Multiple Structural Elements Contribute to the Slow Kinetics of the Ca$_v$3.3 T-type Channel

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Molecular cloning and expression studies established the existence of three T-type Ca$^{2+}$ channel (Ca$_v$3.3) $\alpha_1$ subunits: Ca$_v$3.1 ($\alpha_{1G}$), Ca$_v$3.2 ($\alpha_{1H}$), and Ca$_v$3.3 ($\alpha_{1I}$). Although all three channels are low voltage-activated, they display considerable differences in their kinetics, with Ca$_v$3.1 and Ca$_v$3.2 channels activating and inactivating much faster than Ca$_v$3.3 channels. The goal of the present study was to determine the structural elements that confer the distinctively slow kinetics of Ca$_v$3.3 channels. To address this question, a series of chimeric channels between Ca$_v$3.1 and Ca$_v$3.3 channels were constructed and expressed in Xenopus oocytes. Kinetic analysis showed that the slow activation and inactivation kinetics of the Ca$_v$3.3 channel were not completely abolished by substitution with any one portion of the Ca$_v$3.1 channel. Likewise, the Ca$_v$3.1 channel failed to acquire the slow kinetics by simply adopting one portion of the Ca$_v$3.3 channel. These findings suggest that multiple structural elements contribute to the slow kinetics of Ca$_v$3.3 channels.

Calcium influx via T-type Ca$^{2+}$ channels immediately causes depolarization of the plasma membrane and a rise in intracellular calcium. The calcium rise can mediate diverse physiological functions such as hormone secretion, neurotransmitter release, smooth muscle contraction, fertilization, and cell differentiation (1–3). T-type channels are known to participate in pacemaker activities of heart and many neurons including the thalamic neurons (4–6). Abnormal expression of T-type channels has been implicated in certain pathophysiological conditions such as cardiac hypertrophy and absence epilepsy (7, 8).

T-type channels are distinguished from high voltage-activated (HVA) Ca$^{2+}$ channels by their unique biophysical properties, including low voltage activation, fast activation and inactivation kinetics that produce a criss-crossing pattern between successive traces of a current-voltage (IV) protocol, slow deactivation kinetics, and tiny single channel conductance (2, 9–11). Molecular cloning studies have revealed heterogeneity of T-type channels with the cloning of $\alpha_1$ subunits from three genes (Ca$_v$3.1, $\alpha_{1G}$; Ca$_v$3.2, $\alpha_{1H}$; and Ca$_v$3.3, $\alpha_{1I}$).

Expression studies found that Ca$_v$3.3 channels generate currents with much slower activation and inactivation kinetics than Ca$_v$3.1 and Ca$_v$3.2 channels, which show the more typical transient kinetics described for native T-type channels (2, 12–15).

The inactivation processes of voltage-dependent ion channels including Na$^+$, K$^+$, and HVA Ca$^{2+}$ channels play important roles by preventing continual depolarization, hyperpolarization, and overloading of calcium inside cells, respectively. The fast inactivation process of Na$^+$ channels is attributed to blocking of the ion conduction pathway by the IFM (Ile-Phe-Met) motif contained in the intracellular loop between domains III and IV (16, 17). A similar process occurs in Shaker K$^+$ channels, except in this case the inactivation ball is composed by the first 20 amino acids of the N terminus (18, 19). In contrast to these channels, voltage-dependent inactivation of HVA Ca$^{2+}$ channels appears to be affected by multiple regions, including the S-6 regions in domains II, III, and IV (20–24), the I–II loop (25–28), and the N (29) and C termini (30–32). The kinetics of T-type channels resemble those of Na$^+$ channels, albeit on a slower time scale, suggesting that they may also inactivate by a ball-and-chain mechanism. However, preliminary evidence indicates that T-type channels inactivate by similar processes as HVA Ca$^{2+}$ channels. For example, Marksteiner et al. found that both channel families contain important inactivation determinants in the domain III S6 segment (33). Similarly, intracellular loops also play a role in T-type channel inactivation, as evidenced by the alternatively spliced variants of the III–IV loop in Ca$_v$3.1 and the C terminus in Ca$_v$3.3 (34–36). Chimeric studies using Ca$_v$3.1 and Ca$_v$1.2 (L-type) channels identified a negatively charged region in the C terminus as critical for fast inactivation of Ca$_v$3.1 channels (37). The goal of the present study was to identify structural determinants that confer slow kinetics onto Ca$_v$3.3 channels using chimeras with Ca$_v$3.1. The main finding of these studies is that the more donated by Ca$_v$3.3 to the chimeric channels, the more Ca$_v$3.3-like the currents became. These findings suggest that there is no single inactivation ball, but as for HVA channels, there are multiple structural elements that contribute to inactivation of low voltage activation channels.

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**EXPERIMENTAL PROCEDURES**

**Construction of Ca$_v$3.1/Ca$_v$3.3 Chimeric Ca$^{2+}$ Channels**

The cloning of the rat Ca$_v$3.1 (GenBank™ accession number AF027984) and Ca$_v$3.3 (GenBank™ accession number AF068827) was reported previously (12, 14). Chimeric channel cDNAs were constructed from modifications of cDNAs encoding the Ca$_v$3.1 ($\alpha_{1G}$) and Ca$_v$3.3 ($\alpha_{1I}$) channels.

The plasmids carrying chimeric cDNAs are composed of the following fragments. In some cases, restriction enzyme site(s) were introduced by PCR mutagenesis using modified primers and Pfu DNA polymerase. All

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‡ The abbreviation used is: HVA, high voltage-activated.
fragments derived from PCR were verified by sequence analysis. The restriction enzyme sites are marked by numbers in parentheses by indicating 5'-terminal nucleotide generated by cleavage. The origins of the fragments are also denoted in the parentheses. Silent and nonsilent restriction sites were generated by PCR and are indicated by asterisks and crosses, respectively. The locations of the borders in the Cav3.1 and Ca,3.3 chimeras are shown below and schematically represented in Fig. 2.

\[ pG_{Ca,3.1} \]—The fragments used are BglII (5318, \( a_{1G} \)) - AclI* (5912, \( a_{12D} \)), AclI* (5312, \( a_{12L} \)), SpeI (5496, \( a_{12} \)), and SpeI (5493, \( a_{12} \)) - NotI (3' - polylinker). The plasmid pG_{Ca,3.1} was constructed by ligating these fragments into the BglII-digested (5321, \( a_{12} \)) and NotI-digested (3' - polylinker) plasmid pCDNA3-G.

\[ pG_{Ca,3.2} \]—The fragments used are AnII (4698, \( a_{1I} \)) - AclI* (5312, \( a_{1I} \)), AclI* (5912, \( a_{1I} \)) - KpnI (6170, \( a_{1I} \)), and KpnI (6175, \( a_{1I} \)) - NotI (3' - polylinker). The plasmid pG_{Ca,3.2} was constructed by ligating the above fragments with the AnII-digested (4701, \( a_{1I} \)) and NotI-digested (3' - polylinker) plasmid pCDNA3-I.

\[ pG_{Ca,3.3} \]—The fragments used were HindIII* (5365, \( a_{1I} \)) - BspEI (4246, \( a_{1I} \)), HindIII* (4246, \( a_{1I} \)) - EcoRI (4700, \( a_{1I} \)), EcoRI (4700, \( a_{1I} \)) - BglII (5321, \( a_{1I} \)), and BglII (5318, \( a_{1I} \)) - NotI (3' - polylinker). The plasmid pG_{Ca,3.3} was constructed by ligating the above fragments into the BglII-digested (5321, \( a_{1I} \)) and NotI-digested (3' - polylinker) plasmid pCDNA3-S.

\[ pG_{Ca,3.4} \]—The fragments used were KpnI (5' - polylinker) - NaI (2480, \( a_{1I} \)), NaI (2480, \( a_{1I} \)) - BglII* (2760, \( a_{1I} \)), and BglII* (2760, \( a_{1I} \)) - HindIII* (4249, \( a_{1I} \)). The plasmid pG_{Ca,3.4} was constructed by ligating the above fragments with the KpnI-digested (5' - polylinker) and HindIII-digested (3651, \( a_{1I} \)) plasmid pCDNA3-I.

\[ pG_{Ca,3.5} \]—The fragments used were KpnI (5' - polylinker) - EagI (3987, \( a_{1I} \)), EagI (3987, \( a_{1I} \)) - HindIII* (4249, \( a_{1I} \)), and HindIII* (4249, \( a_{1I} \)) - ClaI (3902, \( a_{1I} \)). The plasmid pG_{Ca,3.5} was constructed by ligating these fragments with the KpnI-digested (5' - polylinker) and ClaI-digested (3901, \( a_{1I} \)) plasmid pCDNA3-I.

\[ pG_{Ca,3.6} \]—The fragments used were KpnI (5' - polylinker) - AvrII (1619, \( a_{1I} \)), AvrII (1619, \( a_{1I} \)) - BspEI (2693, \( a_{1I} \)), and BspEI (2693, \( a_{1I} \)) - BamHI (3625, \( a_{1I} \)). The plasmid pG_{Ca,3.6} was constructed by ligating the above fragments into the KpnI-digested (5' - polylinker) and BamHI-digested (3622, \( a_{1I} \)) plasmid pCDNA3-I.

\[ pG_{Ca,3.7} \]—The fragments used were KpnI (5' - polylinker) - NaI (2480, \( a_{1I} \)), NaI (2480, \( a_{1I} \)) - BglII* (2760, \( a_{1I} \)), and BglII* (2760, \( a_{1I} \)) - HindIII* (4249, \( a_{1I} \)). The plasmid pG_{Ca,3.7} was constructed by ligating the above fragments with the KpnI-digested (5' - polylinker) and HindIII-digested (3651, \( a_{1I} \)) plasmid pCDNA3-I.

\[ pG_{Ca,3.8} \]—The fragments used were KpnI (5' - polylinker) - EagI (3987, \( a_{1I} \)), EagI (3987, \( a_{1I} \)) - HindIII* (4249, \( a_{1I} \)), and HindIII* (4249, \( a_{1I} \)) - ClaI (3902, \( a_{1I} \)). The plasmid pG_{Ca,3.8} was constructed by ligating these fragments with the KpnI-digested (5' - polylinker) and ClaI-digested (3901, \( a_{1I} \)) plasmid pCDNA3-I.

\[ pG_{Ca,3.9} \]—The fragments used were KpnI (5' - polylinker) - AvrII (1619, \( a_{1I} \)), AvrII (1619, \( a_{1I} \)) - BspEI (2693, \( a_{1I} \)), and BspEI (2693, \( a_{1I} \)) - BamHI (7140, \( a_{1I} \)). The plasmid pG_{Ca,3.9} was constructed by ligating the above fragments with the KpnI-digested (5' - polylinker) and BamHI-digested (7137, \( a_{1I} \)) plasmid pCDNA3-G.

\[ pG_{Ca,3.10} \]—The fragments used were KpnI (5' - polylinker) - ClaI (3901, \( a_{1I} \)) - AflII (3651, \( a_{1I} \)) - AclI* (5918, \( a_{12I} \)) - AflII (4698, \( a_{1I} \)) - AclI* (5912, \( a_{1I} \)) - BamHI (7137, \( a_{1I} \)). The plasmid pG_{Ca,3.10} was constructed by ligating these fragments with the KpnI-digested (5' - polylinker) and ClaI-digested (3901, \( a_{1I} \)) plasmid pCDNA3-G, and crosses, respectively. The locations of the borders in the Cav3.1 and Ca,3.3 chimeras are shown below and schematically represented in Fig. 2.

Expression of the Ca,3.1, Ca,3.3, and Chimeras in Xenopus Oocytes

The cDNA constructs were linearized at the 3' end by NotI or AflII. Transcripts were synthesized in vitro using T7 RNA polymerase according to the supplied protocol (Ambion, Austin, TX). The concentrations of the synthesized cRNAs were measured spectrophotometrically.

Several ovary lobes were surgically removed from mature female Xenopus laevis frogs (Xenopus One, Ann Arbor, MI) and torn into small clusters in SOS solution (100 mM NaCl, 2 mM KCl, 1.5 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, 2.5 mM pyruvic acid, 50 µg/ml gentamicin, pH 7.6). The follicular membranes were removed by digestion in Ca++-free OR2 solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 5 mM HEPES, pH 7.6) containing 2 mg/ml collagenase (Sigma). The oocytes were injected with 3–10 ng of cRNA using a Drummond Nanogent pipette injector (Parkway, PA) attached to a Narishige micromanipulator (Tokyo, Japan) under a stereo-microscope.

Electrophysiological Recordings in Oocytes and Data Analysis

Barium currents were measured at room temperature 4–8 days after cRNA injection using a two-electrode voltage-clamp amplifier (OC-725C, Warner Instruments, Hamden, CT). Microelectrodes (Warner Instruments) were filled with 3 M KCl, and their resistances were 0.2–1.0 MΩ. The 10 mM BaCl2 bath solution contained 10 mM Ba(OH)2, 90 mM NaOH, 1 mM KOH, 5 mM HEPES (pH 7.4 with methanesulfonic acid). The currents were sampled at 5 kHz and low pass-filtered at 1 kHz using the pClamp system (Digidata 1320A and pClamp 8; Axon instruments, Foster City, CA). Peak currents and exponential fits to currents were analyzed using Clampfit software (Axon Instruments), and a graphical presentation of the data was obtained using Prism software (GraphPad, San Diego, CA). The data are presented as the means ± S.E. The data were tested for significance using Student’s unpaired t test with p < 0.05, p < 0.01, and p < 0.001 as the levels of significance.

RESULTS

Functional expression of Ca,3.1 and Ca,3.3 channels was measured using the Xenopus oocyte expression system. Their biophysical properties were consistent with previous findings, with Ca,3.1 generating quickly inactivating currents and Ca,3.3 generating slowly inactivating currents (12, 14). Fig. 1A illustrates representative traces of the Ca,3.1 and Ca,3.3 currents evoked during a test pulse to −20 mV. At this potential,
the activation and inactivation rates of Ca,3.1 currents were about 10-fold faster than those of the Ca,3.3 currents (Fig. 1, B and C). Significant kinetic differences were also observed at other test potentials (Fig. 1, D and E).

To determine the structural element(s) in Ca,3.3 channels that lead to these striking kinetic differences, nine chimeras between the Ca,3.1 and Ca,3.3 channels were constructed and expressed in Xenopus oocytes. As shown in Fig. 2, all of the chimeras were constructed by substitution of certain regions of the Ca,3.3 with the corresponding regions of the Ca,3.1 or vice versa. Then we compared the kinetic properties of the nine chimeras with those of the parent channels. Recently, Staes et al. (37) have reported that substitution of the Ca,3.1 C terminus with the same region of Ca,1.2 dramatically slowed down the inactivation kinetic of the chimeric channel currents, suggesting that the C terminus of Ca,3.1 channels contains a critical element that mediates fast inactivation. Accordingly, we first compared the kinetics of Gl,1C and Ig,1C channels constructed by reciprocal exchanges of the C termini of Ca,3.1 and Ca,3.3 (Fig. 2). Fig. 3A illustrates representative current traces of these C-terminal chimeras recorded during test pulses to −20 mV and then normalized for comparison. On average, activation and inactivation time constants of the Gl,1C currents were 2.0 ± 0.3 and 15.4 ± 0.8 ms, whereas those of the Ca,3.1 were 1.4 ± 0.1 and 7.2 ± 0.4 ms, respectively. Thus, substitution of the Ca,3.1 C terminus with that of Ca,3.3 produced a 2-fold slowing of the inactivation rate, with a negligible effect on the activation rate (Fig. 3, B and C). Conversely, substitution of the Ca,3.3 C terminus with that of Ca,3.1 (Ig,1C) slowed activation by 72% and accelerated inactivation by 32% (39.3 ± 5.6 and 66.1 ± 0.9 ms, respectively) when compared with Ca,3.3 currents (22.8 ± 1.5 and 97.0 ± 3.8 ms). The difference in current kinetics was greater at hyperpolarized test potentials, whereas the voltage-independent rates observed at depolarized potentials were more similar (Fig. 3, D and E).

To assess the contribution of the intracellular loops to current kinetics, three loop chimeras, Gl,12L, Ig,12L, and Ig,23L, were constructed (Fig. 2). Fig. 4A illustrates normalized peak currents of the loop chimeras. Unexpectedly, the activation and inactivation of Gl,12L currents became faster than those of Ca,3.1 (Fig. 4, D and E). Replacement of the I–II loop in Ca,3.3 (IG,12L) exerted little effect on inactivation kinetics compared with Ca,3.3 (Fig. 4C). However, activation of IG,12L was much faster than Ca,3.3 and IG,23L at most voltages tested (Fig. 4D). In the case of IG,23L, inactivation kinetics were significantly slower than Ca,3.3 and IG,12L channels, whereas activation kinetics were slightly faster than Ca,3.3 and much slower than IG,12L channels (Fig. 4, C and E). Overall, switching of any loop failed to produce a dramatic transition from a slow phenotype to a fast one and vice versa. Thus, it is unlikely that either the I–II or the I–II–III loop alone is a major structural element determining the activation and inactivation kinetics of T-type channels.

Next, we tested whether kinetics are controlled by multiple structural regions including both membrane spanning domain(s) and intracellular loop(s). Chimeric channels containing each half of Ca,3.1 and Ca,3.3, denoted as IG,12D and IG,34D (Fig. 2), were constructed and expressed in Xenopus oocytes. As illustrated in Fig. 5, both chimeras displayed similar inactivation kinetics with time constants that lie halfway between those of the Ca,3.1 and Ca,3.3 currents. These results suggest that no single half portion determines inactivation kinetics and that each half contributes equally. Notably, IG,34D displayed

Fig. 2. Schematic diagrams of Ca,3.1, Ca,3.3, and their chimeras. For each α1 subunit, domains I–IV are displayed linearly, and the six putative transmembrane segments (S1–S6 from left to right) in each domain are shown by cylinders. Gray cylinders and thin lines indicate the regions from Ca,3.1 (α1)I, whereas white cylinders and thin lines indicate the portions from Ca,3.3 (α1)II. The composition of each α1 subunit is given by the numbers of the corresponding amino acids. The junctional sequences common to the two wild type α1 subunits are represented by amino acid numbers of the Ca,3.1.
much faster activation kinetics than IG_{34D} at low voltage ranges. This finding suggests that the III–IV domain might play a more critical role than the I–II domain of Ca_{3,1} in determining activation kinetics.

Because the experiments using the half/half chimeric channels failed to localize a specific gating particle that determines activation kinetics, we expanded the study to three-quarter:quarter chimeras (IG_{234D}, IG_{34D}, and IG_{4D}). Each chimera derived from Ca_{3,3} contained different structural regions of the Ca_{3,1} channel, i.e., domain IV (IG_{4D}), domains III and IV (IG_{34D}), or domains II through IV (IG_{234D}; Fig. 2). As illustrated in Fig. 6, increasing the proportion of Ca_{3,1} in the chimera steadily shifted the kinetic phenotype toward Ca_{3,1}. These results also show that multiple structural elements are involved in determining the kinetics of Ca_{3,1} channels.

Normalized current-voltage relationships of the Ca_{3,1}, Ca_{3,3}, and different chimeric channels were compared in Fig. 7A. Although the thresholds for all of the chimeras were around −60 mV, differences were noted in the positions of their peak currents. Maximum current amplitudes were detected at potentials ranging between −28 and −16 mV in the following order: GL_{AC} < GL_{1D} < IG_{12L} < IG_{23L} < IG_{234D} < IG_{34D} < IG_{4D}. The thresholds and peak currents of most chimeras tend to be slightly shifted toward negative potentials when compared with their parent channels (Ca_{3,1} and Ca_{3,3}), whereas those of IG_{1D} and IG_{34D} are shifted toward positive potentials. These results were quantitated by calculating the chord conductance and fitting these data with a Boltzmann equation (Fig. 7B). Similar shifts were observed in the steady-state inactivation curves of these channels (Fig. 7C). The mid-points of steady-state inactivation and activation are presented in Table I.

**DISCUSSION**

Although all three recombinant Ca_{3} channels form robust low voltage activation channels, the Ca_{3,3} (a_{12}) currents display slow kinetics that markedly differ from the other two...
isofoms, Ca v,3.1 (α1L) and Ca v,3.2 (α1H) (14, 15). The goal of our study was to determine the structural elements(s) responsible for these differences. To address this question, various G-I chimeric channels were constructed and heterologously expressed in Xenopus oocytes. The major finding in this study was that structural elements conferring the slow kinetics of Ca v,3.3 currents or the fast kinetics of Ca v,3.1 currents are not restricted but spread over the whole α1 subunit.

Reciprocal switching of the C terminus between Ca v,3.1 and Ca v,3.3 channels partially conferred the original inactivation properties of the parent channels to the chimeras (Fig. 3). Previously, Staes et al. (37) proposed that the C terminus contains a negatively charged region that is critical for determining the fast inactivation kinetics of Ca v,3.1 currents. However, sequence alignment reveals that the negatively charged residues are well conserved in the same positions of Ca v,3.1 and Ca v,3.3. Thus, it remained a possibility that the nonconserved portion of the C terminus might determine subtype-specific kinetics. This hypothesis is supported by the recent finding that splice variation at exon 33 in the C terminus of Ca v,3.3 channels alters kinetics (35, 36). In contrast, there were few kinetic effects found in rat splice variants of exon 34 that delete most of the C terminus (36). The fact that substitution of the C terminus induced just partial changes in current kinetics led us to search for additional structural elements.

Our guiding hypothesis was that introduction of different regions from a slow channel into a fast channel might transfer the slow phenotype. Therefore it was quite surprising that the introduction of the I–II loop from Ca v,3.3 into Ca v,3.1 produced the opposite result and accelerated current kinetics (Fig. 4). Similarly, introduction of the Ca v,3.1 II–III loop into Ca v,3.3 conferred faster activation kinetics (IG3234D; Fig. 4). Although these results cannot be explained simply, they do indicate that the I–II loop does not contain the structural region that confers subtype-specific kinetics and suggest other structural portion(s) are also required for establishing channel kinetics. This notion is supported by the fact that substitution of the Ca v,3.1 II–III loop with the corresponding region of Ca v,3.3 decreased the activation time constants but had the opposite effect on inactivation time constants (Fig. 4).

**Fig. 5.** Kinetics of half-half chimeras. A, representative IG12D and IG34D chimeric channel currents evoked by a test pulse to −20 mV. B and C, average activation (B) and inactivation (C) time constants of chimeric channel currents were compared with those of Ca v,3.1 and Ca v,3.3. The activation time constants were 10.8 ± 0.6 and 6.2 ± 0.2 for IG12D and IG34D, respectively, and the inactivation time constants were 27.3 ± 2.5 and 27.5 ± 0.6 for IG12D and IG34D, respectively. Significant differences were evaluated by Student’s t tests. ***, p < 0.001. D and E, average activation (D) and inactivation (E) time constants for Ca v,3.1 (●), Ca v,3.3 (○), IG1234D (▲), and IG3412D (▼) at different test potentials. The data represent the means ± S.E. (n = 5–8).

**Fig. 6.** Contribution of individual domains to kinetics. A, representative currents of IG234D, IG434D, and IG43D chimeric channels during a test pulse to −20 mV. B and C, average activation (B) and inactivation (C) time constants of chimeric channel currents were compared with those of Ca v,3.1 and Ca v,3.3. The activation time constants were 2.2 ± 0.1, 6.2 ± 0.2, and 8.2 ± 0.7 ms for IG234D, IG434D, and IG43D, respectively, and the inactivation time constants were 13.3 ± 0.4, 27.5 ± 0.6, and 50.1 ± 3.0 ms for IG234D, IG434D, and IG43D, respectively. Significant differences of the data were evaluated by Student’s t tests. ***, p < 0.001; **, p < 0.01; *, p < 0.05. D and E, average activation (D) and inactivation (E) time constants for Ca v,3.1 (●), Ca v,3.3 (○), IG234D (▲), IG434D (▼), and IG43D (×) at different test potentials. The data represent the means ± S.E. (n = 5–8).
Structural Determinants of the Slow Ca$_{3.3}$ Currents

In the case of HVA Ca$^{2+}$ channels, auxiliary β subunits (25, 27–28), G-proteins (26, 38), and syntaxin (39, 40) were found to potently modulate the gating of currents by interacting with intracellular loops of the channels. However, conserved binding sites of β subunits, G-proteins, or syntaxin were not found in the intracellular loops of Ca$_{3.3}$ channels. Nevertheless, it is still possible that unidentified auxiliary subunits and diverse signaling molecules including enzymes can regulate T-type channel kinetics. These possible mechanisms might be supported by the following two examples. It is known that the activation and inactivation kinetics of Ca$_{3.3}$ channels are quite different depending on the heterologous expression systems used (14, 36). Recently, we also observed that the current kinetics of Ca$_{3.3}$ channels expressed in HEK cells could be modulated after activation of M1 muscarinic receptors.$^2$

In voltage-activated Na$^+$ channels, the IFM motif of the III–IV loop has been found to play a pivotal role as an inactivating ball (16, 17). However, IFM-like sequences are not present in the III–IV loops of Ca$_3$ channels. The III–IV loops of all three Ca$_3$ channels share high sequence similarity, and their lengths are also similar. According to recent reports (2, 34), three Ca$_{3.1}$ splice variants in the III–IV loop were found, and their expressed currents were not profoundly different in terms of their biophysical properties. Moreover, replacement of the III–IV loop of Ca$_{3.1}$ with the corresponding portion of a slowly inactivating Ca$_{1.2}$ had little effect on inactivation kinetics (37). These findings indicate that the III–IV loops of Ca$_3$ channels do not contain an inactivation ball as described for Na$^+$ channels.

Our results show that replacement of any of the cytoplasmic loops could not confer kinetics from the parental to the donor channel. This finding led us to examine domain(s) including membrane-spanning portions as well as loops, using half-half chimeras (IG$_{34}$ and IG$_{34}$) and serial chimeras (IG$_{42}$, IG$_{34}$, and IG$_{34}$). A general pattern obtained from comparing the current kinetics of the two parent and chimeric channels is that the more Ca$_{3.3}$ donated to the chimeric channels, the more Ca$_{3.3}$-like its kinetics became. This pattern can be more easily appreciated by plotting the percentage of amino acids from Ca$_{3.3}$ in the chimeric channels versus the inactivation or activation time constants (Fig. 8). The general pattern strongly supports our main conclusion that structural elements that determine the kinetics of Ca$_3$ channels are not localized but distributed over the whole structure.

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$^2$ S.-W. Jeong, B.-G. Park, and J.-H. Lee, unpublished data.

### Table 1

Summary of the voltage-dependent properties of Ca$_{3.1}$, Ca$_{3.3}$, and chimeras

| Constructs | $V_{50}$ $\pm$ mV | $k$ $\pm$ ms | $\tau_{act}$ $\pm$ ms | $V_{50}$ $\pm$ mV | $k$ $\pm$ ms | $\tau_{inact}$ $\pm$ ms |
|------------|--------------------|--------------|------------------------|---------------------|-------------|------------------------|
| Ca$_{3.1}$ | $-40.9 \pm 0.4$ (6) | $5.4 \pm 0.4$ | $1.4 \pm 0.1$ (6) | $-63.9 \pm 0.1$ (5) | $5.5 \pm 0.1$ | $7.2 \pm 0.4$ (6) |
| Ca$_{3.3}$ | $-33.5 \pm 0.1$ (7) | $4.9 \pm 0.1$ | $22.8 \pm 1.5$ (8) | $-56.0 \pm 0.2$ (8) | $5.5 \pm 0.2$ | $97.0 \pm 3.8$ (7) |
| Ca$_{3.3}$ | $-41.6 \pm 0.6$ (6) | $4.9 \pm 0.5$ | $2.0 \pm 0.3$ (8) | $-63.9 \pm 0.1$ (4) | $4.1 \pm 0.1$ | $15.4 \pm 0.8$ (7) |
| Ca$_{3.3}$ | $-51.3 \pm 0.4$ (5) | $5.8 \pm 0.4$ | $39.3 \pm 5.6$ (5) | $-51.4 \pm 0.3$ (8) | $4.6 \pm 0.3$ | $66.1 \pm 0.9$ (5) |
| Ca$_{3.3}$ | $-40.6 \pm 0.4$ (5) | $4.8 \pm 0.4$ | $0.9 \pm 0.1$ (8) | $-65.5 \pm 0.2$ (6) | $4.7 \pm 0.1$ | $5.8 \pm 0.3$ (8) |
| Ca$_{3.3}$ | $-40.7 \pm 0.2$ (8) | $4.5 \pm 0.2$ | $10.5 \pm 0.7$ (7) | $-60.4 \pm 0.4$ (8) | $5.1 \pm 0.3$ | $98.1 \pm 3.1$ (7) |
| Ca$_{3.3}$ | $-38.0 \pm 0.5$ (5) | $5.5 \pm 0.4$ | $18.6 \pm 1.1$ (6) | $-57.7 \pm 0.3$ (7) | $5.6 \pm 0.3$ | $124.4 \pm 5.1$ (6) |
| Ca$_{3.3}$ | $-29.6 \pm 0.3$ (6) | $5.9 \pm 0.3$ | $10.8 \pm 0.6$ (7) | $-51.7 \pm 0.3$ (5) | $4.9 \pm 0.3$ | $27.3 \pm 2.5$ (7) |
| Ca$_{3.3}$ | $-42.2 \pm 0.4$ (8) | $6.5 \pm 0.4$ | $6.2 \pm 0.2$ (8) | $-63.6 \pm 0.3$ (7) | $4.7 \pm 0.2$ | $27.5 \pm 0.6$ (8) |
| Ca$_{3.3}$ | $-44.1 \pm 0.5$ (5) | $6.6 \pm 0.4$ | $2.2 \pm 0.1$ (7) | $-66.2 \pm 0.2$ (6) | $4.4 \pm 0.1$ | $13.3 \pm 0.4$ (7) |
| Ca$_{3.3}$ | $-42.3 \pm 0.4$ (8) | $5.1 \pm 0.4$ | $8.2 \pm 0.7$ (7) | $-64.4 \pm 0.3$ (7) | $6.1 \pm 0.2$ | $50.1 \pm 3.0$ (7) |
Fig. 8. Correlation between Cav3.3 content in the chimeras and the activation or inactivation kinetics. The percentage of amino acids donated by Cav3.3 in each chimera was plotted against the activation or inactivation time constants. Most chimeric channels have the tendency to display current kinetics that correlate well with the relative percentage of Cav3.3 they contain. The data represent the means ± S.E. ○, activation tau; ●, inactivation tau.

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