Roles of Molecular Regions in Determining Differences between Voltage Dependence of Activation of CaV3.1 and CaV1.2 Calcium Channels*

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Voltage-dependent calcium channels are classified into low voltage-activated and high voltage-activated channels. We have investigated the molecular basis for this difference in voltage dependence of activation by constructing chimeras between a low voltage-activated channel (CaV3.1) and a high voltage-activated channel (CaV1.2), focusing on steady-state activation properties. Wild type and chimeras were expressed in oocytes, and two-electrode voltage clamp recordings were made of calcium channel currents. Replacement of domains I, III, or IV of the CaV3.1 channel with the corresponding domain of CaV1.2 led to high voltage-activated channels; for these constructs the current/voltage (I/V) curves were similar to those for CaV1.2 wild type. However, replacement of domain II gave only a small shift to the right of the I/V curve and modulation of the activation kinetics but did not lead to a high voltage-activating channel with an I/V curve like CaV1.2. We also investigated the role of the voltage sensor S4 by replacing the S4 segment of CaV3.1 with that of CaV1.2. For domain I, there was no shift in the I/V curve as compared with CaV3.1, and there were relatively small shifts to the right for domains III and IV. Taken together, these results suggest that domains I, III, and IV (rather than domain II) are apparently critical for channel opening and, therefore, contribute strongly to the difference in voltage dependence of activation between CaV3.1 and CaV1.2. However, the S4 segments in domains I, III, and IV did not account for this difference in voltage dependence.

By regulating the influx of calcium, voltage-dependent calcium channels play important roles in a wide range of cellular processes, such as neurotransmitter release, second messenger cascades, cardiac excitation and contraction, and gene regulation. The main pore-forming subunit (α1) of calcium channels has four homologous domains (I-IV), each comprising six transmembrane segments (S1-S6) (1, 2). Calcium channels have been classified into three families according to their electrophysiological, pharmacological, and amino acid sequences. The CaV1 family displays L-type currents and includes α1G, α1C, α1D, and α1S, the CaV2 family shows P/Q-, N-, and R-type currents and comprises the corresponding α1A, α1B, and α1E calcium channels, and the CaV3 family displays T-type currents and includes α1G, α1M, and α1J (3–7). CaV3 channels have markedly different biophysical characteristics from the other families; CaV3 channels are low voltage-activating and have fast inactivation and small single channel conductance. On the other hand, CaV1 channels, for instance, are high voltage-activating and show little inactivation (with Ba2+ as the charge carrier) and large single channel conductance (2, 8, 9).

Voltage-dependent ion channels form a huge family comprising potassium and sodium as well as calcium channels. For this family, the S4 segments have a conserved amino acid sequence with four to eight positively charged residues (arginines or lysines), each separated by two hydrophobic residues. This segment plays a vital role in voltage-dependent activation (10). The suggested crystal structure for the K+AP potassium channel shows a possible "paddle-like" movement of the S4 segment during depolarization (11, 12). Most research on the molecular basis of activation has previously been carried out with potassium channels and, to a lesser extent, sodium channels. For calcium channels there are very few studies on the molecular mechanisms of activation (13, 14), and voltage gating in the calcium channels is not yet clear. However, because the four S4 segments in the calcium channel have similar conserved motifs of charged amino acids as in sodium and potassium channels, it is thought that the S4 segment in calcium channels has a similar function as in sodium and potassium channels (2, 15).

For calcium channels, it is not understood at a molecular level why CaV3 channels are low voltage-activating, whereas CaV1 and CaV2 channels are high voltage-activating. One might perhaps expect that these differences in activation are due to differences in S4 segments. In the present work we have investigated the molecular basis for these differences in voltage dependence of activation between CaV3 channels and CaV1 channels, where there are striking differences between the two types of wild type channels. For this, we have created chimeras between CaV3.1 and CaV1.2 channels, swapping the four domains in CaV3.1 separately with the corresponding region in CaV1.2 to study the effects of each domain on channel activation. Similarly, we have investigated the role of the S4 segments by swapping these segments between the two channels. In this way we have aimed to locate key molecular regions underlying the differences in activation properties, focusing on the striking differences (which make a chimeric study possible) in voltage dependence of steady-state activation between these high and low voltage-activated channels.

EXPERIMENTAL PROCEDURES

Materials—The following cDNA clones were used: mouse brain CaV3.1 (α3A), GenBank™ accession number AJ012569 (7); rabbit cardiac CaV1.2 (α1C), GenBank™ accession number X15539 (19); rat car-

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The four transmembrane domains (I-IV) are represented by four letter codes. GGGG refers to wild type CaV3.1 (thin lines), and CCCC refers to CaV1.2 (thick or shaded lines). Four chimeras were constructed by swapping domains I-IV, represented by CGGG, GCGG, GGCG, and GGCC respectively. Another four chimeras were made by replacing the S4 segment in CaV3.1 by the corresponding segment in CaV1.2 for domains I-IV, represented by IS4C, IIS4C, IIIS4C, and IVS4C, respectively.

For the domain I chimera, CGGG, amino acids 81–398 of CaV3.1 were replaced by residues 464–672 in CaV1.2. The PCR fragment was digested with these enzymes to yield a 1188-bp product and ligated into the similarly digested wild type CaV3.1-PGEM-HEL. For the domain II chimera, GCGG, residues 399–967 of CaV3.1 were replaced by residues 439–786 of CaV1.2. The final chimeric PCR fragment included Csp45I and PinAI sites in the CaV3.1 sequences on either side of the CaV1.2 sequence; after digestion (3008-bp product) it was ligated into CaV3.1-pGEM-HEL using these two enzymes. For the domain III chimera, GGCG, residues 968–1541 of CaV1.2 were replaced by residues 786–1199 of CaV3.1. The final chimeric PCR product contained BamHI and XhoI sites; after digestion (2573-bp product), it was inserted with the same enzymes into CaV3.1-HindIII-KpnI-pUC18. The latter is a subclone of wild type CaV3.1, which had been made previously by inserting the HindIII-KpnI subfragment of CaV3.1 (4543 bp) into the vector pUC18. For the domain IV chimera, GGCC, residues 1542–1861 of CaV3.1 were replaced by residues 1200–1568 of CaV1.2. The chimeric PCR product included EcoRI and KpnI sites (1499 bp after digestion); it was inserted into CaV3.1-pcDNA3 between the above sites. For the S4 chimera in domain I, IS4C, residues 175–199 of CaV3.1 were replaced by residues 262–286 of CaV1.2. The chimeric PCR product included KspI and Csp45I sites; the digested product (500 bp) was inserted into CaV3.1-pGEM-HEL using these two enzymes. For the S4 chimera in domain II, IIS4C, residues 830–856 of CaV3.1 were replaced by residues 644–672 in CaV1.2. The PCR fragment contained BamHI and BsaAI sites and was digested, after digestion (741-bp product) into CaV3.1-HindIII-KpnI-pUC18. For the S4 chimera in domain III, IIIS4C, residues 1377–1401 of CaV3.1 were replaced by residues 1023–1047 of CaV1.2. The PCR product contained Stul and PinAI sites and, after digestion (961-bp product), was ligated into CaV3.1-HindIII-KpnI-pUC18. For the S4 chimera in domain IV, IVS4C, residues 1716–1740 of CaV1.2 were replaced by residues 1355–1379 of CaV3.1. The PCR fragment included EcoRI and KpnI sites and, after digestion (1532 bp), was inserted into CaV3.1-pGEM-HEL using these two enzymes. Finally, the above inserts in pcDNA3 were subcloned into CaV3.1-pGEM-HEL using EcoRI and NotI digestion; the inserts in CaV3.1-HindIII-KpnI-pUC18 were transferred to CaV3.1-pGEM-HEL by HindIII and XhoI digestion. The sequences of the chimeras were verified by DNA sequencing; the joins and the entire PCR inserts were sequenced. Ca3.1 and chimeric cDNAs (in pGEM-HEL) were linearized with MluI; CaV1.2 (in pcDNA3) was linearized with Asp718; $\beta_2$ and $\alpha_2\delta$ (both in pcDNA3) were linearized with NotI. Capped cRNAstrans were synthesized in vitro using T7 MEGAAscript (Ambion).

Electrophysiological Recording and Data Analysis—DuPont stage VI oocytes were prepared from Xenopus laevis frogs using standard techniques (24, 25). Each oocyte was injected with 10–20 ng of cRNA in a volume of 50 nl. For co-injection, the ratio of $\alpha_2\beta_2\gamma\delta$ was about 3:1:1:1 by weight. Oocytes were incubated at 19 °C for 2–4 days in modified Barth’s solution (88 mm NaCl, 1 mm KCl, 2.4 mm NaHCO3, 0.82 mm
For electrophysiological recording, oocytes were held in a 50-μl recording chamber and perfused with barium solution (40 mM Ba(OH)\textsubscript{2}, 50 mM NaOH, 2 mM KOH, and 5 mM HEPES, adjusted to pH 7.4 with methanesulfonic acid). The calcium channel currents (i.e. with Ba\textsuperscript{2+} as charge carrier) were measured at 22–25°C by the 2-electrode voltage-clamp technique using a Geneclamp\textsuperscript{500} amplifier (Axon Instruments) as described previously (24, 25). Currents were filtered at 2 kHz and sampled at 4 kHz using a CED1401Plus interface with CED data acquisition software. The membrane potential of oocytes was held at -80 mV. To create the current-voltage (I/V) relationship, Ba\textsuperscript{2+} currents were elicited by a series of 500-ms pulses every 10 s, from -70 mV to +70 mV in a 10-mV step. This was followed by twenty 200-ms hyperpolarizing pulses every 2 s to -90 mV for subsequent leak and capacity current subtractions. In cases where channel currents were particularly small (i.e. for small voltage steps with high voltage-activating channels/chimeras), a low signal to background ratio did not always allow reliable determination of channel currents.

Electrophysiology data was acquired and analyzed by CED software, with Origin 5.0 used for further analysis and curve-fitting. The I/V curves were obtained for peak current amplitudes and were fitted with the Boltzmann equation, \( I = (V - V_{rev})G_{max}/(1 + \exp(V_{0.5} - V/k)) \), where \( I \) is the measured peak current, \( V \) is test potential, \( V_{rev} \) is the reversal potential, \( G_{max} \) is the maximum conductance, \( V_{0.5} \) is the potential for half-maximal activation, and \( k \) is the slope parameter. The activation times were taken as the times from 20 to 80% of maximum current (\( t_{20-80} \)). Inactivation time courses were fit with a single exponential of the form \( t_{inact} = \tau_{inact} \exp(-t/\tau_{inact}) \). Experiments were conducted at 22–25°C and 95% humidity, with 5% CO\textsubscript{2} and 5% O\textsubscript{2}.

**RESULTS**

Voltage Dependence of Activation for Wild Type Channels—The properties of the wild type Ca\textsubscript{v}1.2 and Ca\textsubscript{v}3.1 were first compared when expressed in oocytes using two-electrode voltage-clamp recordings of calcium channel currents with Ba\textsuperscript{2+} as charge carrier using identical laboratory conditions. As expected (7, 25), Ca\textsubscript{v}3.1 wild-type currents were transient and activated at low voltages, whereas Ca\textsubscript{v}1.2 wild-type currents were sustained and high voltage-activating (Fig. 2, A–C). The difference in voltage dependence of activation between these channels can also be seen for the normalized currents (Fig. 2D) and for the Boltzmann parameters shown in Fig. 2E. The fact that there are marked differences in the voltage dependence of activation between Ca\textsubscript{v}1.2 and Ca\textsubscript{v}3.1 allows a chimeric ap-
approach to the study of molecular regions that contribute to the differences in activation between these two channels.

When CaV3.1 was expressed in the presence of \(\alpha_2\delta/\beta_2\) auxiliary subunits, there was no change in the voltage dependence of the normalized I/V curves compared with expression of CaV3.1 alone (Fig. 2). For the CaV1.2 channel, co-expression with auxiliary subunits increased the current by about 2-fold and shifted the I/V curves to the left (Fig. 2). For a clean approach, we would have preferred to carry out experiments throughout in the absence of auxiliary subunits. For the S4 chimeras, we indeed carried out comparisons with wild type in the absence of auxiliary subunits both for test and control. However, for chimeras CGGG, GGGC, and GCGG, comparisons with wild type were mainly made in the presence of subunits because currents were rather small in the absence of subunits, although a very limited number of experiments were also carried out in the absence of subunits. For GCGG, where currents are larger, we carried out detailed comparisons with and without subunits, because the II/II linker (where the \(\beta\) subunit binds) is swapped in this chimera.

Effects of Domain I and Its S4 Segment on Voltage Dependence of Activation—To investigate the role of domain I in determining the differences in voltage dependence of activation between CaV1.2 and CaV3.1 we have replaced the S1 to S6 region of this domain in CaV3.1 with the corresponding domain in CaV1.2 to form chimera CGGG (Fig. 1). Upon expression in oocytes (with \(\alpha_2\delta/\beta_2\)), the calcium channel currents for this chimera were like CaV1.2, and the I/V curves were shifted to higher voltages, similar to the I/V curves for CaV1.2 (Fig. 3A). For comparison, the curves for wild type channels (with \(\alpha_2\delta/\beta_2\)) are also shown using the curves in Fig. 2D (dashed line, CaV3.1; dotted line, CaV1.2). A sample current trace for the CGGG chimera with a voltage step to +10 mV is shown in the inset. B, the normalized I/V curve is shown for chimera IS4C (\(n = 8\), \(\text{V}_0\), no auxiliary subunits), with normalization to the value at +30 mV (0.55 ± 0.12 A). The curves for wild type channels (no auxiliary subunits) are also shown using the curves from Fig. 2D (dashed line, CaV3.1; dotted line, CaV1.2). A sample current trace for IS4C is shown in the inset (voltage step to +30 mV). C, the Boltzmann parameters \(V_{0.5}\) and \(k\) are shown for CGGG and IS4C in comparison with appropriate wild type controls. *, significant difference \((p < 0.05)\) compared with wild type CaV3.1. +, significant difference from wild type CaV1.2.

Effect of Domain II and Its S4 Segment on Voltage Dependence of Activation—The contribution of domain II to determining differences in voltage dependence of activation was studied
using chimera GCGG. More precisely, this chimera has the second domain and the I/II linker in CaV3.1 replaced by the corresponding sequence for CaV1.2 (Fig. 1). GCGG and wild type channels were first compared in the presence of auxiliary subunits. As compared with CaV3.1 wild type channel, the I/V curve was not shifted for the chimera (Fig. 4A); the Boltzmann parameters for the chimera remained as for CaV3.1 (Fig. 4D). However, because the auxiliary β subunit binds to the I/II linker in the CaV1.2 wild type channel, giving a leftward shift in the I/V curve, in the above experiments the β subunit may have also caused an underlying shift for the chimera (removal of the auxiliary subunits would, therefore, be expected to uncover a shift to the right). Indeed, when experiments were carried out in the absence of auxiliary subunits, there was a shift to the right in the I/V curve for the chimera as compared with CaV3.1 but not so far to the right as to be similar to CaV1.2 (Fig. 4B, Boltzmann parameters Fig. 4D). Therefore, taken together, the data for this chimera indicate that domain II only contributes to a small extent to the differences in voltage dependence of activation between the two channels. To determine whether this effect is due to the S4 segment of domain II, we replaced this segment in CaV3.1 with that for CaV1.2. We found that this replacement gave a similar shift in the I/V curves (Fig. 4C) as for chimera GCGG without auxiliary subunits. Also, the Boltzmann parameters were not significantly different from those for chimera GCGG without subunits. This suggests that the (smaller) effect of domain II on voltage differences of activation is due largely if not entirely to the S4 segment.

**Effect of Domains III and IV and Their S4 Segments on Voltage Dependence of Activation**—The results for domains III and IV were similar and, therefore, are collected together here. For chimeras GCGG and GGGC (in the presence of αδβ2), the I/V curves were shifted to the right as compared with CaV3.1 and in fact were similar to the I/V curves for CaV1.2 (Figs. 5A and 6A), with Boltzmann parameters not significantly different from those for CaV1.2 (Figs. 5C and 6C) (except for the k value for GGGC, which was somewhat larger than CaV1.2). Thus, both domains III and IV contribute strongly to the differences in voltage dependence of activation, as for domain I. Regarding the contribution of the S4 segments in domains III and IV, for the chimeras with S4 segments of CaV3.1 replaced by CaV1.2, the I/V curves were shifted to the right (as compared with

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**Fig. 4. The role of domain II and its S4 segment in determining differences in voltage dependence of activation between CaV3.1 and CaV1.2.** A, the normalized I/V curves are shown for GCGG with αδβ2 subunits (n = 10), normalized at −30 mV (−0.99 ± 0.17 μA). The curves for wild type channels (in the presence of αδβ2) are also shown, again from the curves in Fig. 2D (dashed line, CaV3.1; dotted line, CaV1.2). A sample current trace for GCGG (with αδβ2) with voltage step to −30 mV is shown in the inset. B, the normalized I/V curve for GCGG in the absence of αδβ2 subunits is shown (n = 11), normalized at −10 mV (−0.30 ± 0.03 μA). Curves for wild type channels (in the absence of αδβ2) are again as in Fig. 2D. A sample current trace for GCGG without αδβ2 (step to −10 mV) is shown in the inset. C, the normalized I/V curve is shown for IIS4C (n = 7, no auxiliary subunits), normalized at 0 mV (−0.32 ± 0.04 μA). Wild type I/V curves (no auxiliary subunits) are shown as before (dashed line, CaV3.1; dotted line, CaV1.2). A sample current trace for IIS4C with voltage step to 0 mV is shown in the inset. D, the Boltzmann parameters V50 and k are shown for GCGG with and without αδβ2, and for IIS4C in comparison with appropriate wild type controls. *, significant difference (p < 0.05) from appropriate wild type CaV1.2. +, significant difference (p < 0.05) from appropriate wild type CaV3.1.
The role of domain III and its S4 segment in determining differences in voltage dependence of activation between CaV3.1 and CaV1.2. A, the normalized I/V curve is shown for chimera GGCG (with $\alpha_2$/$\beta_3$ subunits, $n = 6$) with normalization to the current at +10 mV ($-0.31 \pm 0.05 \mu A$). The curves for wild type channels (with $\alpha_2$/$\beta_3$) are also shown as before (dashed line, CaV3.1; dotted line, CaV1.2). A sample current trace for the GGCG chimera with a voltage step to +10 mV is shown in the inset. B, the normalized I/V curve is shown for chimera IIIS4C ($n = 7$), no auxiliary subunits) with normalization to the value at -20 mV ($-0.41 \pm 0.06 \mu A$). The curves for wild type channels (no auxiliary subunits) are again taken from Fig. 2D (dashed line, CaV3.1; dotted line, CaV1.2). A sample current trace for IIIS4C is shown in the inset (voltage step to -20 mV). C, the Boltzmann parameters $V_{0.5}$ and $k$ are shown for GGCG and for IIIS4C in comparison with appropriate wild type controls. *, significant difference ($p < 0.05$) compared with wild type CaV3.1; **, significant difference from wild type CaV1.2.

CaV3.1 wild type) but not so far to the right as CaV1.2 (Figs. 5B and 5B). The Boltzmann parameters indicated only relatively small but significant differences from values for CaV3.1 wild type (Figs. 5C and 6C). Taken together, the data suggest that domains III and IV contribute strongly to the difference in voltage dependence of activation but that the S4 segments in these domains do not contribute strongly.

When we carried out limited experiments for the GGCG and GGGG chimeras (as well as for CGGG) in the absence of subunits, we found mean values for $V_{0.5}$ of 7 mV ($n = 2$), 12 mV ($n = 2$), and -3 mV ($n = 3$), respectively, and $k$ values of 15, 16, and 11 mV. These are qualitatively similar to the values found in the presence of subunits. Because the auxiliary subunits had little effect on these chimeras and as little effect was expected because the I/II linker was G rather than C, we did not consider it useful to pursue detailed experiments further with CGGG, GGGG, and GGGG without subunits, particularly given the small size of currents.

Time Courses of Activation and Inactivation—Activation time courses were analyzed, and sample time courses are shown in Figs. 2–6. In some cases, measurements could not be carried out because of interfering capacitative spikes that could not always be reliably subtracted from the relatively small calcium channel currents. Fig. 7A shows that activation times were somewhat smaller for CaV3.1 than for CaV1.2. For chimera GCGG, activation times were larger than for CaV3.1 (Fig. 7A). This suggests that for the kinetics of activation, domain II is also important, in contrast to the results for steady-state activation obtained above. However, for kinetics the data are more difficult to interpret given the small difference in activation time between CaV3.1 and CaV1.2, and the differences in activation thresholds between the constructs. For the chimera with S4 swapped in domain II, activation times were somewhat larger than for CaV3.1 but still smaller than for GGGG. This indicates that IIIS4 makes some contribution to activation kinetics but does not explain the whole effect. For the remaining S4 chimeras (Fig. 7B), the time courses of activation were not slower than for CaV3.1 (for IS4C, the time course was even faster than for CaV3.1 at low voltages). Thus, the data indicate that the S4 regions in domains I, III, and IV do not contribute to slowing the activation kinetics of wild type CaV1.2.

As shown in the sample currents in Figs. 3A, 4A and B, 5A, and 6A, currents for chimeras CGGG, GGGG, and GGGG, were non-inactivating, whereas for GGGG there was no fast inactivation. These observations were also found for every single recording that we made for these chimeras. Therefore, all four domains must contribute to the fast inactivation seen with CaV3.1. On the other hand, for the S4 chimeras there was always fast inactivation, similar to CaV3.1, although there was some variability in inactivation time constants (Fig. 7C), which were always vastly different from the non-inactivating CaV1.2. Thus, taken together, the data indicate that the S4 regions in...
DISCUSSION

This study has investigated the contribution of molecular regions to the difference in voltage dependence of activation between a low and a high voltage-activating channel (CaV3.1 and CaV1.2, respectively), and we have focused mainly on the steady-state voltage-dependent differences in the I/V curves, where there are striking differences between the two types of channel. Our data showed that, for these steady-state data, replacing domains I, III, and IV of the CaV3.1 channel with the corresponding domain for CaV1.2 led to high voltage-activated channels (40–45-mV shifts); for these constructs the I/V curves were similar to those for CaV1.2 wild type. However, replacement of domain II of CaV3.1 with the corresponding domain of CaV1.2 did not give a high voltage-activating channel like CaV1.2, although the I/V curve for this chimera was shifted to the right somewhat (around 15 mV) as compared with CaV3.1. These results suggest that domains in the calcium channel play different roles in the channel activation process; domains I, III, and IV are apparently critical for channel opening and, therefore, contribute strongly to the difference in voltage dependence of activation between CaV3.1 and CaV1.2, whereas domain II is less important in regulating the voltage dependence of activation between CaV3.1 and CaV1.2.

How might these differences in voltage dependence of activation be explained? Current models of ion channel function involve movement of the voltage sensor followed by channel opening (10). Therefore, for the CaV3.1 channel, voltage sensors would move at low voltages, whereas for the CaV1.2 channel they would move at high voltages. The simplest model would be that the voltage sensors in all four domains should move before the channel opens. In that case, replacing any one of the domains in CaV3.1 with CaV1.2 should produce a high voltage-activating channel (because the voltage sensor in a single CaV1.2 domain in the chimera would not move at low voltages). Our results for chimeras CGGG, GGCG, and GGGC (i.e. domains I, III, and IV) are consistent with this. However, surprisingly, the domain II chimera was not activated at high voltages. The simplest explanation of our results would be that channel opening is produced by the necessary prior movement of the voltage sensors in domains I, III, and IV; if any of these are in the resting position, the channel cannot be opened. The role of the voltage sensor in domain II is less critical, although movement of the voltage sensor in domain II does seem to modulate the voltage dependence of steady-state activation.

Fig. 6. The role of domain IV and its S4 segment in determining differences in voltage dependence of activation between CaV3.1 and CaV1.2. A, the normalized I/V curve is shown for chimera GGGC (with α,β subunits) (n = 6 (A)) with normalization to the current at +10 mV (−0.36 ± 0.05 μA). The curves for wild type channels (with α,β subunits) are also shown as before (dashed line, CaV3.1; dotted line, CaV1.2). A sample current trace for the GGGC chimera with a voltage step to +10 mV is shown in the inset. B, the normalized I/V curve is shown for chimera IVS4C (n = 9 (V), no auxiliary subunits), with normalization to the value at −10 mV (−0.40 ± 0.09 μA). The curves for wild type channels (no auxiliary subunits) are again taken from Fig. 2D (dashed line, CaV3.1; dotted line, CaV1.2). A sample current trace for IVS4C is shown in the inset (voltage step to −10 mV). C, the Boltzmann parameters V0.5 and k are shown for GGGC and for IVS4C in comparison with appropriate wild type controls. *, significant difference (p < 0.05) compared with wild type CaV3.1. †, significant difference from wild type CaV1.2.
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The auxiliary subunits did not give a shift in the voltage dependence for whole domain swapping of domains III and IV. The voltage shift due to the S4 segment makes a contribution of some 8 or 15 mV to the shift in the I/V curve as compared with CaV3.1 wild type. This would suggest that other regions in domain I contribute to the difference in voltage dependence of activation. On the other hand, for domain II, replacement of the S4 region in CaV3.1 with CaV1.2 gave similar (though not identical) shifts in I/V curves as for replacement of the whole of domain II. This would suggest that the relatively small shifts observed for domain II are entirely due to the S4 region. Finally, for domains III and IV, the S4 makes a contribution of some 8 or 15 mV to the shift in the I/V curves, but this does not explain the much larger shifts (40–45 mV) observed for whole domain swapping of domains III and IV. This suggests that other regions of domains III and IV must also contribute to determining differences between the two channels.

Although the time courses of activation of CaV1.2 and CaV3.1 are different, the magnitude of the difference in activation kinetics is not so striking as the differences in voltage dependence of steady-state activation, so the time course did not lend itself so readily to a detailed chimeric analysis. However, our results suggested that, in contrast to the steady-state results outlined above, domain II might make a contribution to activation kinetics because the GCGG chimera had slower activation than for CaV3.1 wild type. There was a partial contribution to this effect from the S4 region in domain II (which also produced some slowing of activation kinetics), but this did not explain the whole effect of the GCGG chimera. The apparent effect of the GCGG chimera in producing a slowing of activation could arise from other parts of domain II, such as the pore region. We might also perhaps expect slowing of activation kinetics for the chimeras of the other domains, but the small size of the chimeric currents did not allow detailed analysis. However, we did observe that, for the S4 chimeras of domains I, III, and IV, there was no contribution to slowing of activation of CaV3.1 (IS4 was even faster), again indicating that the S4 regions in these domains do not primarily determine the differences in activation kinetics between the two wild type channels.

Although the S4 segment is of primary importance in gating, other regions must also contribute in determining the voltage dependence. For instance, the S2 and S3 regions also form the voltage-sensing in calcium channels and, hence, may determine differences in voltage dependence of activation between high voltage-activating and low voltage-activating calcium channels. Further...
thermore, point mutations in the pore (P) region of Ca₃.1 showed that shifts in I/V curves can occur (32, 33). Thus, it will be interesting in future studies to investigate the possible role of the pore helices S5-P-S6 as well as the voltage sensor domain S2/ S3/S4 in determining differences in voltage dependence of activation. Other regions that might contribute are the intracellular linkers between the domains; our chimeras GCCG, GGCG, and GGCC also involved swapping linkers (I/II, II/III, III/IV respectively). However, isoforms of Ca₃.1 that are alternatively spliced in the II/III and III/IV linker do not show shifts in I/V curves that are greater than some 5 mV, suggesting that these linkers do not contribute to determining whether the channel is low or high voltage-activating (34). Also, by studying other splice variants of Ca₃.3 (35), it similarly appears that the C terminus does not contribute to the voltage dependence of activation.

Another possible reason why the S4 regions did not contribute much to determine voltage-dependent differences in activation could be because of the marked homology of the S4 regions that were swapped in our chimeras. The alignments are shown in Fig. 8 and compared with the S4 region of the Shaker potassium channel. For the latter channel, the key region, which contributes to gating charge movement and is involved in movement across the membrane upon gating (10), involves charged residues 362, 365, 368, and 371 (Shaker residue numbering, Fig. 8). Over this latter region there is even more homology between S4 regions of the different domains of the two calcium channels studied here. In particular, there is the most similarity between the S4 of domains I and III and between domains II and IV. This mirrors our results where S4 chimeras in domains I and III gave no shift or a small shift in I/V curves, whereas S4 chimeras in domains II and IV gave larger shifts.

For calcium channels, unlike potassium and sodium channels, there are few papers that study the molecular mechanism for channel activation. Garcia et al. (13) made point mutations of charged residues in S4 segments of domains I-IV of a high voltage-activated L-type calcium channel. They showed that significant changes in activation properties were obtained for S4 regions of domains I and III rather than domains II and IV, indicating a differential role for these domains in activation. Furthermore, S4 regions of domains I and III each possess a proline residue (Fig. 8), and point mutations at these residues also show greater effects on activation than for mutations of the corresponding residues of domains II and IV (14). Again, this led to the conclusion that S4 regions in domains I and III contribute more to channel opening than domains II and IV. From our present results, we have also shown qualitative differences between the effects of S4 chimeras in domains I and III from the effects in IIVS4 and IVS4. However, our results are quantitatively different from the above papers (we found smaller shifts for IIVS4 and IIIS4), which probably reflects the fact that we did not change either charged or proline residues in our S4 chimeras.

There have been more previous studies on sodium channel activation than for the calcium channel, and it is interesting to compare our results with those for the sodium channel, which has a similar molecular structure formed from four homologous domains I-IV. As for the calcium channel, mutation of charged residues of the S4 segments in the four domains of the sodium channel shows that each S4 segment does not contribute equally to the voltage-dependent properties of the channel (16–18). Further studies of gating currents and of S4 movement using fluorescent tags indicate that S4 segments of domains I, II, and III of the sodium channel move first upon depolarization, leading to an open state; the IVS4 moves later to reveal a further open state. The IVS4 segment is then strongly involved in inactivation (36, 37). Our results in the present study suggest that the calcium channel may have some similarities with the activation of the sodium channel. Because we have shown that regions in domains I, III, and IV of the calcium channel are critical for channel opening, we suggest that the S2-S4 voltage-sensor regions of domains I, III, and IV may activate first in parallel, then the voltage sensor in domain II may be triggered to activate. Thus, domain II is less important in determining the voltage dependence of steady-state calcium channel activation, although it can be important in modulating the kinetics of activation. It will be very interesting to test this hypothesis by further experiments using fluorescence techniques.

Finally, as regards inactivation, we have shown that replacing any one of the domains I-IV of CaV₃.1 (fast inactivating) by CaV₁.2 (non-inactivating) led to a chimeric channel that was not fast-inactivating. Therefore, all four domains I-IV of the calcium channel seem to participate in the mechanism for fast inactivation of the CaV₃.1 calcium channel. This is consonant with previous work for many calcium channel types showing that regions from all four domains contribute to inactivation (38–43). Indeed, regions at the C terminus and segment III/S6 of the CaV₃.1 channel have already been shown to be involved in inactivation (42, 43). We have also shown that replacement of each of the S4 segments in domains I-IV of CaV₃.1 by CaV₁.2 does not remove the fast inactivation, although there was some modulation of the inactivation time courses. The fact that the chimeric S4 substitutions did not contribute markedly to the inactivation could again be because of their marked homology, or more likely, the data suggest that the S4 segments have no obvious role in inactivation of the calcium channel.

In summary, by domain-swapping experiments between the low voltage-activating channel CaV₃.1 and the high voltage-activating channel CaV₁.2 channel, we have shown that domains I, III, and IV rather than domain II are of key importance in determining the difference in voltage dependence of activation between these two channels. Chimeras with the S4 segments swapped did not explain the whole effects, suggesting roles for segments S1-S3 and/or S5-S6.

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