± 1.8 | 14.3 ± 0.8 | 7 |
| 86 | -27.5 ± 1.3 | 11.2 ± 0.3 | 3 |

\(^a\) n, number of tested oocytes.

properties of the \(\alpha_{1C,86}\) channel. Fig. 5A shows representative trace of \(I_{\text{Ba}}\) through \(\alpha_{1C,77M1,3}\) elicited by step depolarization to +20 mV from \(V_h = -90\) mV. Approximately 90% of the current inactivated with \(\tau_f\) of 40 to 45 ms, a distinct characteristic of \(\alpha_{1C,86}\) channel (Table II). Furthermore, the time constant of inactivation of \(I_{\text{Ba}}\) through \(\alpha_{1C,77M1}\) and \(\alpha_{1C,77M2}\) was strongly voltage-dependent (Fig. 5C). As compared with \(\alpha_{1C,77M1}\) and \(\alpha_{1C,77M2}\), the voltage dependence of \(I_{\text{Ba}}\) through the double mutated channel was shifted by about +15 mV (Fig. 5C) but all other parameters were essentially identical to those of \(\alpha_{1C,86}\) channel (Table III).

Double-mutated \(\alpha_{1C,77M1,3}\) channel did not show Ca\(^{2+}\)-dependent inactivation as evidenced by the absence of characteristic U-shaped voltage dependence of \(\tau_f\) for \(I_{\text{Ca}}\) (Fig. 5E). Unlike \(\alpha_{1C,77M1}\) channel (Fig. 5D), inactivation kinetics of \(I_{\text{Ca}}\) through \(\alpha_{1C,77M1,3}\) was strongly voltage dependent (Fig. 5E). Taken together, these data suggest that motifs M1 and M3 complement each other in disrupting the functional site of Ca\(^{2+}\)-dependent inactivation. Thus, two 5-amino acid motifs located at positions 1572–1576 and 1600–1604 of \(\alpha_{1C,77}\) cooperatively participate in the gating function of the channel.

**DISCUSSION**

Unlike Ba\(^{2+}\), the magnitude of inactivation of \(I_{\text{Ca}}\) depends on the size of \(I_{\text{Ba}}\) so that the time constant of inactivation exhibits a U-shaped voltage dependence. Ca\(^{2+}\) accelerates inactivation of the L-type Ca\(^{2+}\) channel by reducing its open probability by interacting with the \(\alpha_{1C}\) subunit (4, 14) in equimolar ratio (15). The Ca\(^{2+}\)-binding site for Ca\(^{2+}\)-dependent inactivation has been postulated to be very close to the internal opening of the pore (4) but outside of the conduction pathway (16). The Ca\(^{2+}\)-dependent inactivation was thought to be linked (17) to a 29-amino acid domain (1506–1534) with homology to Ca\(^{2+}\)-binding EF-hand motif (18). Recent experiments using mutation analysis (5), however, have excluded amino acids

FIG. 4. Electrophysiological properties of \(\alpha_{1C,77M1}\) channel. A, representative traces of \(I_{\text{Ba}}\) through \(\alpha_{1C,77M1}\), \(\alpha_{1C,77M2}\), \(\alpha_{1C,77L}\), and \(\alpha_{1C,77}\) channels scaled to the same amplitude. B, averaged I-V curve (●, \(n = 10\)) and voltage dependence of \(\tau_f\) (○, \(n = 13\)) for \(I_{\text{Ba}}\) through \(\alpha_{1C,77M1}\) channel. The time constant of inactivation decreased from \(\tau_f(0) = 84.9 ± 6.4\) ms (61.1 ± 3.6% of \(I_{\text{Ba}}\)) to \(\tau_f(40) = 56.7 ± 4.7\) ms (75.7 ± 5.3% of \(I_{\text{Ba}}\)). C, I-V curve (●) and voltage dependence of \(\tau_f\) (○) for \(I_{\text{Ca}}\) through \(\alpha_{1C,77M1}\) channel (\(n = 0\)).
1506–1534 from this function. Other observations (5, 8) suggest that the location of the site for Ca$^{2+}$-dependent inactivation may be at least 40 amino acids toward the carboxyl terminus of the polypeptide chain.

None of the mutations described in this paper have caused appreciable changes in the kinetics of $I_{Ca}$. However, the kinetics of $I_{Ba}$ decay was changed in a gradual manner (Table II). Since $t_f$ decreases in the following order among the mutants: $\alpha_{1C,77M1}^C > \alpha_{1C,77K}^C > \alpha_{1C,77L}^C > \alpha_{1C,86}^C$, it is likely that retardation of inactivation can be eased through specific structural changes not requiring Ca$^{2+}$. The values of $t_f$ for the decay of $I_{Ba}$ through the fast mutants are almost identical to those for $I_{Ca}$ through the slow channel, suggesting that both modes of inactivation may be thermodynamically similar.

It would be reasonable to assume that mutations leading to faster decay rates of $I_{Ba}$ disrupt intraprotein interactions responsible for retardation of inactivation, which otherwise may be achieved through interaction with Ca$^{2+}$. First, inactivation time constants of $I_{Ba}$ through fast channels ($\alpha_{1C,77K}, \alpha_{1C,77L}, \alpha_{1C,77M1,3}$, and $\alpha_{1C,86}$) do not exhibit a U-shaped voltage dependence, and therefore Ba$^{2+}$ does not influence inactivation in a current-dependent manner. Second, all fast channels are deprived of Ca$^{2+}$-dependent inactivation.

At least two partially overlapping sequences, 1572–1598 in $\alpha_{1C,77L}$ and 1572–1576 plus 1600–1604 in $\alpha_{1C,77M1,3}$, can transform a conventional slow $\alpha_{1C,77}$ channel into the fast ($\alpha_{1C,86}$-like) one. The motif in the 77L mutation can apparently be narrowed to 1572–1587 because its partial mutants, $\alpha_{1C,77M2}$ and $\alpha_{1C,77M5}$, exhibit properties of the slow channel. It is the 77M1 motif (1572–1576, Fig. 1) that is common to both fast mutants. However, when each motif is introduced alone, it causes only partial disruption of Ca$^{2+}$ sensitivity and acceleration of $I_{Ba}$. Therefore, for the full effect to occur, M1 must be supplemented with another determinant. The latter may be mobilized either from the adjacent sequence (1577–1587) of the 77L motif or from a distant 77M3 motif. These segments in $\alpha_{1C,77}$ channel are responsible for Ca$^{2+}$-dependent inactivation and, in the absence of Ca$^{2+}$, for the accelerated kinetics of $I_{Ba}$ inactivation. Thus, these motifs are involved in the gating function of the channel possibly through a direct interaction with the pore.

It is possible that 8 out of 11 amino acids (1574–1584, Fig. 1) of $\alpha_{1C,77}$ represent residues that may form coordination bonds with Ca$^{2+}$. However, disruption of this motif by insertion of 19 additional amino acids at position 1576 neither changed the

FIG. 5. Electrophysiological properties of $\alpha_{1C,77M3}$ channel and double-mutated $\alpha_{1C,77M1,3}$ channel. A, representative traces of $I_{Ba}$ through $\alpha_{1C,86}, \alpha_{1C,77M1}, \alpha_{1C,77M3}$, and double mutated $\alpha_{1C,77M1,3}$ channels scaled to the same amplitude. Averaged I-V curves and voltage dependence of $\gamma$ (C) for $I_{Ba}$ through $\alpha_{1C,77M3}$ (B, $n = 5$) and $\alpha_{1C,77M1,3}$ (C, $n = 5$) channels. Time constant of inactivation of $\alpha_{1C,77M3}$ decreased from $t_f(0) = 57.1 \pm 6.3$ ms (60.6 \pm 3.8% of $I_{Ba}$) to $t_f(40) = 40.6 \pm 7.5$ ms (80.8 \pm 4.5% of $I_{Ba}$). Time constant of inactivation of $\alpha_{1C,77M1,3}$ decreased from $t_f(0) = 66.0 \pm 19.3$ ms (73.8 \pm 15.0% of $I_{Ba}$) to $t_f(40) = 29.6 \pm 1.7$ ms (93.6 \pm 1.7% of $I_{Ba}$). Averaged I-V curves and voltage dependence of $\gamma$ (C) for $I_{Ca}$ through $\alpha_{1C,77M3}$ (D, $n = 4$) and $\alpha_{1C,77M1,3}$ (E, $n = 4$) channels.
kinetics of $I_{Ba}$ decay, nor the Ca$^{2+}$-dependent inhibition of otherwise invariant channel isoform, $\alpha_{1C,72}$ (8). Moreover, $\alpha_{1C,77M1}$ mutant retained some Ca$^{2+}$-induced inactivation property. Since segment 1577–1584 in both the slow $\alpha_{1C,72}$ and the fast $\alpha_{1C,77M1,3}$ channels is intact (Fig. 1), the remaining segment (1572–1575) is apparently critical for gating. Coordination of Ca$^{2+}$ in Ca$^{2+}$-binding sites involves seven bonds that are spatially distributed as an octahedron (19) and can be separated into two vertices. Our data suggest that either of the two motifs, 1577–1587 or 1600–1604, may serve as the second critical element for the channel gating. Additional point mutation analysis is needed to make a final judgment about critical amino acids in the proposed Ca$^{2+}$-coordination motif responsible for the channel gating.

Our data point to the complexity of molecular determinants for Ca$^{2+}$ dependence of inactivation. In addition to motifs discussed above, there may be other sequences which determine fast inactivation kinetics and loss of Ca$^{2+}$-induced inactivation of $\alpha_{1C,77K}$ channel. Investigation of these motifs is in progress.

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