Fast and Slow Inactivation Kinetics of the Ca\(^{2+}\) Channels ECaC1 and ECaC2 (TRPV5 and TRPV6)

ROLE OF THE INTRACELLULAR LOOP LOCATED BETWEEN TRANSMEMBRANE SEGMENTS 2 AND 3*

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The Ca\(^{2+}\) channels ECaC1 and ECaC2 (TRPV5 and TRPV6) share several functional properties including permeation profile and Ca\(^{2+}\)-dependent inactivation. However, the kinetics of ECaC2 currents notably differ from ECaC1 currents. The initial inactivation is much faster in ECaC2 than in ECaC1, and the kinetic differences between Ca\(^{2+}\) and Ba\(^{2+}\) currents are more pronounced for ECaC2 than ECaC1. Here, we identify the structural determinants for these functional differences. Chimeric proteins were expressed heterologously in HEK 293 cells and studied by patch clamp analysis. Both channels retained their phenotype after exchanging the complete N termini, the C termini, or even both N and C termini. The ECaC1 phenotype with the ECaC2 N or C terminus still showed a delay of inactivation. In the substitution of the intracellular loop between the transmembrane domains 2 and 3 of ECaC2, the ECaC1 kinetics were analogous to ECaC2. In conclusion, the TM2-TM3 loop is a critical determinant of the inactivation in ECaC1 and ECaC2.

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The abbreviations used are: TRP, transient receptor potential; CaT1, calcium transporter 1; HEK, human embryonic kidney; TM, transmembrane; GFP, green fluorescent protein; N1 or N2, N-terminal tail of ECaC1 or ECaC2; C1 or C2, C-terminal tail of ECaC1 or ECaC2; ICL, intracellular linker; ANOVA, analysis of variance; NMDG, N-methyl-D-glucamine.
Surprisingly, the N and C termini of both channels are not responsible for the distinctive kinetic differences between ECaC1 and ECaC2. However, we identified a region in the intracellular linker between transmembrane domains 2 and 3 (TM2 and TM3) that determines the initial current decay and the degree of inactivation.

**MATERIALS AND METHODS**

**Construction of Mannomannal Expression Vectors**—The entire open reading frame from mouse ECaC2/CaT1 was cloned as a EcoRI fragment from pSPORT-mECaC2 in the pCINEo/IRES-GFP vector. This bicistronic expression vector pCINEo/IRES-GFP/ECaC2 was used to co-express ECaC2 and green fluorescent protein (GFP). The entire open reading frame from rbECaC1 was cloned as a BspEI fragment in the pCINEo/IRES-GFP vector as described previously (12). To exchange the ECaC1 N terminus for the ECaC2 N terminus, a unique BspEI site has been incorporated at amino acid position Ser221-Tyr222 without affecting the amino acid composition. The N terminus of ECaC1 was cloned as a Nhel-BspEI fragment into the ECaC2 expression vector, resulting in a chimeric transcript ECaC1-N2, and vice versa for ECaC2, resulting in ECaC2-N1. A similar strategy was applied to swap the amino acids in the intracellular loop between TM2 and TM3 of ECaC1 and ECaC2. However, we identified a region in the intracellular linker between transmembrane domains 2 and 3 (TM2 and TM3) that determines the initial current decay and the degree of inactivation.

**Cell Culture and Transfection**—HEK 293 were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) human serum, 2 mM l-glutamine, 2 units/ml penicillin, and 2 mg/ml streptomycin at 37 °C in a humidity-controlled incubator with 10% (v/v) CO₂. HEK 293 cells were loaded with 10 mM BAPTA. The insets show currents normalized to the peak, C, pooled data comparing the time constants of the monoexponential fit of the last 1.5 s of the voltage step as in A and B (τ₁,ₐ) between ECaC1 and ECaC2. The number of cells is indicated in parentheses. D, pooled data comparing the time constants of the monoexponential fit of the last 1.5 s of the voltage step as in A and B (τ₁,ₐ) between ECaC1 and ECaC2. The time to 10% decay for Ba²⁺ and Ca²⁺ currents through ECaC1, F, same as in E but through ECaC2.
ried by either Ca$^{2+}$ or Ba$^{2+}$. The initial rapid phase of inactivation of ECaC2 assessed from the time to 10% current decay was significantly faster than that of ECaC1 (Fig. 1C). The late decay of the current assessed from the time constant of a monoexponential fit to the last 1.5 s of the voltage step to −100 mV was not significantly different between the two channels (Fig. 1D). If Ba$^{2+}$ was used as the charge carrier, the time to 10% inactivation for ECaC1 was only modestly prolonged compared with the Ca$^{2+}$ current (Fig. 1E). However, for ECaC2, these differences were significantly more pronounced (Fig. 1F) as is obvious from Fig. 1, insets in A and B, which shows the currents normalized to the peak values with Ca$^{2+}$ as the charge carrier. Time to 10% inactivation was 112 ± 14 ms (n = 41) for Ca$^{2+}$ currents through ECaC1 and 169 ± 21 ms (n = 15) for Ba$^{2+}$ currents. The same measurements for ECaC2 resulted in 45 ± 5 ms (n = 24) for Ca$^{2+}$ and 830 ± 95 ms (n = 17) for Ba$^{2+}$. The kinetic difference between both channels can be more impressively described by the ratio between these times, t$_{10\%,Ca}^{10\%,Ba}$, which was 0.66 for ECaC1 but 0.05 for ECaC2. The ratio of the Ca$^{2+}$ and Ba$^{2+}$ current densities was also significantly different between ECaC1 (0.69 ± 0.05 (n = 12)) and ECaC2 (0.39 ± 0.03 (n = 15)).

Exchange of the N and C Termini between ECaC1 and ECaC2—The N-terminal tails of ECaC1 (N1) and ECaC2 (N2), which share 81.3% homology, were exchanged to identify the structural determinants for the functional differences between these two channels. Fig. 2 shows typical traces of the inactivation of Ca$^{2+}$ and Ba$^{2+}$ currents for both chimeras. For ECaC1-N2, the chimera in which the ECaC1 N terminus was substituted by that of ECaC2, the time for 10% inactivation of the Ca$^{2+}$ current remained long (210 ± 55 ms, n = 6) as is typical for ECaC1 (Fig. 2A) (see Fig. 4 for a summary of the analysis). In contrast, the time for 10% inactivation of the Ba$^{2+}$ current was prolonged compared with that of wild-type ECaC1 (313 ± 79 ms, n = 6) but was still much shorter than that of ECaC2. The kinetic coefficients, t$_{10\%,Ca}^{10\%,Ba}$, were nearly identical to ECaC1. The ratio I$_{10\%,Ba}$/I$_{10\%,Ca}$ was 1.03 ± 0.12 (n = 6, same cells). This ratio reflects the ECaC1 more than the ECaC2 phenotype. However, Ba$^{2+}$ currents through this chimera were significantly larger compared with ECaC1.

The phenotype of ECaC2-N1 obtained by exchanging the N terminus of ECaC2 for that of ECaC1 was very similar to that of wild-type ECaC2. However, the decay of the Ba$^{2+}$ current is significantly slower compared with the Ca$^{2+}$ current (Fig. 2B). The time to 10% decay of the Ca$^{2+}$ current was not significantly altered compared with wild-type ECaC2 (69 ± 11, n = 9), whereas the Ba$^{2+}$ current is again prolonged (529 ± 136, n = 9). The ratio I$_{10\%,Ca}$/I$_{10\%,Ba}$ was 0.45 ± 0.08 (n = 7, values only from the same cells) and not significantly different from wild-type ECaC2.

Because the exchange of the N-terminal tails did not affect the inactivation kinetics of the ECaC channel, we exchanged their C-terminal tails, which display a homology of 61%. It is obvious from the current traces in Fig. 2C that the time course of inactivation of the ECaC1-C2 chimera in which the ECaC1 C terminus is substituted by the complete C-terminal tail of ECaC2 is not significantly different from wild-type ECaC1 (t$_{10\%,Ca}$ = 230 ± 49, n = 9, and t$_{10\%,Ba}$ = 379 ± 66, n = 10). However, the ratio I$_{10\%,Ba}$/I$_{10\%,Ca}$ was significantly increased to 1.15 ± 0.17 (n = 8) compared with wild-type ECaC1. Similarly, the kinetic properties of ECaC2-C1 obtained by replacing the ECaC2 C terminus with the C-terminal tail of ECaC1 were identical to those of wild-type ECaC2 (Fig. 2D). The time to 10% inactivation was 35 ± 7 ms (n = 11) for Ca$^{2+}$ currents and 918 ± 112 ms (n = 12) for Ba$^{2+}$ currents. Similarly, the I$_{10\%,Ba}$/I$_{10\%,Ca}$ ratios were not significantly different, i.e. 0.43 ± 0.04 (n = 10) for ECaC2-C1 compared with 0.49 for the wild type.

In the next experiment, the effects of exchanging both N and C termini were studied. Fig. 3 summarizes the obtained results. The time to 10% inactivation of both Ca$^{2+}$ and Ba$^{2+}$ currents was significantly prolonged if ECaC1 contained both the N and the C terminus of ECaC2 (t$_{10\%,Ca}$ = 512 ± 167 ms, n = 6, and t$_{10\%,Ba}$ = 825 ± 182 ms, n = 4). However, as for all chimeras, the differences between the kinetics of the Ca$^{2+}$ and Ba$^{2+}$ currents were small (t$_{10\%,Ca}$/t$_{10\%,Ba}$ = 0.82), and the fast initial phase of inactivation was lacking (Fig. 3, A and B). The I$_{10\%,Ba}$/I$_{10\%,Ca}$ ratio of 1.06 ± 0.09 (n = 4, same cells) was increased but matched again the ECaC1 rather than the ECaC2 phenotype. As for the other chimeras, the fast component of inactivation and the large differences in kinetics of the Ca$^{2+}$ and Ba$^{2+}$ currents were conserved whether the N- and the C-terminal tail of ECaC2 was exchanged with those of ECaC1 (t$_{10\%,Ca}$ = 94 ± 18 ms, n = 5, and t$_{10\%,Ba}$/t$_{10\%,Ca}$ = 678 ± 87 ms, n = 5) (Fig. 3, C and D). The ratio between the Ba$^{2+}$ and Ca$^{2+}$ cur-
rents, $I_{\text{Ba}}/I_{\text{Ca}}$, was 0.56 ± 0.08 ($n = 5$, same cells) and similar to the ECaC2 phenotype. Both chimeras exhibit similar expression levels, i.e. the density of the monovalent cation current in the absence of divalent cations at $-80$ mV was $-692 ± 46$ pA/pF ($n = 8$) for ECaC1-N2C2 and $-827 ± 114$ pA/pF ($n = 6$) for ECaC2-N1C1.

Fig. 4 summarizes the data concerning the initial fast inactivation. The time to 10% decay of the Ca$^{2+}$ current for all ECaC1 chimeras ranged between 100 and 500 ms. For ECaC2 and its chimeric proteins, the 10% current decay occurred in <100 ms (Fig. 4A). The initial decay of Ba$^{2+}$ currents was slower than that of the Ca$^{2+}$ currents. The ratio of the time to 10% decay of the Ca$^{2+}$ current and the Ba$^{2+}$ current ($t_{10\% Ca}/t_{10\% Ba}$) ranged between 0.5 and 0.9 for ECaC1 and its corresponding chimeras and was <0.25 for ECaC2 and the corresponding chimeras.

The fraction of open channels at the end of a 3-s hyperpolarizing step expressed as the ratio of the current densities at the end of the pulse and the peak current was similar for all of the chimeras and for both charge carriers. This ratio, which scatters substantially and also depends on the size of the current (8), was <15% at the end of the pulse (Fig. 5), suggesting that the swapping of the N and C termini did not significantly affect the inactivation behavior. Therefore, the exchange of either or both N and C termini between ECaC1 and ECaC2 did not change severely the inactivation kinetics of the wild-type channel, indicating that other domains or even the core structure may determine the discriminating inactivation kinetics of ECaC1 and ECaC2.

Substitution of the Intracellular Linker Domain between the Transmembrane Regions TM2 and TM3—Because the exchange of the cytoplasmic N- and C-terminal tails did not affect the phenotype of the channel kinetics, we replaced the intracellular linker between TM2 and TM3 (ICL1ECaC1 for the ECaC1 linker and ICL1ECaC2 for the ECaC2 linker). Intrigu-
ingly, the inactivation properties of the ECaC2 channel were drastically affected by substitution of its first intracellular loop with that of ECaC1 (Fig. 6). Panel A shows the main kinetic features of ECaC2 including fast initial inactivation, delayed inactivation with Ba\(^{2+}\) as the charge carrier, dramatic differences in the time to 10% inactivation between Ca\(^{2+}\) and Ba\(^{2+}\) currents, and a smaller current density of Ba\(^{2+}\) compared with the Ca\(^{2+}\) currents. The inactivation of both Ca\(^{2+}\) and Ba\(^{2+}\) currents was delayed in the loop1 ECaC2/ICL1 ECaC2 chimera (Fig. 6, B and C). In addition, the fraction of channels inactivated at the end of the 3-s pulse was much less in ECaC2—compared with wild-type ECaC2 (Fig. 6D), indicating a defect of ECaC2 inactivation in this chimeric protein. The times to 10% inactivation were 929 ± 150 ms (n = 11) for Ca\(^{2+}\) and 2878 ± 40 ms (n = 8) for Ba\(^{2+}\). In this analysis, the t\(_{50}\) of the current that did not inactivate at all was set at 3 s, which is identical to the step duration. Thus, these quantitative data even represent an underestimation. The ratio between Ba\(^{2+}\) and Ca\(^{2+}\) current, I\(_{Ba}/I_{Ca}\), was 0.36 ± 0.03 (n = 9, same cells), reflecting the ECaC2 phenotype. Similar results were obtained with 10 mM Ba\(^{2+}\) and Ca\(^{2+}\) as the charge carrier (data not shown, n = 12). Whether the opposite chimera, the first intracellular loop of ECaC2, ICL1 ECaC2, was introduced in ECaC1 (chimera ECaC1/ICL1 ECaC2), no dramatic changes were observed, although it displayed some features of the ECaC2 phenotype. Fig. 6E showed the kinetic behavior of this chimera. In contrast to wild-type ECaC1, the initial inactivation was significantly accelerated, e.g., from 112 ± 14 ms (n = 41) to 15 ± 3 ms (n = 9) for ECaC1/ICL1 ECaC2 (Fig. 6F). This value is much closer to ECaC2 (45 ± 5 ms, n = 24). Also in contrast to ECaC1, which does not strongly differentiate during the initial phase of inactivation between Ca\(^{2+}\) and Ba\(^{2+}\), the ECaC1/ICL1 ECaC2 chimera has now a strikingly delayed time to 10% inactivation for Ba\(^{2+}\) as compared with Ca\(^{2+}\) (130 ± 25 ms, n = 8). The ratio between both times, t\(_{50,Ca}/t_{50,Ba}\), was 0.18 ± 0.05, which was closer to the ECaC2 than to the ECaC1 phenotype. In addition, the ratio between the peak Ba\(^{2+}\) and Ca\(^{2+}\) currents, I\(_{peak,Ba}/I_{peak,Ca}\), was 0.47 ± 0.06, which is intermediate between the ECaC1 and ECaC2 phenotypes. The fraction of channels, which are open at the end of the 3-s step, was dramatically changed in the ECaC2/ICL1 ECaC2 chimera. However, the same change from ECaC2 to ECaC1 resulted again in the ECaC2 phenotype. The inactivation of the Ca\(^{2+}\) currents was nearly complete in both ECaC1 and ECaC2 (0.05 ± 0.02, n = 12, and 0.06 ± 0.03, n = 10). The inactivation of Ba\(^{2+}\) currents through ECaC2 was diminished as compared with ECaC1 (0.13 ± 0.03, n = 10, for ECaC2 and 0.07 ± 0.01, n = 10, for ECaC1). Similar data were measured for the ECaC1/ICL1 ECaC2 chimera, e.g., 0.11 ± 0.03 (n = 8) for Ba\(^{2+}\) and 0.030 ± 0.004 (n = 9) for Ca\(^{2+}\). Thus, the kinetic behavior of this chimera further supports the essential role of the intracellular linker between TM2 and TM3 as a critical determinant of the kinetic behavior of the ECaC channels.

To identify the amino acid residues, which might be crucial for this dramatic change in phenotype, we have exchanged the divergent residues in TM2-TM3 of ECaC2 by the corresponding amino acids from the TM2-TM3 loop in ECaC1, i. e. L409V, V411A, T412S, and F414Y (Fig. 7). The effects of swapping the entire TM2-TM3 loop are depicted at the top rows of the four panels. The mutations at positions 409, 411, and 412 cause a significant delay of the initial decay of the Ca\(^{2+}\) current and a more pronounced prolongation of the inactivation of the Ba\(^{2+}\) current. These mutations resulted in a highly significant decrease of inactivation assessed from the ratio between the current at the end of the pulse and the peak current. An exchange of Phe-414 into a tyrosine had no effect. These data indicate that the N-terminal part of this intracellular linker is critically involved in controlling ECaC2 inactivation.

**DISCUSSION**

This study demonstrates that the intracellular loop between TM2 and TM3, particularly Leu-409, Val-411, and Thr-412, determines the initial rapid inactivation kinetics of ECaC1 and ECaC2, whereas the cytoplasmic N- and C-terminal tails do not contribute significantly.

Voltage-operated Ca\(^{2+}\) channels contain in the N and C termini major determinants of the inactivation process (17–23). Likewise, the N and C termini of the ECaC\(_2\)s contain many putative regulatory domains including protein kinase C and calmodulin-activating kinase phosphorylation sites, postsynaptic density protein, Disc large tumor suppressor, Zonula occludens protein motifs, and ankyrin repeats (2). It has been recently shown that the human isoform CaT-L is highly homologous to ECaC2 and inactivates in a Ca\(^{2+}\)-dependent...
manner via a multiphasic process. This process is characterized by a rapid Ca\(^{2+}\)-dependent phase followed by a Ca\(^{2+}\)/CaM-dependent phase (24). The calmodulin-dependent inactivation can be counteracted by the phosphorylation of a protein kinase C site in the C terminus (24). However, the proposed calmodulin binding site in human CaT-L is not conserved, and also the protein kinase C site, which counteracts Ca\(^{2+}\)-dependent inactivation in CaT-L, is not present in CaC1. Therefore, the swapping of the C termini of both ECaC channels could further evaluate such a mechanism. The cytoplasmic tails of ECaC1 and ECaC2 were exchanged, and the chimeric proteins expressed in HEK 293 cells were studied by patch clamp analysis. Surprisingly, the swapping of the entire intracellular N and C termini did not basically alter the inactivation kinetics of ECaC1 and ECaC2. Of note, Suzuki et al. (25) demonstrated that in the C terminus, the Q579H mutation in ECaC1 and the corresponding H587Q mutation in ECaC2 play a role in the inactivation process. However, in this latter study, the inactivation process was not studied in detail, and the information on the fast and subsequent slower inactivation process is lacking.
However, this study indicates that the cytoplasmic tails of ECaC1 and ECaC2 are not responsible for the difference in inactivation behavior of both channels.

A detailed comparison of the intracellular loops of ECaC1 and ECaC2 demonstrated that the domain between TM3 and TM4 is identical, whereas the loop between TM2 and TM3 contains four conserved amino acid differences (26). Chimeras were constructed in which the discriminating TM2-TM3 loop was exchanged. A subsequent electrophysiological analysis demonstrated that the characteristic fast inactivation of the ECaC2 current was absent in the ECaC2/ICL11 chimera and therefore mimicked the inactivation kinetics of ECaC1. The point mutations of the discriminating four amino acids revealed that Leu-409, Thr-412, and to a lesser extent Val-411 were exchanged. A subsequent electrophysiological analysis demonstrated that the fast inactivation process occurring in ECaC2 is accelerated, and the kinetic discrimination between Ca$^{2+}$ and Ba$^{2+}$ is accentuated. Interestingly, this critical motif is not present in other channel proteins and does not resemble the classical calmodulin-binding domain.

Interestingly, we have identified a short stretch of amino acids that is involved in the fast inactivation of ECaC2 when Ca$^{2+}$ is the charge carrier. This situation is similar to Ca$^{2+}$-dependent inactivation of L-type α1C Ca$^{2+}$ channels (27–29). In the study by Pitt et al. (29), it has been shown that Ca$^{2+}$-dependent inactivation requires the tethering of the Ca$^{2+}$ sensor calmodulin to an effector region, which is supported by other stretches of amino acids. These stretches contain putative calmodulin-binding sequences. It is intriguing to speculate a similar role for the linker identified here. This linker is a critical motif, which is not present in other channel proteins. It does not resemble the classical calmodulin-binding domain but might contain a new calmodulin binding sequence or another inactivation mechanism, including interaction with another Ca$^{2+}$ sensor, could be operative.

Another distinctive feature of both Ca$^{2+}$ channels is that the ratio of Ba$^{2+}$ and Ca$^{2+}$ currents, $I_{Ba}/I_{Ca}$, through both channels is different (8), e.g. the ratio is significantly larger for ECaC1 than for ECaC2. This ratio may result from a smaller single channel conductance for Ba$^{2+}$ than for Ca$^{2+}$ for ECaC2 in comparison with ECaC1 or a decreased open probability of ECaC2 if Ba$^{2+}$ is the charge carrier. This feature is again conserved for all chimeras including the loop chimeras. The current ratio for all chimeras with the ECaC1 core was in a range between 0.69 and 1.15, whereas for all chimeras with the ECaC2 core, the ratio scattered between 0.39 and 0.56. This feature is also not changed by the translocation of the first intracellular linker and the mutations within this linker. This conserved behavior indicates that the mechanism by which both channels differentially discriminate between Ba$^{2+}$ and Ca$^{2+}$ is not located in the swapped domains and rather may be related to other region of the channels including the pore.

In conclusion, the present observations suggest that the residues Leu-409, Val-411, and Thr-414 present in the intracellular loop between TM2 and TM3 determine the fast inactivation of ECaC2. The ECaC2 residues may form part of an inactivation gate receptor region, and the definition of the state-dependent events may elucidate the molecular interactions important for gating transitions among closed, open, and inactivated states of the epithelial Ca$^{2+}$ channel.

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