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Molecular Structures Involved in L-type Calcium Channel Inactivation

ROLE OF THE CARBOXYL-TERMINAL REGION ENCODED BY EXONS 40–42 IN \( \alpha_{1C} \) SUBUNIT IN THE KINETICS AND \( \text{Ca}^{2+} \) DEPENDENCE OF INACTIVATION

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The pore-forming \( \alpha_{1C} \) subunit is the principal component of the voltage-sensitive L-type \( \text{Ca}^{2+} \) channel. It has a long cytoplasmic carboxyl-terminal tail playing a critical role in channel gating. The expression of \( \alpha_{1C} \) subunits is characterized by alternative splicing, which generates multiple isoforms. cDNA cloning points to a diversity of human hippocampus \( \alpha_{1C} \) transcripts in the region of exons 40–43 that encode a part of the 662-amino acid carboxyl terminus. We compared electrophysiological properties of the well defined 2139-amino acid \( \alpha_{1C,77} \) channel isoform with two splice variants, \( \alpha_{1C,77} \) and \( \alpha_{1C,86} \). They contain alterations in the carboxyl terminus due to alternative splicing of exons 40–42. The 2157-amino acid \( \alpha_{1C,72} \) isoform contains an insertion of 19 amino acids at position 1575. The 2139-amino acid \( \alpha_{1C,86} \) has 80 amino acids replaced in positions 1572–1651 of \( \alpha_{1C,77} \) by a non-identical sequence of 81 amino acids. When expressed in Xenopus oocytes, all three splice variants retained high sensitivity toward dihydropyridine blockers but showed large differences in gating properties. Unlike \( \alpha_{1C,77} \) and \( \alpha_{1C,72} \), \( \text{Ba}^{2+} \) currents (\( I_{\text{Ba}} \)) through \( \alpha_{1C,86} \) inactivated 8–10 times faster at +20 mV, and its inactivation rate was strongly voltage-dependent. Compared to \( \alpha_{1C,77} \), the inactivation curves of \( I_{\text{Ba}} \) through \( \alpha_{1C,86} \) and \( \alpha_{1C,72} \) channels were shifted toward more negative voltages by 11 and 6 mV, respectively. Unlike \( \alpha_{1C,77} \) and \( \alpha_{1C,72} \), the \( \alpha_{1C,86} \) channel lacks a \( \text{Ca}^{2+} \)-dependent component of inactivation. Thus the segment 1572–1651 of the cytoplasmic tail of \( \alpha_{1C} \) is critical for the kinetics as well as for the \( \text{Ca}^{2+} \) and voltage dependence of L-type \( \text{Ca}^{2+} \) channel gating.

DHP\(^{2+}\)-sensitive \( \text{Ca}^{2+} \) channels of class C (1) are voltage-gated channels, which start to open at membrane voltages more positive than −40 mV and slowly inactivate if \( \text{Ba}^{2+} \) is the charge carrier. Inactivation is usually greatly accelerated if \( \text{Ba}^{2+} \) is replaced by \( \text{Ca}^{2+} \) (2). The channels are also designated as L-type and are multisubunit proteins composed of the pore-forming \( \alpha_{1C} \) subunit, which contains high affinity binding sites for DHPs (3–7), and of the auxiliary \( \beta \) and \( \alpha_{2} \delta \) subunits (8, 9). Analysis of the hydrophobicity profile of \( \alpha_{1C} \) indicates four repetitive motifs of similarity (I–IV), each composed of six transmembrane segments (S1–S6) (10). Both, the short amino-terminal tail encoded by exons 1 and 2, and the long carboxyl-terminal tail encoded by exons 38–50 of the human \( \alpha_{1C} \) gene (11) are located in the cytoplasm.

Expression of \( \alpha_{1C} \) is regulated through alternative splicing (12), which has primarily been detected in the membrane-spanning regions of the molecule. However, there is evidence that the carboxyl-terminal tail is also affected by alternative splicing. Two partial transcripts have been identified in a cDNA library of human hippocampus (11, 13). They show that exons 40–43 encoding the second quarter of the putative cytoplasmic tail of the \( \alpha_{1C} \) molecule are subject to alternative splicing and may give rise to new \( \alpha_{1C} \) splice variants in the brain.

The functional role of the carboxyl-terminal tail attracts much attention because of its potential involvement in channel gating. Removal of approximately 70% of the tail causes an increase in the opening probability of the rabbit cardiac \( \alpha_{1C} \) channel (14). A similar deletion mutant of the human cardiac \( \alpha_{1C} \) showed faster inactivation of the channel as compared to the wild-type channel (15). It has been concluded that this tail part of \( \alpha_{1C} \) may serve as a critical component of the gating structure that influences inactivation properties of the channel (15).

In this report we describe two recombinant plasmids, pHLC72 and pHLC86, which contain alternative exons encoding parts of the carboxyl-terminal tails that are found in human hippocampus transcripts. After expression in Xenopus oocytes, we have analyzed electrophysiological properties of \( \alpha_{1C,72} \) and \( \alpha_{1C,86} \) channels and compared them with the reference \( \alpha_{1C,77} \) channel (16). The results of our study show that amino acids encoded by exons 40–42 are important for the voltage dependence of activation and inactivation of the current through these channels, as well as for the kinetics and \( \text{Ca}^{2+} \) dependence of inactivation.

MATERIALS AND METHODS

Preparation of cDNAs Encoding \( \alpha_{1C} \) Subunit Splice Variants—All splice variants were constructed within the frame of pHLC77 (16) composed of exons 1–20, 22–30, 32–44, and 46–50 using the pBlue-Script SK(−) vector (Stratagene) flanked at the 5′-end with HindIII/BgII and at the 3′-end with BglII/BamHI fragments of the Xenopus \( \beta \)-globin gene untranslated region sequences, respectively (17, 18). The recombinant plasmid pHLC86 was prepared by replacing nucleotides 5104–5482 of pHLC77, encoding exons 41 and 42, with the BsaI/BglII

This paper is available on line at http://www-jbc.stanford.edu/jbc/
Expression of Ca\textsuperscript{2+} Channels in Xenopus Oocytes—Xenopus laevis oocytes were defolliculated 1 day before injection (20). cRNA samples were dissolved in 5 mM HEPES, pH 6.8, and oocytes were injected with 50–100 nl of a mixture containing cRNAs (0.5 \mu g/\mu l) for an \alpha\textsc{c1} splice variant, and for \alpha\delta (21) and \beta\textsubscript{1} (22, 23) subunits in equimolar ratio. In some experiments \beta\textsubscript{1}\textalpha or \beta\textsubscript{1} (24) subunits instead of \beta\textsubscript{1} were used. Injected oocytes were stored for 5–6 days at 18 °C in sterile Barths medium supplemented with 100 units of penicillin/ml and 100 \mu g of streptomycin/ml (Boehringer Mannheim, Rotkreuz, Switzerland). The medium was changed daily. Whole-cell Ba\textsuperscript{2+} currents (I\textsubscript{Ba}) were recorded by a two-electrode voltage clamp method using an Axoclamp 2-A amplifier (Axon Instruments, Burlingame, CA) or a Warner Oocyte Clamp OC-725C (Warner Instrument Corp., Hamden, CT). Glass pipettes (50–60 M\Omega) were filled with 3 M CsCl and had resistances between 0.2 and 1 megohms. Throughout the experiments oocytes were continuously superfused at 5–15 ml/min. The Ba\textsuperscript{2+} bathing solution contained (in mM): Ba\textsubscript{2}OH\textsubscript{2} 40, NaOH 50, KOH 1, HEPES 10 (pH 7.4 with methanesulfonic acid). Irsadipine-containing solutions were prepared freshly from a stock solution.

In some experiments Ca\textsuperscript{2+} was used as charge carrier through the channels. One to 5 h prior to the recording of Ca\textsuperscript{2+} currents (I\textsubscript{Ca}), oocytes were injected with 50 nl of a BAPTA solution containing 40 mM Na\textsubscript{2}BAPTA and 10 mM HEPES (pH 7 with KOH). The bathing solution contained (in mM): Ca(NO\textsubscript{3})\textsubscript{2} 40, NaOH 50, KOH 1, HEPES 10 (pH 7.4 with methanesulfonic acid).

Voltage-clamp commands, current recordings and leak current subtraction were performed by means of the EPC software (Cambridge Electronic Design, Cambridge, UK). The EPC software analysis module, the KaleidaGraph software (Abelbeck, Reading, CA), and the FigP software (Biosoft, Ferguson, MO) were used for the data analysis.

Statistical values are given as means ± S.E. Membrane currents, filtered at 0.5–1 KHz and sampled at 2 KHz, were triggered by 0.25- or 1-s step depolarizations applied from V\textsubscript{p} of −90 mV at a frequency of 0.033 Hz. All experiments were performed at room temperature (20–22 °C).

Inactivation characteristics of I\textsubscript{Ba} through the three \alpha\textsc{c1} splice variants were measured with 2-s conditioning pre-pulses. An increase in the duration of conditioning pre-pulses from 2 s to 20 s produced, in all tested \alpha\textsc{c1} splice variants, an additional shift of the inactivation curves by 6–2 mV toward more negative potentials without changes in their steepness, indicating that a steady state had not been reached with 2-s pre-pulses. However, the long pre-pulses were poorly tolerated by many oocytes; therefore, all inactivation curves reported in this paper have been obtained with a 2-s pre-pulse protocol and consequently are called “isochronic” inactivation curves.

To compare the sensitivities of \alpha\textsc{c1} splice variants toward DHPs, we have measured the fractional inhibition of I\textsubscript{Ba} at V\textsubscript{p} = −90 mV by different concentrations of (±)-isradipine ranging from 10 nm to 1 \mu M. After application of isradipine, I\textsubscript{Ba} was monitored at 30-s intervals until an equilibrium of the inhibition was reached. In these experiments endogenous, DHP-insensitive Ca\textsuperscript{2+} or Ba\textsuperscript{2+} currents (20) were not subtracted from recorded peak I\textsubscript{Ba} amplitudes.

RESULTS

Structural Features of the Studied Splice Variants—We have compared electrophysiological properties of human \alpha\textsc{c1} splice variants: \alpha\textsc{c1,72} and two of its homologues, \alpha\textsc{1c,72,2} and \alpha\textsc{1c,54,6}. Both homologues contain substitutions in the region of exons 40–42 encoding the second quarter of the putative cytoplasmic tail of \alpha\textsc{c1} (Fig. 1 , upper panel, see diagram). The recombinant plasmids for \alpha\textsc{c1,72} (pHLCC72) and for \alpha\textsc{1c,54,6} (pHLCC86) were prepared by incorporation of partial cDNA clones (h2,05 and h54) into the nucleotide sequence of pHLCC77. These partial clones have been isolated earlier from the human hippocampus cDNA library (11, 13) and proved to be products of alternative splicing of one and the same \alpha\textsc{c1} gene (11). The nucleotide sequence of exon 41 in pHLCC72 is extended by 57 nt in the upstream direction and thus produces an insertion of 19 residues into the amino acid sequence of the \alpha\textsc{c1,72} channel at position 1575. In pHLCC86, 17 nt are deleted from the 3’-end of exon 40, the 102-nt exon 41 is replaced by the 118-nt exon 40B, and the 128-nt exon 42 is replaced by a 132-nt extension of exon 43 in the upward direction. Thus, 247 nt of the original pHLCC77 cDNA are replaced in pHLCC86 by 250 nt of a new coding sequence. At the amino acid level, this results in the arrangement of exons, numbered according to Ref. 10, in three \alpha\textsc{c1} splice variants. Exons incorporated into the coding sequences are shown in bold lines. Deleted exons are shown in light lines. Shaded boxes point to exons found in human hippocampus transcripts.

The inset shows the test pulse protocol for the traces of I\textsubscript{Ba} through \alpha\textsc{c1,54,6} (A), \alpha\textsc{1c,72,2} (B), and \alpha\textsc{1c,54,6} (C). Averaged isochronic inactivation curves (12–16 experiments) of I\textsubscript{Ba} through \alpha\textsc{1c,54,6} (D), \alpha\textsc{1c,72,2} (E), and \alpha\textsc{1c,77} (F) were obtained with 2-s conditioning pre-pulses (protocol not shown). The equation for fitting the data is given in Table II. G–I, averaged current-voltage relationships (10–18 experiments) of I\textsubscript{Ba} through \alpha\textsc{1c,54,6}, \alpha\textsc{1c,72,2}, and \alpha\textsc{1c,77}. Test pulses were applied at 30-s intervals. The equation for fitting the data is given in Table III. V\textsubscript{i} = −90 mV. Pare-forming \alpha\textsubscript{1c} subunits are co-expressed with auxiliary \beta\textsubscript{1} and \alpha\delta subunits at a 1:1:1 molar ratio.

FIG. 1. Electrophysiological properties and location of variable parts of three \alpha\textsc{c1} splice variants. Schematic diagrams (upper panel) show the arrangement of exons, numbered according to Ref. 10, in three \alpha\textsc{c1} splice variants. Exons incorporated into the coding sequences are shown in bold lines. Deleted exons are shown in light lines. Shaded boxes point to exons found in human hippocampus transcripts. The inset shows the test pulse protocol for the traces of I\textsubscript{Ba} through \alpha\textsc{c1,54,6} (A), \alpha\textsc{1c,72,2} (B), and \alpha\textsc{1c,54,6} (C). Averaged isochronic inactivation curves (12–16 experiments) of I\textsubscript{Ba} through \alpha\textsc{1c,54,6} (D), \alpha\textsc{1c,72,2} (E), and \alpha\textsc{1c,77} (F) were obtained with 2-s conditioning pre-pulses (protocol not shown). The equation for fitting the data is given in Table II. G–I, averaged current-voltage relationships (10–18 experiments) of I\textsubscript{Ba} through \alpha\textsc{1c,54,6}, \alpha\textsc{1c,72,2}, and \alpha\textsc{1c,77}. Test pulses were applied at 30-s intervals. The equation for fitting the data is given in Table III. V\textsubscript{i} = −90 mV. Pare-forming \alpha\textsubscript{1c} subunits are co-expressed with auxiliary \beta\textsubscript{1} and \alpha\delta subunits at a 1:1:1 molar ratio.
involvement of sequences encoded by exons 40–42 in important gating properties of the channel.

**Differences between α1C,77, α1C,72, and α1C,86 in Inactivation, Current-Voltage Relations, and Sensitivity to DHP Blockers**—When cRNAs for α1C,77, α1C,72, or α1C,86 were co-injected into Xenopus oocytes with cRNAs for auxiliary α,δ (21) and β1 subunits (22, 23), they gave rise to functional Ca$^{2+}$ channels with significantly different electrophysiological properties. Fig. 1 (A–C) show traces of $I_{Ba}$ through splice variants of the poreforming α1C subunit recorded in response to depolarizing voltage clamp steps to $+20$ mV (1 s) from $V_h = -90$ mV. The inactivation kinetics of $I_{Ba}$ through α1C,86 was much faster than that through α1C,77 and α1C,72 channels. A direct comparison of time constants (τ) of inactivation obtained from exponential fits of the current traces is shown in Table I. In the case of α1C,77 and α1C,72, the kinetics of the $I_{Ba}$ decay was fitted best by a single-exponential function. For α1C,86 an exponential fit indicated two time constants, where the slow time constant, τs, was approximately 4 times that of the fast time constant, τf (Table I). Subtraction of the DHP-insensitive, endogenous $I_{Ba}$ did not change significantly the absolute τ values. With β1 co-expressed, the fast component of the inactivation phase of $I_{Ba}$ through α1C,86 comprised 84.6 ± 1.3% (n = 12) of the total current recorded with a 1-s pulse, while the slow component was 15.4 ± 1.3% (n = 12) (Table I). The slow component of $I_{Ba}$ through α1C,86 was still significantly faster than that through α1C,77 or α1C,72 channels (Table I).

The time constants of inactivation of $I_{Ba}$ through α1C,77, α1C,72, and α1C,86 showed different voltage dependences (Fig. 2A). In the case of α1C,77, the time constant of inactivation of $I_{Ba}$ decreased only slightly from $\tau_f(0) = 455 ± 38$ ms at $0$ mV to $\tau_f(-40) = 347 ± 18$ ms at $+40$ mV (n = 15), i.e. by a factor of 1.3 (Fig. 2, B, and C). Similarly, only a small voltage dependence of inactivation time constants was observed for α1C,72. In the α1C,86 channel, however, a more than 3.5-fold decrease of the fast inactivation time constant, from $\tau_f(0) = 113 ± 14$ ms (n = 10) to $\tau_f(-40) = 29 ± 1$ ms (n = 14) was measured (Fig. 2, B, and C).

To characterize further the inactivation properties of the three splice variants of α1C, we examined the rate of recovery of $I_{Ba}$ from inactivation. Fig. 3 shows the ratio of maximum amplitudes of $I_{Ba}$ elicited by two consecutive test pulses with different intervals. The duration of the first pulse was 0.4, 2, or 3 s for α1C,86, α1C,72, and α1C,77, respectively, a time required to reach 80–90% of inactivation of the currents through these channels. The second pulse lasted 400 ms. In Fig. 3 the ratios of $I_{Ba}$ at pulse 2 divided by $I_{Ba}$ at pulse 1 are plotted as function of the time intervals between the two pulses. This represents the fractional recovery of $I_{Ba}$ from inactivation. Only the initial phase of recovery of $I_{Ba}$ from inactivation could be fitted with a single-exponential function. This phase had approximately the same time constant for all three α1C splice variants (Fig. 3). However, $I_{Ba}$ through the α1C,86 channel reached full recovery much faster than $I_{Ba}$ through α1C,77 and α1C,72. At the 0.5-s interval between pulses, when 93 ± 1% (n = 4) of $I_{Ba}$ through α1C,86 had recovered, only 57 ± 2% of $I_{Ba}$ through α1C,72 and 56 ± 2% for α1C,77 were available. With 16-s intervals between pulses, all measured $I_{Ba}$ had almost completely recovered from inactivation.

As reported previously (21, 24), auxiliary β-subunits affect, among other properties, the kinetics of the Ca$^{2+}$ channel current. We have found that $\beta_1$, $\beta_{2A}$, or $\beta_3$ subunits, when co-expressed with α,δ and the splice variants of α1C subunits, caused modulatory effects on the inactivation kinetics of $I_{Ba}$, which, however, were smaller than the differences between α1C,86 and α1C,77 or α1C,72 (Table I).

**TABLE I**

| α1C subunit (τ) | β subunit | τ | $I_{Ba}$ fractions | n |
|----------------|-----------|---|-------------------|---|
| α1C,77         | $\beta_1$ | 484 ± 22 | 13 | 10 |
|                | $\beta_2$ | 590 ± 62 | 5  | 10 |
|                | $\beta_3$ | 1.341 ± 144 | 2 | 10 |
| α1C,72         | $\beta_1$ | 382 ± 13* | 14 | 10 |
|                | $\beta_2$ | 721 ± 90 | 4  | 10 |
| α1C,86 (fast component) | $\beta_1$ | 47.5 ± 2.6* | 12 | 10 |
|                | $\beta_2$ | 71.3 ± 6.7* | 7  | 10 |
|                | $\beta_3$ | 55.8 ± 3.9* | 2  | 10 |
| α1C,86 (slow component) | $\beta_1$ | 11.6 ± 1.0* | 10 | 10 |
|                | $\beta_2$ | 272.2 ± 6.8* | 2  | 10 |
|                | $\beta_3$ | 202.1 ± 7.6* | 2  | 10 |

![Fig. 2. Dependence of $I_{Ba}$ inactivation kinetics on membrane potential.](http://www.jbc.org/)

A. traces of $I_{Ba}$ recorded at 0, +20, and +40 mV. The voltage dependence of the inactivation time constant (τ) was determined by fitting current traces of $I_{Ba}$ in the range of 0 to +40 mV with exponential functions (B). Values of $\tau$ for α1C,72 (C, n = 10) and α1C,77 (C, n = 15) were determined by mono-exponential fitting. A bi-exponential approximation was used to obtain $\tau$ values for α1C,86 only the fast component has been plotted in B and C, n = 10–14. To illustrate differences in the voltage dependence of $\tau$ for the three α1C splice variants, the values of $\tau$ at each potential were normalized with respect to $\tau$ at +40 mV (C). The subunit composition of the analyzed channels was α1C,β,α,δ (1:1:1:1, mol).
pre-pulses) inactivation characteristics of \( I_{\text{Ba}} \)
through the three \( \alpha_{1C} \) splice variants. Isotoxic inactivation curves were shifted
toward negative potentials by 5 mV (\( \alpha_{1C-72} \)) and 11 mV (\( \alpha_{1C-86} \)) with respect
to that of \( \alpha_{1C-77} \) (Fig. 1, D–F; Table II, see \( V_{0.5} \)
values). The slopes of isoxic inactivation curves were less
steep for \( \alpha_{1C-86} \) and \( \alpha_{1C-72} \) channels than for \( \alpha_{1C-77} \) (Table II).
Thus, cooperativity in the mechanism leading to inactivation of
\( I_{\text{Ba}} \) may be different for \( \alpha_{1C-86} \) and \( \alpha_{1C-72} \) than for \( \alpha_{1C-77} \).

Current-voltage relationships also point to differences in
the voltage dependence of \( I_{\text{Ba}} \) through \( \alpha_{1C-77} \) as compared to the
other two splice variants (Table III, Fig. 1, G–I). In contrast to
the negative shift of the inactivation curves of \( I_{\text{Ba}} \) through
\( \alpha_{1C-77} \) and \( \alpha_{1C-86} \), their values for half-maximal activation
were shifted toward more positive potentials by 6 mV and 11 mV,
respectively (Fig. 1, G–I, and Table III). These data suggest
that structural changes produced by alternative splicing of
exons 40–42 in \( \alpha_{1C} \) influence the voltage sensors of the
channels for activation and inactivation in different ways. Since the
reversal potentials of the current flowing through \( \alpha_{1C-77} \), \( \alpha_{1C-72} \),
and \( \alpha_{1C-86} \) channels are not significantly different (Table III),
the pore region determining the selectivity of the channel is
probably the same in the studied splice variants (25).

All three splice variants retain a high affinity for DHP block-

![Figure 3](image-url)

**Figure 3. Effect of \( \alpha_{1C} \) subunit structures on the recovery of \( I_{\text{Ba}} \)
from inactivation.** Recovery of \( I_{\text{Ba}} \) through \( \alpha_{1C-86} \) (○), \( \alpha_{1C-72} \) (○),
and \( \alpha_{1C-77} \) (●) was measured with +20 mV pre-pulses of 0.4, 2, and 3 s in
duration, respectively, and 0.4-s test pulses, both applied from \( V_{h} = -90 \) mV. The different pre-pulse durations for \( \alpha_{1C-86} \), \( \alpha_{1C-72} \),
and \( \alpha_{1C-77} \) were necessary to achieve 80–90% inactivation of \( I_{\text{Ba}} \) through each
channel. Pre-pulses and test pulses were separated by increasing
intervals. *Smooth lines* represent fits of the mean data by single
exponentials with time constants \( \tau = 27.2 \pm 0.9 \) ms (\( \alpha_{1C-77} \)), \( \tau = 30.0 \pm 1.6 \) ms
(\( \alpha_{1C-72} \)), \( \tau = 24.0 \pm 2.1 \) ms (\( \alpha_{1C-86} \)) (\( n = 4 \)). The pore-forming \( \alpha_{1C} \) subunit
was co-expressed with auxiliary \( \beta \) and \( \delta \) subunits at equimolar ratio.

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**Table II**

| \( \alpha_{1C} \) subunit | \( \beta \) subunit | \( V_{0.5} \) | Slope | \( n \) |
|--------------------------|-------------------|----------|-------|-----|
| \( \alpha_{1C-77} \)     | \( \beta_{1} \)    | 1.9 ± 0.7 | 7.8  ± 0.3 | 12  |
|                         | \( \beta_{2A} \)   | 3.2 ± 0.3 | 8.6  ± 0.3* | 2*  |
|                         | \( \beta_{3} \)    | 4.0 ± 0.3 | 7.7  ± 0.1 | 2   |
| \( \alpha_{1C-72} \)     | \( \beta_{1} \)    | 2.7 ± 0.2 | 8.7  ± 0.1* | 2   |
| \( \alpha_{1C-86} \)     | \( \beta_{2A} \)   | 3.2 ± 0.3 | 8.6  ± 0.3* | 2*  |
|                         | \( \beta_{3} \)    | 4.0 ± 0.3 | 7.7  ± 0.1 | 2   |

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When measured at \( V_{h} = -90 \) mV, the IC\( _{50} \) value for
(+)-isradipine inhibition of \( I_{\text{Ba}} \) through \( \alpha_{1C-77} \) is about 3.5 times
higher than those for the other splice variants (Table III).

**Differences between Splice Variants in \( Ca^{2+} \)-dependent Inactivation**—Besides voltage-dependent inactivation, many L-type
\( Ca^{2+} \) channels exhibit \( Ca^{2+} \)-dependent inactivation (2). This
latter mode of inactivation has also been shown for heterolo-
gously expressed L-type \( Ca^{2+} \) channels (26–28). In view of the
marked kinetic differences in inactivation between \( \alpha_{1C-86} \),
\( \alpha_{1C-77} \), and \( \alpha_{1C-72} \), we studied their respective \( Ca^{2+} \)-dependent
inactivation properties.

To buffer intracellular \( Ca^{2+} \) ions and to minimize contaminating
\( Ca^{2+} \)-dependent \( CI^{-} \) currents, 50 nl of 40 mM BAPTA
solution were injected into the oocytes prior to the recordings.
The BAPTA injection did not affect properties of \( I_{\text{Ba}} \). However,
it could have reduced the response to \( Ca^{2+} \) of \( Ca^{2+} \)-dependent
inactivation, although Neely et al. (26) have shown that the
time course of \( Ca^{2+} \)-dependent inactivation remains virtually
unchanged over a 20-fold range of buffering capacity. In Fig. 4,
representative current traces recorded from oocytes during
superfusion with 40 mM Ba\(^{2+} \) solution and after switching to 40
mM Ca\(^{2+} \) solution were superimposed. Ca\(^{2+} \) current (\( I_{Ca} \))
amplitudes were much smaller in all three channels than \( I_{\text{Ba}} \)
amplitudes. This is consistent with a lower conductance for
\( Ca^{2+} \) than for Ba\(^{2+} \) ions of L-type calcium channels (29).
The reduction was less pronounced in \( \alpha_{1C-86} \) compared to \( \alpha_{1C-77} \)
and \( \alpha_{1C-72} \). The accelerated inactivation rate seen in \( \alpha_{1C-77} \)
and \( \alpha_{1C-72} \), when \( Ca^{2+} \) was the charge carrier, was absent in \( \alpha_{1C-86} \).
This is illustrated in Fig. 4B, where peak \( I_{Ca} \) has been scaled up
to the level of peak \( I_{\text{Ba}} \). The scaling factors for \( \alpha_{1C-77} \), \( \alpha_{1C-72} \),
and \( \alpha_{1C-86} \) were 3.3, 2.9, and 1.8, respectively. In contrast to \( I_{inact} \)
inactivation kinetics of \( \alpha_{1C-77} \) and \( \alpha_{1C-72} \), \( I_{Ca} \) kinetics could not
be fitted by a single exponential. With a bi-exponential fitting
procedure, at +20 mV the fast time constants, \( \tau_{f} \), of \( I_{inact} \)
inactivation were 27.7 ± 1.9 ms (\( n = 7 \)) and 34.4 ± 5.5 ms (\( n = 4 \)) for
\( \alpha_{1C-77} \) and \( \alpha_{1C-72} \), respectively. The \( I_{inact} \) inactivation time
constants were 398.7 ± 39.6 ms (\( n = 7 \)) and 348.1 ± 22.1 ms (\( n = 7 \)) in these experiments. Thus, an acceleration of the
inactivation kinetics by a factor of 13 and 10 was observed if \( Ca^{2+} \) ions
were the charge carriers through \( \alpha_{1C-77} \) and \( \alpha_{1C-72} \). By contrast,
inactivation kinetics of \( \alpha_{1C-86} \) were only slightly influenced by
\( Ca^{2+} \) ions. The time constant, \( \tau_{f} \), observed at +20 mV in \( Ca^{2+} \)-
containing solution was 78.2 ± 8.0 ms (\( n = 13 \)) compared to
59.0 ± 3.0 ms (\( n = 10 \)) in Ba\(^{2+} \). The apparent slowing of the
inactivation kinetics of \( \alpha_{1C-86} \) by \( Ca^{2+} \) ions could be explained by
a different surface potential with \( Ca^{2+} \) ions in the solution
(30). This is also indicated by a slight shift toward more positive
potentials of the current-voltage curve of all three calcium
channel constructs when switching from Ba\(^{2+} \) to Ca\(^{2+} \) solution.
Table III
Dependence of parameters of current-voltage relationships on \( a_{1C} \) and \( \beta \) subunits

| Subunit | \( I_{Ba} \)(max) | \( E_{rev} \) | \( V_{0.5} \) | \( k_{IV} \) | \( n \) | \( IC_{50} \) for isradipine (nM) |
|---------|----------------|---------|----------|--------|------|-------------------------|
| \( a_{1C,77} \) | \( \beta_1 \) | 1.47 ± 0.14 | 59.8 ± 0.9 | -0.3 ± 1.1 | -5.6 ± 0.3 | 18 | 146.2 ± 12.2 (34) |
| \( a_{1C,77} \) | \( \beta_{1A} \) | 0.58 ± 0.11 | 58.0 ± 0.7 | 2.2 ± 1.0 | 7.0 ± 0.4 | 4 | |
| \( a_{1C,72} \) | \( \beta_1 \) | 1.13 ± 0.02 | 59.3 ± 0.2 | 0.4 ± 1.1 | 4.8 ± 0.2 | 2 | |
| \( a_{1C,72} \) | \( \beta_{1A} \) | 0.99 ± 0.12 | 60.3 ± 0.8 | 5.7 ± 2.0 | -6.6 ± 0.3 | 12 | 39.8 ± 6.2 (10)* |
| \( a_{1C,86} \) | \( \beta_1 \) | 1.23 ± 0.36 | 63.7 ± 2.3 | 5.9 ± 5.7 | -6.5 ± 0.6 | 3 | |
| \( a_{1C,86} \) | \( \beta_{1A} \) | 0.78 ± 0.15* | 62.5 ± 1.2 | 11.5 ± 1.8* | -7.5 ± 0.3* | 10 | 37.7 ± 5.1 (9)* |

DISCUSSION

Alternative splicing of the \( a_1 \) subunit of voltage-dependent \( Ca^{2+} \) channels contributes to the structural diversity of these ion channels, but only little is known about its functional importance. It has been shown that alternative splicing of the gene encoding the \( a_{1C} \) subunit of \( L \)-type \( Ca^{2+} \) channels contributes to differences in the voltage dependence of the sensitivity toward DHPs (16) and to the DHP tissue selectivity (31). In this study we have investigated electrophysiologically three putative splice variants of the human class C \( L \)-type \( Ca^{2+} \) channel.

We show that a segment of 80 amino acids replaced in \( a_{1C,77} \) by a nonidentical sequence of 81 amino acids of \( a_{1C,86} \) in the second quarter of the 662-amino acid carboxyl-terminal tail (1572–1651) caused a 10-fold increase in the rate of inactivation, an 11-mV hyperpolarizing shift in the voltage dependence of inactivation, and elimination of \( Ca^{2+} \)-dependent inactivation, as well as an increase in the affinity of the channel to the DHP blocker (+)-isradipine. Some but not all of these effects were also partially visible in the \( a_{1C,72} \) channel. It is structurally identical to the reference 2138-amino acid \( a_{1C,77} \) channel, except for an insertion of 19 amino acids at position 1575 between sequences encoded by exons 40 and 41. There was a 5-mV hyperpolarizing shift of the voltage dependence of inactivation, the kinetics of inactivation of \( I_{Ba} \) through \( a_{1C,72} \) was only 20% faster than that through \( a_{1C,77} \) (Table I), and the DHP sensitivity of \( a_{1C,72} \) was the same as that of \( a_{1C,86} \) but about 4 times higher than that of \( a_{1C,77} \) (Table II).

Over the last few years, a multitude of studies have shed light on the molecular basis of \( Ca^{2+} \)-channel inactivation. It has been suggested that voltage- and \( Ca^{2+} \)-dependent inactivation are regulated by distinct sites on the \( a_{1C} \) subunit (32). The structural determinants for voltage-dependent inactivation have been attributed to sequences near or in the S6 segments of domains I, III, and IV of the \( a_{1} \) subunit (33, 34). Substituting as few as 9 amino acids from a rapidly inactivating class A \( Ca^{2+} \) channel near the transmembrane region IS6 for homologous residues in the \( a_{1C} \) subunit was sufficient to transform \( a_{1C} \) into a fast inactivating channel (33). More recently several studies have implicated carboxyl-terminal seg-

Fig. 4. \( Ca^{2+} \)-dependent inactivation occurs in \( a_{1C,72} \) and \( a_{1C,77} \) but not in \( a_{1C,86} \). \( I_{Ba} \) and \( I_{Ca} \), through \( a_{1C,77} \), \( a_{1C,72} \), and \( a_{1C,86} \) were recorded in \( Xenopus \) oocytes after injection of 50 nl of 40 mM BAPTA. Current traces were evoked by 400-ms depolarizing steps from \( V_h \) = -90 mV to +20 mV. The oocytes were superfused by a bath solution containing 40 mM \( Ba^{2+} \) or 40 mM \( Ca^{2+} \). \( I_{Ba} \) and \( I_{Ca} \) recorded from the same oocyte expressing either \( a_{1C,77} \), \( a_{1C,72} \), or \( a_{1C,86} \). B, \( I_{Ca} \) traces from panel A normalized to peak \( I_{Ba} \) show that \( Ca^{2+} \)-dependent inactivation is present in \( a_{1C,77} \), but absent in \( a_{1C,72} \) and \( a_{1C,86} \). The individual \( a_{1C} \) subunits were co-expressed with auxiliary \( \beta_1 \) and \( \delta \) subunits in an equimolar ratio.

(Fig. 5, B–D, filled triangles). \( Ca^{2+} \)-induced inactivation is dependent upon the size of \( Ca^{2+} \) influx through the channel pore (2). This can be studied by applying a double-pulse protocol as shown in Fig. 5A (upper panel). The duration of the pre-pulse was 400 ms. A pulse interval of 50 ms was chosen, which was long enough to minimize incomplete recovery from partial inactivation during the pulse intervals. The test pulse was always at +20 mV and also lasted 400 ms. The interval between cycles was 30 s. Representative current traces for \( a_{1C,77} \) at the pre-pulse potentials -40 mV, +20 mV, and +80 mV for \( I_{Ba} \) (middle panel) and \( I_{Ca} \) (bottom panel) are shown in Fig. 5A. In \( Ba^{2+} \) solution, increasing pre-pulse potentials led to a persistent reduction (23.1%) of \( I_{Ba} \) through \( a_{1C,77} \) at the test pulse (Fig. 5, A and B). This is due to incomplete recovery from partial inactivation under these experimental conditions (data not shown). By contrast, with \( Ca^{2+} \) as charge carrier, the test pulse current \( (I_{TP}) \) exhibited a bell-shaped relation as a function of the pre-pulse potential (Fig. 5B). It was inversely related to the current amplitudes at the pre-pulse potentials \( (I_{TP}) \). The maximal current reduction of \( I_{TP} \) in \( Ca^{2+} \) solution was 53.5%, and became less with further depolarization during pre-pulses (Fig. 5, A and B). This is a strong indication for \( Ca^{2+} \)-dependent inactivation triggered by \( Ca^{2+} \) influx through \( a_{1C,77} \) channels. Application of the same protocol to \( a_{1C,72} \) (Fig. 5C) resulted in a similar relationship between \( I_{TP} \) and \( I_{TP} \) as with \( a_{1C,77} \). The maximal current reductions were 15.9% in \( Ba^{2+} \) solution and 48.9% in \( Ca^{2+} \) solution. However, for \( a_{1C,86} \) (Fig. 5D) the relationships between \( I_{TP} \) and \( I_{TP} \) in \( Ba^{2+} \) and \( Ca^{2+} \) solutions (maximal current reductions were 22.8% and 24.3%, respectively) were almost identical and comparable to those obtained with \( a_{1C,77} \) and \( a_{1C,72} \) in \( Ba^{2+} \) solution. This provides further evidence that \( a_{1C,86} \) lacks \( Ca^{2+} \)-dependent inactivation.

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ments as being involved in voltage-dependent inactivation (15, 35).

The membrane-spanning regions, and consequently the voltage sensor in the S4 segments (36), are structurally identical in all three splice variants of α1C studied in our work. The new amino acid sequences in the cytoplasmic carboxy-terminal tail, encoded by alternative exons in α1C,72 and α1C,86, do not show hydrophobic stretches that would suggest their insertion into the plasma membrane. The differences in the voltage dependence of gating between α1C,77 α1C,72, and α1C,86 may, therefore, be due to an altered interaction of cytoplasmic amino acid sequences with the intramembrane voltage sensor in the S4 segments of the α1C protein. For example, a direct interaction of the amino acids encoded by exons 40–42 with the cytoplasmic ends of the charged transmembrane segments S4 may affect the mobility of the charged regions in response to a change in the transmembrane electric field (37). This could influence the transitions between open and closed states of the channel depending on the conformational flexibility of the cytoplasmic polypeptide chains, which may be highest for the α1C,86 channel. However, we cannot rule out that the fast inactivation observed in α1C,86 may be due to some modulatory effect on the interaction with auxiliary subunits, which are known to influence Ca\(^{2+}\) channel inactivation properties (38).

Ca\(^{2+}\)-dependent inactivation seems to be mediated directly by binding of Ca\(^{2+}\) ions to the channel (39). Elimination of the Ca\(^{2+}\) selectivity by the E1145Q mutation in the pore region of repeat III of rabbit cardiac α1C was associated with a loss of Ca\(^{2+}\)-dependent inactivation (27). A Ca\(^{2+}\) binding site has also been implicated for a carboxy-terminal segment near the transmembrane region IVS6 that includes a putative Ca\(^{2+}\)-binding EF-hand motif (40–42). This motif is essential for Ca\(^{2+}\)-dependent inactivation, although additional residues downstream to the EF-domain are required to exhibit the full effect (42–45). On the other hand, neither truncation of up to 70% of the carboxy-terminal tail of cloned α1C subunits (15, 28) nor cytoplasmic modification by trypsin of cloned cardiac Ca\(^{2+}\) channels in HEK 293 cells (15) and endogenous Ca\(^{2+}\) channels in ventricular myocytes (35) had any effect on Ca\(^{2+}\)-dependent inactivation. However, in another study, Ca\(^{2+}\)-dependent inactivation in ventricular myocytes was abolished by trypsin digestion (46), indicating that there is a limit to the extent by which the carboxyl terminus can be shortened before inactiva-
tion is impaired.

Our data show that substituting a stretch of 81 amino acids of α1C,86 for a segment of 80 amino acids in α1C,77 not only affects voltage-dependent inactivation, it also eliminates Ca\(^{2+}\)-dependent inactivation. This substitution left the four putative transmembrane domains and the EF-hand motif intact. These structural regions are identical in both α1C constructs. Thus, our study supports recent observations (43–45) suggesting that the EF-hand motif is not the only determinant of Ca\(^{2+}\)-dependent inactivation (42). We could narrow down a carboxy-terminal regulatory domain to a segment of maximally 81 amino acids.

Our studies have shown an alternatively spliced segment in the carboxyl terminus of the human α1C subunit, which determines inactivation properties of the Ca\(^{2+}\) channel. It remains to be elucidated which parts and residues encoded by exons 40–42 are involved in voltage-dependent inactivation and whether these same sites are responsible for abolishing Ca\(^{2+}\)-dependent inactivation of α1C,86. Furthermore, it will be of great importance to clarify whether an α1C,86-like splice variant is a functional class C Ca\(^{2+}\) channel in the brain.

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