Characterization of C-terminal Splice Variants of Cav1.4 Ca\(^{2+}\) Channels in Human Retina*

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Voltage-gated Ca\(^{2+}\) channels (Ca\(_v\)) undergo extensive alternative splicing that greatly enhances their functional diversity in excitable cells. Here, we characterized novel splice variants of the cytoplasmic C-terminal domain of Ca\(_v\)1.4 Ca\(^{2+}\) channels that regulate neurotransmitter release in photoreceptors in the retina. These variants lack a portion of exon 45 and/or the entire exon 47 (Ca\(_v\)1.4Δex p45, Ca\(_v\)1.4Δex 47, Ca\(_v\)1.4ΔexΔex p45,47) and are expressed in the retina of primates but not mice. Although the electrophysiological properties of Ca\(_v\)1.4Δex p45 are similar to those of full-length channels (Ca\(_v\)1.4FL), skipping of exon 47 dramatically alters Ca\(_v\)1.4 function. Deletion of exon 47 removes part of a C-terminal automodulatory domain (CTM) previously shown to suppress Ca\(^{2+}\)-dependent inactivation (CDI) and to cause a positive shift in the voltage dependence of channel activation. Exon 47 is crucial for these effects of the CTM because variants lacking this exon show intense CDI and activate at more hyperpolarized voltages than Ca\(_v\)1.4FL. The robust CDI of Ca\(_v\)1.4Δex 47 is suppressed by CaBP4, a regulator of Ca\(_v\)1.4 channels in photoreceptors. Although CaBP4 enhances activation of Ca\(_v\)1.4FL, Ca\(_v\)1.4Δex 47 shows similar voltage-dependent activation in the presence and absence of CaBP4. We conclude that exon 47 encodes structural determinants that regulate CDI and voltage-dependent activation of Ca\(_v\)1.4, and is necessary for modulation of channel activation by CaBP4.

In the retina, voltage-gated Ca\(_v\)1.4 (L-type) Ca\(^{2+}\) channels are localized in the synaptic terminals of rod and cone photoreceptors where they mediate Ca\(^{2+}\) signals that trigger glutamate release at the first synapse in the visual pathway (1). In mice lacking expression of Ca\(_v\)1.4, there is a complete loss of photoreceptor synaptic transmission and a failure in photoreceptor synapse maturation (2–6). The importance of Ca\(_v\)1.4 for vision in humans is illustrated by the disorders associated with mutations in the CACNA1F gene encoding the pore-forming α\(_1\) subunit of Ca\(_v\)1.4. These include congenital stationary night blindness type 2 (CSNB2 (7)), X-linked cone-rod dystrophy (8, 9), and Åland island eye disease (10).

Compared with Ca\(_v\)1.2 channels that are prominent in the brain and heart, Ca\(_v\)1.4 channels activate at more negative voltages, and show very little inactivation during sustained depolarizations (11–13). It is thought that these properties support tonic glutamate release at the membrane potential of photoreceptors in darkness (−30 to −40 mV (14, 15)). Unlike other Ca\(_v\) channels, Ca\(_v\)1.4 does not undergo Ca\(^{2+}\)-dependent inactivation (CDI) (11–13), a negative feedback regulation by incoming Ca\(^{2+}\) ions. For Ca\(_v\)1 and Ca\(_v\)2 channels, CDI is mediated by calmodulin (CaM) binding to site(s) in the proximal C-terminal domain of the pore-forming α\(_1\) subunit (reviewed in Refs. 16 and 17)). In Ca\(_v\)1.4, a sequence in the distal C-terminal domain (C-terminal automodulatory domain, CTM) suppresses CDI through an intramolecular interaction with the proximal C-terminal domain (18–20). In addition to effects on CDI, the CTM inhibits voltage-dependent activation of Ca\(_v\)1.4. A CSNB2-causing mutation deletes the CTM from Ca\(_v\)1.4 (K1591X (21)), unmasks strong CDI, and causes a hyperpolarizing shift in the voltage dependence of activation (22), both of which would be expected to decrease the dynamic range of photoreceptor signal transmission (15).

Like other Ca\(_v\) channels, Ca\(_v\)1.4 undergoes alternative splicing that can greatly alter the functional properties of the channel. For example, a splice variant that removes a large fraction of the C-terminal domain including the CTM (Ca\(_v\)1.4 ex43*) is expressed in human retina and exhibits robust CDI and hyperpolarized activation voltages in transfected HEK-293 cells (23). Such properties, as in K1591X (22), might be expected to cause pathological changes in visual signaling. However, photoreceptors in the retina express CaBP4, a member of a family of Ca\(^{2+}\)-binding proteins (CaBPs) related to CaM (24). CaBP family members prevent CDI of Ca\(_v\)1 channels (25–27), in part by competing with CaM for binding sites on the channel (28–30). CaBP4 binds to the C-terminal domain of Ca\(_v\)1.4 channels containing the CTM, and enhances voltage-dependent activation. CaBP4 does not affect CDI, which is already nullified in full-length Ca\(_v\)1.4 channels (31, 32). Coexpression of CaBP4 with Ca\(_v\)1.4 channels lacking the CTM strongly suppresses CDI as in full-length channels (32). Therefore, splice variants lacking the

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2The abbreviations used are: CSNB2, congenital stationary night blindness type 2; CDI, Ca\(^{2+}\)-dependent inactivation; CTM, C-terminal automodulatory domain; CaM, calmodulin; CaBP, Ca\(^{2+}\)-binding protein; CT, C terminal domain; aa, amino acid; qPCR, quantitative PCR; ANOVA, analysis of variance; FL, full-length.
CTM may exhibit properties consistent with native photoreceptor Ca\(_2\).1 channels (i.e., no CDI (33)) in contrast to their properties in transfected HEK-293 cells (23).

Electrophysiological analysis of Ca\(_2\).1.4 is challenged by the small current densities produced by these channels in heterologous expression systems. One strategy to overcome this hurdle is to fuse a portion of Ca\(_2\).1.4 (e.g., the CTM) to the core of Ca\(_2\).1.2 or Ca\(_2\).1.3 channels, giving rise to more robust currents (23, 34). Another caveat is that virtually all studies to date have used auxiliary Ca\(_2\).1/Ca\(_2\).1.3 subunits that may not be associated with native Ca\(_2\).1.4 channel complexes in photoreceptors. Of the 4 Ca\(_2\).1/\(_2\).1.4 variants that have been characterized (35, 36), \(_2\).1.4ex45, \(_2\).1.4ex47, and \(_2\).1.4ex48 variants that have been characterized (23, 34), those lacking exon 47 have not.

To gain insights into how alternative splicing affects Ca\(_2\).1.4 channels containing \(_2\).1.4ex47, we analyzed the electrophysiological properties of new Ca\(_2\).1.4 splice variants that we discovered while isolating cDNAs encoding Ca\(_2\).1.4 from human retina. Unlike Ca\(_2\).1.4ex43*, which lacks the C-terminal 256 amino acids of the channel (23), these variants lack only exon 47, which deletes part of the CTM, but leaves the remaining C-terminal domain intact. We show that these variants are expressed at significant levels in human retina, and exhibit hyperpolarized voltages of activation and CDI similar to Ca\(_2\).1.4ex43*. CaBP4 binds to and inhibits CDI of channels lacking exon 47, although it does not further enhance the hyperpolarized voltage-dependent activation of these channels. We conclude that exon 47 encodes critical determinants for regulating CDI and activation in a heterologous expression system, but that the presence of CaBP4 would likely nullify the CDI while not affecting the activation properties of these variants in vivo. Our results highlight the importance of analyzing Ca\(_2\).1.4 channels in the presence of known modulators for understanding the impact of alternative splicing on the properties of the native channels.

Results

Identification of Novel Ca\(_2\).1.4 Distal C-terminal Splice Variants in Human Retina—Using RT-PCR to generate a cDNA construct corresponding to human Ca\(_2\).1.4 from retinal RNA, we amplified a shorter product than expected with primers flanking nucleotides 3913 to 5934 of the cDNA encoding the full-length channel (Ca\(_2\).1.4FL). Sequencing revealed that this fragment lacked the first 21 amino acids of exon 45 (p45) due to an alternative 3′ splice site in exon 45. This fragment was also missing exon 47 (Ca\(_2\).1.4ex45,47; Figs. 1, A–C, and 2, A and B). Interestingly, Ca\(_2\).1.4ex45,47 was detected in retinal samples from both human and monkey but not from mouse (Fig. 2A). The inability to measure Ca\(_2\).1.4ex45,47 in mouse retina could be due to expression of Ca\(_2\).1.4ex45,47 primarily in cone photoreceptors, which are more abundant in the retina of primates than mice. To test this, we compared the expression of Ca\(_2\).1.4FL and Ca\(_2\).1.4ex45,47 in the cone-rich macula of monkey retina. No significant difference was observed between the ratio of Ca\(_2\).1.4FL and Ca\(_2\).1.4ex45,47 in the macula compared with the peripheral retina of the monkey (Fig. 2, C and D). Therefore, Ca\(_2\).1.4ex45,47 is unlikely to be more enriched in cones compared with rods.

Quantitative PCR revealed that Ca\(_2\).1.4ex45,47 was highly expressed in human and monkey retina, albeit at 20–150 lower levels than Ca\(_2\).1.4FL (Fig. 2B–D; see Table 1 for primer sequences). In additional experiments, we detected Ca\(_2\).1.4 transcripts that had the single partial deletion of exon 45 (Ca\(_2\).1.4ex45) or full deletion of exon 47 (Ca\(_2\).1.4ex47). Of these, Ca\(_2\).1.4ex45 was the most abundant (Fig. 3, A–C).

Ca\(_2\).1.4 Variants Lacking Exon 47 Exhibit Robust CDI—Although C-terminal splice variants including Ca\(_2\).1.4ex45 have been characterized (23), those lacking exon 47 have not. Because deletion of exon 47 removes part of the CTM (Fig. 1, A and B), we predicted that its deletion might affect CDI and voltage-dependent activation. We tested this in whole cell patch clamp recordings of HEK293T cells transfected with Ca\(_2\).1.4FL, Ca\(_2\).1.4ex45, Ca\(_2\).1.4ex47, or Ca\(_2\).1.4ex45,47. Cells were cotransfected with cDNAs encoding the auxiliary \(_2\).1.4ex45,47, and \(_2\).1.4ex47 subunits that co-assemble with Ca\(_2\).1.4 in the retina (40). To study CDI, we compared inactivation of Ca\(_2\)\(_{2+}\) currents (\(I_{Ca}\)) with that of Ba\(_{2+}\) currents (\(I_{Ba}\)). Inactivation was measured as the residual current amplitude at the end of the pulse normalized to the peak current amplitude (Fractional I); CDI was calculated as \(F_{CDI}\) (difference in Fractional \(I_{Ca}\) and mean Fractional \(I_{Ba}\) at ∼20 mV).

As shown previously, Ca\(_2\).1.4FL currents showed little inactivation during 1-s depolarizations regardless of whether Ca\(_2\)\(_{2+}\) or

| Variant/species | Forward primer | Location forward | Reverse primer | Location reverse |
|-----------------|----------------|-----------------|----------------|-----------------|
| 1 Ca\(_2\).1.4FL/human | 5′-GAAGAATTCCTGATCCCTGTC-3′ | Ex44/45 jct | 5′-CCCTCTGAGATGACCCACCC-3′ | Ex47/48 jct |
| 2 Ca\(_2\).1.4ex45/47/monkey | 5′-GAAGAATTCCTGATCCCTGTC-3′ | Ex44/45 jct | 5′-CCCTCTGAGATGACCCACCC-3′ | Ex47/48 jct |
| 3 Ca\(_2\).1.4ex45/47/mouse | 5′-GAAGAATTCCTGATCCCTGTC-3′ | Ex44/45 jct | 5′-CCCTCTGAGATGACCCACCC-3′ | Ex47/48 jct |
| 4 Ca\(_2\).1.4ex45/47/human | 5′-GAAGAATTCCTGATCCCTGTC-3′ | Ex44/45 jct | 5′-CCCTCTGAGATGACCCACCC-3′ | Ex47/48 jct |
| 5 Ca\(_2\).1.4ex45/47/mouse | 5′-GAAGAATTCCTGATCCCTGTC-3′ | Ex44/45 jct | 5′-CCCTCTGAGATGACCCACCC-3′ | Ex47/48 jct |
| 6 Ca\(_2\).1.4ex45/47/human | 5′-GAAGAATTCCTGATCCCTGTC-3′ | Ex44/45 jct | 5′-CCCTCTGAGATGACCCACCC-3′ | Ex47/48 jct |
| 7 Ca\(_2\).1.4ex45/47/mouse | 5′-GAAGAATTCCTGATCCCTGTC-3′ | Ex44/45 jct | 5′-CCCTCTGAGATGACCCACCC-3′ | Ex47/48 jct |
| 8 Ca\(_2\).1.4ex45/47/human | 5′-GAAGAATTCCTGATCCCTGTC-3′ | Ex44/45 jct | 5′-CCCTCTGAGATGACCCACCC-3′ | Ex47/48 jct |
| 9 Ca\(_2\).1.4ex45/47/human | 5′-GAAGAATTCCTGATCCCTGTC-3′ | Ex44/45 jct | 5′-CCCTCTGAGATGACCCACCC-3′ | Ex47/48 jct |
| 10 Ca\(_2\).1.4ex45/47/human | 5′-GAAGAATTCCTGATCCCTGTC-3′ | Ex44/45 jct | 5′-CCCTCTGAGATGACCCACCC-3′ | Ex47/48 jct |
| 11 Ca\(_2\).1.4ex45/47/human | 5′-GAAGAATTCCTGATCCCTGTC-3′ | Ex44/45 jct | 5′-CCCTCTGAGATGACCCACCC-3′ | Ex47/48 jct |
| 12 Ca\(_2\).1.4ex45/47/human | 5′-GAAGAATTCCTGATCCCTGTC-3′ | Ex44/45 jct | 5′-CCCTCTGAGATGACCCACCC-3′ | Ex47/48 jct |
Ba\(^{2+}\) was used as the permeant ion (Fig. 4A). The same was true for Cav1.4Δex p45 (Fig. 4B). There was no significant difference in \(F_{\text{CDI}}\) for Cav1.4FL (0.05 ± 0.02, \(n = 4\)) and Cav1.4Δex p45 (0.03 ± 0.02, \(n = 8; p = 0.08\)). In contrast, deletion of exon 47 caused robust inactivation of \(I_{\text{Ca}}\), whereas \(I_{\text{Ba}}\) inactivation was unchanged (Fig. 4C). \(F_{\text{CDI}}\) was significantly greater (−12-fold) for Cav1.4Δex47 (0.60 ± 0.02, \(n = 13; p < 0.01, \text{by} \ t\) test) compared with Cav1.4FL. We next determined if exon 45 might act synergistically with exon 47 in regulating CDI. Although CDI was robust for Cav1.4Δex p45 (0.65 ± 0.03, \(n = 13\)), it was not significantly different from that for Cav1.4Δex47 (\(p = 0.08, \text{by} \ t\) test). These results indicate that exon 47 but not exon 45 contains critical determinants for suppressing CDI of Cav1.4FL.

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**FIGURE 1. Alternative Cav1.4 splice variants lacking portions of the C-terminal domain.** A, schematic of Cav1.4FL, highlighting CDI-regulatory motifs (IQ, EF), CTM, and exons 45 and 47. B, alignment of the sequence in the C-terminal domain of human Cav1.4 from amino acids 1693 to 1977 and corresponding sequence in Cav1.4Δex p45,47. The amino acids deleted in the variant are indicated by dots. Black line, CTM described by Wahl-Scott et al. (19). Dashed line, additional N-terminal sequence of the CTM described by Singh et al. (22). Gray line, previously reported determinants for CDI inhibition (19, 22). C, exon-intron junctions of exon 44 to exon 48 in Cav1.4FL, Cav1.4Δex p45, Cav1.4Δex47, and Cav1.4Δex p45,47.
C-terminal Ca,1.4 Splice Variants

Ca,1.4 Variants Lacking Exon 47 Exhibit Activation at More Negative Voltages Than Ca,1.4FL—In plots of current density against voltage (I–V), only Ca,1.4 channels lacking exon 47 exhibited an increase in current density, although both Ca,1.4Δex47 and Ca,1.4Δex p45,47 activated at more negative voltages than Ca,1.4FL (Table 2; Fig. 5, A–C). To more rigorously characterize the voltage dependence of channel activation, we plotted the normalized tail current amplitudes against voltage (I–V), only Cav1.4 channels lacking exon 47 exhibited steeper slopes for Cav1.4FL (Fig. 5, A). To test if splicing of exons 45 and 47 affected the interaction of CaBP4 with the channel, we used FLAG antibodies to immunoprecipitate FLAG-tagged Ca,1.4 variants in HEK-293 cells transfected alone or cotransfected with CaBP4. Western blotting analysis with anti-CaBP4 antibodies revealed the co-immunoprecipitation of CaBP4 with both FLAG-Ca,1.4FL and FLAG-Ca,1.4Δex p45,47 (Fig. 6A). As we have found previously for FLAG-tagged Ca,1.3 (42), channel protein was detected by Western blotting only after immunoprecipitation and not in the cell lysates presumably due to limited sensitivity of the FLAG antibodies. These co-immunoprecipitated proteins were not detected when control mouse IgG was used instead of anti-FLAG antibodies. Although these results suggested that CaBP4 binds equally well to both Ca,1.4 and Ca,1.4Δex p45,47, it was possible that our co-immunoprecipitation assay did not report subtle changes in CaBP4 binding affinity. Therefore, we compared CaBP4 binding to these variants in an ELISA binding assay. For these experiments, we generated SUMO-tagged fusion proteins corresponding to the CT of Ca,1.4FL and Ca,1.4Δex p45,47 and compared their binding to 96-well plates coated with GST-tagged CaBP4. Binding of Ca,1.4Δex p45,47 CT to CaBP4 was similar to that by Ca,1.4FL CT (Fig. 6B). Signals representing CaBP4 binding were specific.

Deletion of Exon 47 Does Not Affect Binding of Ca,1.4 to CaBP4, but Prevents Effects of CaBP4 on Activation—CaBP4 is a CaM-like modulator of Ca,1.4 in photoreceptors, and binds to the same site(s) as CaM in the C-terminal domain (CT) of Ca,1.4 (31, 32). To test if splicing of exons 45 and 47 affected the interaction of CaBP4 with the channel, we used FLAG antibodies to immunoprecipitate FLAG-tagged Ca,1.4 variants in HEK-293 cells transfected alone or cotransfected with CaBP4. Western blotting analysis with anti-CaBP4 antibodies revealed the co-immunoprecipitation of CaBP4 with both FLAG-Ca,1.4FL and FLAG-Ca,1.4Δex p45,47 (Fig. 6A). As we have found previously for FLAG-tagged Ca,1.3 (42), channel protein was detected by Western blotting only after immunoprecipitation and not in the cell lysates presumably due to limited sensitivity of the FLAG antibodies. These co-immunoprecipitated proteins were not detected when control mouse IgG was used instead of anti-FLAG antibodies. Although these results suggested that CaBP4 binds equally well to both Ca,1.4 and Ca,1.4Δex p45,47, it was possible that our co-immunoprecipitation assay did not report subtle changes in CaBP4 binding affinity. Therefore, we compared CaBP4 binding to these variants in an ELISA binding assay. For these experiments, we generated SUMO-tagged fusion proteins corresponding to the CT of Ca,1.4FL and Ca,1.4Δex p45,47 and compared their binding to 96-well plates coated with GST-tagged CaBP4. Binding of Ca,1.4Δex p45,47 CT to CaBP4 was similar to that by Ca,1.4FL CT (Fig. 6B). Signals representing CaBP4 binding were specific.

FIGURE 2. Expression of Ca,1.4FL and Ca,1.4Δex p45,47 in the retina. A, RT-PCR analysis of Ca,1.4 and Ca,1.4Δex p45,47 expression in human, monkey, and mouse retina with primers as indicated in Table 1 (rows 1 to 6). B, qPCR analysis of Ca,1.4 and Ca,1.4Δex p45,47 expression in human retina. Values represent mean ΔCt values (Ct values for Ca,1.4 and Ca,1.4Δex p45,47 normalized to the Ct values of the internal standard GAPDH) for all RNA preparations. (***, p < 0.001, unpaired t test; n = 3). Primers are indicated in Table 1 (rows 1, 2). C, qPCR analysis of Ca,1.4FL and Ca,1.4Δex p45,47 expression in monkey retina and fovea. Legend as described for B. Primers are indicated in Table 1 (rows 3, 4). D, fold-difference in transcript levels of Ca,1.4 and Ca,1.4Δex p45,47 in human retina and in monkey retina and macula. qPCR data in C were analyzed using the 2^−ΔΔCt as described by Livak et al. (41). Fold-differences in Ct values between Ca,1.4 and Ca,1.4Δex p45,47 were calculated after normalization to the GAPDH control. Data represent mean ± S.D. (n = 3).
C-terminal Cav1.4 Splice Variants

FIGURE 3. Comparison of expression of Cav1.4 variants with C-terminal splice variants in the human retina. A, qPCR analysis of the expression of Cav1.4FL and exon 45/47 variants in human retina. Legend as described in Fig. 2B, primers as indicated in Table 1 (rows 2, 7, to 9). B, fold-difference in transcript levels between Cav1.4FL and exon 45/47 variants in human retina. Legend as described in Fig. 2D. (***, p < 0.008; ***, p < 0.001; by unpaired t test, n = 3.) No significant difference between transcript levels of Cav1.4pex45,47 and Cav1.4pex 47. C, RT-PCR analysis of Cav1.4 and exon 45/47 variants in human retina.

TABLE 2

Parameters for Boltzmann fits of I-V relationships

| Construct | Vθ | k | n |
|-----------|----|---|---|
| Cav1.4FL | 7 ± 1.0 | 9 ± 0.4 | 6 |
| Cav1.4Δex p45 | 4 ± 1.0 | 10 ± 0.3 | 7 |
| Cav1.4Δex 47 | −1 ± 1.0p | −10 ± 0.3 | 11 |
| Cav1.4Δex p45,47 | 0.03 ± 1.0p | −10 ± 0.3 | 7 |

*p < 0.001; compared with Cav1.4FL (one-way ANOVA followed by Bonferroni post test).

We next determined if deletion of exon 47 affected modulation by CaBP4. For these experiments, we used Cav1.4Δex 47 because the splicing of exon 45 had no effect on the electrophysiological properties of the channel (Figs. 4 and 5). Although CaBP4 causes a negative shift in voltage-dependent activation of Cav1.4FL (31, 32), it did not similarly affect Cav1.4Δ ex47. There was no significant difference in I-V or normalized tail current-voltage relationships in cells expressing Cav1.4Δ ex47 alone and those co-expressing CaBP4 (Fig. 7A and B; Table 4).

However, CaBP4 did blunt the strong inactivation of Cav1.4Δ ex47 Ica. CaBP4 caused a significant 2-fold reduction in the amount of Ica inactivation of Cav1.4Δ ex47 (Fractional Ica = 0.30 ± 0.1, n = 5 for Cav1.4Δ ex47 alone versus 0.56 ± 0.10, n = 7 for Cav1.4Δ ex47 + CaBP4; p < 0.02 by t test; Fig. 7C). We in that no binding was detected on plates coated with GST alone (not shown) or with Cav1.4 CT lacking the CaM-binding region in the proximal CT (Cav1.4ΔpCT Fig. 6B). We also tested how deletion of exons 45 and 47 affect CaM binding to the CT. Consistent with previous in vitro analyses of CaM binding to the Cav1.4FL CT (19), purified CaM bound to the Cav1.4FL CT. CaM bound similarly to the CT of Cav1.4Δex p45,47, but binding was abolished by deletion of the CaM/CaBP4 binding site (Fig. 6C). Taken together, our results show that deletion of exon 47 and part of exon 45 does not affect binding of CaM or CaBP4 to Cav1.4.
C-terminal Cav1.4 Splice Variants

FIGURE 5. Splicing of exon 47 but not exon 45 significantly affects voltage-dependent activation of Cav1.4. A–C, normalized current-voltage (I–V) plots for ICa. Currents were evoked by 50-ms pulses from −80 mV to various test voltages. D–F, normalized tail current − voltage plots for Cav1.4FL (lanes 3, 6, and 7). Tail currents were evoked by 10-ms pulses from −80 mV to various test voltages with 2-ms repolarization to −60 mV during which the peak tail current amplitude was measured. Current amplitudes were normalized to that evoked by a +60-mV pulse and plotted against the test voltage. Parentheses indicate the number of cells.

TABLE 3 Parameters for Boltzmann fits of normalized tail-current voltage curves

| Construct          | V0   | k     | n    |
|--------------------|------|-------|------|
| Cav1.4FL           | −4 ± 1.8 | −10 ± 0.3 | 6    |
| Cav1.4Δex p45      | −7 ± 1.7 | −8 ± 1.0 | 7    |
| Cav1.4Δex 47       | −18 ± 1.0a | −6 ± 1.2b | 13   |
| Cav1.4Δex p45,47   | −15 ± 2.0c | −7 ± 1.0b | 10   |

*p < 0.001 compared with Cav1.4FL (one-way ANOVA followed by Bonferroni post-test).

*p < 0.05 compared with Cav1.4FL (one-way ANOVA followed by Bonferroni post test).

conclude that exon 47 is dispensable for the ability of CaBP4 to antagonize effects of CaM on CDI of Cav1.4FL, but is required for CaBP4 modulation of voltage-dependent activation.

Discussion

Our study reveals new insights into how splice variation of the C-terminal domain affects the properties of Cav1.4 channels in the retina. First, we report that novel Cav1.4 splice variants that lack exon 47 alone (Cav1.4Δex 47) and/or partial deletion of exon 45 (Cav1.4Δex p45,47) are highly expressed in primate but not mouse retina. Second, exon 47 contains crucial determinants within the CTM for suppressing CDI and voltage-dependent activation. Finally, exon 47 is also needed for the functional modulation of channel activation by CaBP4, but not for the physical interaction of CaBP4 with the C-terminal domain. These findings underscore the importance of the distal C-terminal domain in controlling the intrinsic biophysical properties of Cav1.4 as well as its modulation by CaM and CaBP4.

Exon 47 as a Modulator of Cav1.4 CDI and Voltage-dependent Activation—The distal CT domain has emerged as a key regulator of CDI of Cav1.3 and Cav1.4 (19, 22, 43). Alternative splice variants of Cav1.3 and Cav1.4 lacking this domain exhibit stronger CDI than the corresponding full-length channels (23, 43, 44). For both Cav1.3 and Cav1.4, CDI suppression is mediated by a CTM corresponding to the final ~100–150 amino acids.
C-terminal Ca\textsubscript{v}1.4 Splice Variants

By contrast, deletion of exon 47 had dramatic effects on CDI. For Ca\textsubscript{v}1.4\Delta ex 47 and Ca\textsubscript{v}1.4\Delta ex p45,47 variants (Fig. 4), CDI was as robust as that caused by removal of the entire CTM (19, 22). At first glance, this result may seem at odds with previous findings that truncation of the final 55 aa distal to exon 47 disabled the ability of the CTM to suppress CDI (22). Because deletion of the last 32 amino acids was ineffective in this regard, it was concluded that the stretch of 20 aa between aa −55 and −32 from the C terminus contains the molecular determinants for CDI suppression (19, 22). Our results show that these 20 aa are not sufficient to support the function of the CTM because their presence in Ca\textsubscript{v}1.4\Delta ex 47 and Ca\textsubscript{v}1.4\Delta ex p45,47 was not able to suppress CDI (Fig. 4, C and D). The region enclosed by exon 47 may enable proper folding of the CTM and/or provide key contact points required for the intramolecular interaction with the proximal CT. Consistent with both possibilities, deletion of portions of exon 47 prevented binding of the CTM to the proximal CT (20).

In addition to suppressing CDI, the CTM inhibits voltage-dependent activation of Ca\textsubscript{v}1 channels. For Ca\textsubscript{v}1.2, the distal CT is proteolytically cleaved but remains covalently attached to the proximal CT, causing a positive shift in \( V_h \) (45). Although there is no evidence that the distal CT is cleaved in vivo for Ca\textsubscript{v}1.3 (46) or Ca\textsubscript{v}1.4, the distal CT of these channels autoinhibits voltage-dependent activation. Ca\textsubscript{v}1.3 or Ca\textsubscript{v}1.4 mutants or splice variants lacking the CTM exhibit negative shifts in \( V_h \) compared with full-length channels (19, 20, 22, 23, 43, 44). Our findings indicate that exon 47 is a key element within the CTM that regulates activation because \( V_h \) for Ca\textsubscript{v}1.4 variants lacking exon 47 were −15 mV more negative than that of Ca\textsubscript{v}1.4FL (Fig. 5, Table 3). How the CTM regulates voltage-dependent activation of Ca\textsubscript{v}1.4 is not entirely clear but could involve inhibition of movement of the voltage-sensing domains. However, for Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3, the positive shift in \( V_h \) due to autoinhibition by the CTM is attributed to weaker coupling of voltage sensor movement to opening of the channel pore (45, 47). Addressing the underlying mechanism for Ca\textsubscript{v}1.4 would require analysis of the voltage dependence of gating charges representing movement of the voltage sensors (48). These experiments would be technically quite challenging for Ca\textsubscript{v}1.4 channels, which produce very modest current density in heterologous expression systems compared with Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 (40).

Exon 47 and CaBP4 Modulation of Ca\textsubscript{v}1.4—CaBP4 and other CaBP family members are potent suppressors of CDI of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 channels (reviewed in Ref. 49). Compared with the analogous CTM regions of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3, the CTM of Ca\textsubscript{v}1.4FL more completely abolishes CDI such that an effect of CaBP4 on suppressing CDI can only be observed in Ca\textsubscript{v}1.4FL channels lacking the CTM (32). Nevertheless, CaBP4 does bind to Ca\textsubscript{v}1.4FL and enhances activation through a −10 mV shift in \( V_h \) (31, 32). The inability of CaBP4 to similarly promote voltagedependent activation of channels lacking exon 47 (Fig. 7, A and B) indicates a key role for this exon in supporting CaBP4 modulation. CaBP4 still binds to the CT (Fig. 6B) and markedly suppresses CDI of channels lacking exon 47 (Fig. 7, C and D), which argues against the possibility that deletion of exon 47 prevents the physical interaction of CaBP4 with the channel. Our results agree with previous findings that deletion of the entire previously defined CTM does not abrogate the physical interaction of CaBP4 with Ca\textsubscript{v}1.4FL, despite preventing effects of CaBP4 on voltage-dependent activation (32). As discussed for its role in regulating CDI, exon 47 may contribute to the structure and/or function of the CTM, which is necessary for

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**TABLE 4**

Parameters for Boltzmann fits of I-V and normalized tail-current voltage curves

| Construct | \( V_h \) | \( K \) | \( n \) |
|-----------|-----|-----|-----|
| 1-Vs     |     |     |     |
| Ca\textsubscript{v}1.4\Delta ex 47 alone | 2 ± 0.2 | −11 ± 5 | 6 |
| Ca\textsubscript{v}1.4\Delta ex 47 + CaBP4 | −0.2 ± 1.6 | −10 ± 0.3 | 8 |
| Normalized tail-voltage |     |     |     |
| Ca\textsubscript{v}1.4\Delta ex 47 alone | −13 ± 3.0 | −10 ± 1.0 | 6 |
| Ca\textsubscript{v}1.4\Delta ex 47 + CaBP4 | −14 ± 1.3 | −8 ± 1.0 | 7 |

\( * p < 0.001 \) compared with Ca\textsubscript{v}1.4\Delta ex 47 (control) (Student’s t test).

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**FIGURE 7.** CaBP4 modulates CDI but not voltage-dependent activation of Ca\textsubscript{v}1.4\Delta ex 47. A and B, normalized I-V (A) and tail current-voltage plots (B) for \( I_c \), in cells transfected with Ca\textsubscript{v}1.4\Delta ex 47 alone or with CaBP4. Data were obtained as described in Fig. 5. A-F. C, representative traces for \( I_c \) evoked by a 1-s test pulse from −80 to 0 mV in cells transfected as in A and B. D, fractional I was obtained as in Fig. 4 and plotted against test voltage. Parentheses indicate the number of cells.

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Acids (aa) of the channel protein (19, 22, 43). The underlying mechanism is controversial and involves binding of the CTM to a site in the proximal CT, which may physically displace CaM from the channel (22, 43). Alternatively, the CTM binding to the proximal CT is not competitive, but allosterically alters the binding of CaM in a way that weakens CDI (20). This interaction of the CTM with the proximal CT could be affected by sequences between the two domains, including exon 45 (Fig. 1). However, we did not find that splicing out part of exon 45 affected CDI or voltage-dependent activation (Figs. 4 and 5). These results are consistent with previous analyses of the partial deletion of exon 45 in chimeric Ca\textsubscript{v}1.2-Ca\textsubscript{v}1.4 (23), and with the inconsequential effects of alternative splicing of the analogous exon 44 of Ca\textsubscript{v}1.3 (43).

By contrast, deletion of exon 47 had dramatic effects on CDI. For Ca\textsubscript{v}1.4\Delta ex 47 and Ca\textsubscript{v}1.4\Delta ex p45,47 variants (Fig. 4), CDI was as robust as that caused by removal of the entire CTM (19, 20, 22). At first glance, this result may seem at odds with previous findings that truncation of the final 55 aa distal to exon 47...
transducing the effect of CaBP4 binding on activation. Alternatively, the negative shift in activation of Ca_{1.4}\Delta ex\ p45,47 compared with Ca_{1.4}\ p45 could be developmentally uncoupled from activation of CaBP4.

**Physiological Relevance of C-terminal Splicing of Exons 45 and 47 in the Retina**—Our electrophysiological recordings utilized channels containing \( \beta_2 \) subunits and CaBP4, and exon 47-lacking Cav1.4 variants would provide channels containing CaBP4, and exon 47-lacking Cav1.4 variants would be expected for Cav1.4 channels here (Tables 2–4) should be 20 mV more positive than would be expected for Cav1.4 channels in vivo. Taking this into account, the channels lacking exon 47 would be expected to support 3-fold higher levels of Ca\(^{2+}\) influx compared with Ca_{1.4}\ p45, at the photoreceptor membrane potential in darkness (−30 to −40 mV (14, 15)). However, this difference may be offset by the presence of CaBP4 in photoreceptor terminals. As a consequence of modulation by CaBP4 (31, 32), Ca_{1.4}\ p45 would exhibit hyperpolarized voltage dependence of activation similar to that of Ca_{1.4}\Delta ex\ 47 and Ca_{1.4}\Delta ex\ p45,47 (Fig. 5, E and F). The negative activation properties of Ca_{1.4}\ p45 modulated by CaBP4, and exon 47-lacking Cav1.4 variants would promote presynaptic Ca\(^{2+}\) influx to support glutamate release at the photoreceptor membrane potential in darkness. This in turn would ensure mGluR6-mediated closure of nearly all postsynaptic TRPM1 channels, which at the rod-bipolar cell synapse, is necessary for the optimal encoding of dim light signals (53, 54). At the same time, CaBP4 would suppress CDI of Ca_{1.4}\ p45, the HindIII-NotI insert of pcDNA-Ca_{1.4}\ p45, covering fragment F5 was replaced with a fragment HindIII-Sacl covering exon 33 to exon 45 from pcDNA-Ca_{1.4}\Delta ex\ p45,47, the Sacl restriction site being located just after the deletion, with a fragment Sacl-NotI (exon 45 to exon 48) from pcDNA-Ca_{1.4}\. To clone Ca_{1.4}\Delta ex\ 47, the HindIII-NotI insert of pcDNA-Ca_{1.4}\ p45, covering fragment F5 was replaced with a fragment HindIII-Sacl covering exon 33 to exon 45 from pcDNA-Ca_{1.4}\Delta ex\, with a fragment Sacl-NotI (exon 45 to exon 48) from pcDNA-Ca_{1.4}\Delta ex\ p45,47.

To subclone the CT domain of Ca_{1.4}\ p45, and Ca_{1.4}\Delta ex\ p45,47, a fragment encoding amino acids 1441 to the terminal stop codon was amplified by PCR with Pfx polymerase (Life Technologies). To remove the CaM binding site(s), a fragment HindIII-TGA stop codon, covering nucleotides 3913 to 5934 in Ca_{1.4}\ p45, harboring these deletions was cloned into pcDNA3.1 containing F1 to F4 of Ca_{1.4}\ p45. To clone Ca_{1.4}\Delta ex\ p45, the HindIII-NotI insert of pcDNA-Ca_{1.4}\ p45 covering fragment F5 was replaced with a fragment HindIII-Sacl covering exon 33 to exon 45 from pcDNA-Ca_{1.4}\Δ ex\ p45,47, the Sacl restriction site being located just after the deletion, with a fragment Sacl-NotI (exon 45 to exon 48) from pcDNA-Ca_{1.4}\Δ ex\ p45,47.

To subclone the CT domain of Ca_{1.4}\ p45, and Ca_{1.4}\Δ ex\ p45,47, a fragment encoding amino acids 1441 to the terminal stop codon was amplified by PCR with Pfx polymerase (Life Technologies). To remove the CaM binding site(s), a fragment encoding amino acids 1603 to the stop codon was also amplified. These PCR products were cloned into the pET-SUMO vector (Life Technologies) for fusion to both a His\(_6\) and a SUMO tag. The fusion proteins were expressed in BL21(DE3)pLysS Escherichia coli after induction with 0.5 mM isopropyl-1-thio-\( \beta\)-D-galactopyranoside and purified on nickel-nitrilotriacetic acid columns according to the manufacturer's protocol.

CaBP4 was amplified by PCR from a human retina cDNA library and cloned into pentr-D-TOPO vector (Life Technologies). After sequencing, the cDNA was transferred by recombination into the pDest15 vector using the Gateway Technology System (Life Technologies) for fusion to both a GST tag and expression in bacteria. The GST fusion proteins were expressed in BL21(DE3) pLysS E. coli after induction with 0.5 mM isopropyl-1-thio-\( \beta\)-D-galactopyranoside and purified on a glutathione column according to the manufacturer's protocol.

**Quantitative PCR Analysis of Human and Monkey Ca_{1.4} and Their Splice Variants**—All procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington. Retinas from Macaca nemestrina were obtained at the University of Washington Regional Primate Center (Seattle, WA). Human retinas were obtained from...
donors without known eye disease from the Lions Eye Bank of Oregon 4–10 h after death. Total RNA was isolated from the retina of human, monkey, or mouse using a RNAeasy kit (Qiagen). The relative expression of splice variants was determined by a two-step quantitative PCR. Total RNA (1 μg) was subjected to first strand cDNA synthesis using SuperScript III reverse transcriptase and oligo(dT) in a volume of 20 μl according to the manufacturer’s protocol (Life Technologies). For the qPCR analysis of the Cav1.4Δex p45,47, primers were designed on the exon 44-alternate exon 45 joint (forward) and ~200 bp downstream on the exon 46-exon 48 joint (reverse, Table 1, row 2). For the Cav1.4FL primers were designed on the exon 44-exon 45 junction (forward) and ~460 bp downstream on the exon 47-exon 48 junction (reverse, Table 1, row 1). For the RT-PCR analysis of Cav1.4Δex p45,47 in mouse, human, and monkey, similar primers were used as indicated in Table 1 (rows 1 to 6).

For comparison by qPCR of all the C-terminal variants with Cav1.4FL, primers were designed to exon 45, exon 47, exon 44-alternate exon 45 joint, or exon 46-exon 48 joint (Table 1, rows 2 and 7–9). For normalization, primers were used to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Table 1, rows 10 to 12). Reactions were carried out in triplicate using 0.5 μl of cDNA, 400 nm of each primer, and 10 μl of QuantiTect SYBR Green PCR mix (Qiagen) in a 20-μl total reaction volume. After an initial incubation at 95 °C for 15 min, the qPCR was carried out for 40 cycles of denaturation at 95 °C for 15 s, annealing at 68 °C for 30 s, and extension at 72 °C for 1 min on an ABI PRISM 7000 (Applied Biosystems). Single bands for the expected size were verified by agarose gel electrophoresis. Threshold cycle was determined using the ABI Prism 7000 software. Data were analyzed by comparing cycle threshold (Ct) normalized to the Ct values of the internal control, GAPDH (ΔCt = Ct value of WT or variant − Ct value of GAPDH); standard deviation of ΔCt = (S.D. of variant or WT)2 + (S.D. of GAPDH)2. The normalized Ct values of the Cav1.4 variants and Cav1.4FL were compared by determining ΔΔCt = ΔCt Cav1.4Δex − ΔCt Cav1.4FL. Fold-induction was calculated as 2–ΔΔCt (41).

Co-immunoprecipitation of Cav1.4 and CaBP4—HEK-293 cells were transfected with cDNAs encoding Ca1.4Δx or Ca1.4Δex p45,47, δp, and CaBP4. Three days later, whole cell lysates were prepared by incubation of transfected cells at 4 °C for 1 h in 20 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1 mM MgCl2, 0.1 mM CaCl2 and inhibitors of proteases (Sigma). Lysates were subject to centrifugation at 22,000 g for 30 min and incubated with mouse IgG (purified on protein G plus agarose from mouse serum) or anti-FLAG antibodies (Sigma). After 1 h incubation at 4 °C, protein G-magnetic beads (Life Technologies) were added and the incubation proceeded for 3 h at 4 °C. After 4 washes with lysis buffer, proteins were eluted with SDS-sample buffer and analyzed by Western blotting with specific antibodies.

Enzyme-linked Immunosorbent Assay (ELISA)—Purified GST, GST-CaBP4, or GST-CaM fusion proteins (2 μg/ml in 100 mM sodium bicarbonate, pH 9.0) were bound to 96-well ELISA plates (200 ng/well) overnight at 4 °C. The wells were blocked with animal-free blocker (Vector laboratories) for 1 h at room temperature. 2-Fold dilutions of SUMO-Ca1.4 C-terminal domain (CT) fusion proteins in TBST_MC (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 0.05% Tween 20) were added and reactions were incubated for 1 h at room temperature. After 3 washes in TBST-MC, bound Cav1.4_CT was detected with rat anti-SUMO antibodies (raised in rats and purified with SUMO proteins using a previously described method (40)) for 1 h at room temperature, followed by incubation with alkaline phosphatase-conjugated anti-rat antibodies. Reactions were incubated with p-nitrophenyl phosphate substrate (diluted in 100 mM Tris, pH 9.0, 50 mM MgCl2, 100 mM NaCl) for 30 min at room temperature and the absorbance was measured at 405 nm with a microplate reader (Bio-Rad). The absorbance data of nonspecific binding of Ca1.4 to GST (negative control for binding to GST) was subtracted from that for binding of SUMO-Cav1.4 CT to GST-CaBP4.

Electrophysiology—HEK-293T cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies) with 10% fetal bovine serum (Atlantic Biologicals) at 37 °C in 5% CO2, and grown to 70–80% confluence. Cells plated in 35-mm dishes were co-transfected with cDNAs encoding human Cav1.4 α1 (1.8 μg; Cav1.4FL, Cav1.4Δex p45, Cav1.4Δex p45,47, or Cav1.4Δex 47), β2×13 (0.6 μg), δpδp (0.6 μg), and enhanced green fluorescent protein (0.1 μg). FuGENE 6 transfection reagent (Promega) was used according to the manufacturer’s protocol. Cells treated with the transfection mixture were incubated at 37 °C for 24 h prior to dissociation and maintenance at 30 °C prior to recording.

We have found that overexpression of CaBP4 has inhibitory effects on Ca1.4 current density, perhaps through dampening of channel expression levels. To offset these effects, we used an ecdysone-inducible system to co-express CaBP4 with Cav1.4 (27). Cells were co-transfected with Cav1.4 subunits as described above, but cotransfected with CaBP4 subcloned into an ecdysone-inducible expression (pIND) vector (Invitrogen; 3 μg) and pVgRXR (1 μg), which encodes a heterodimeric retinoid X receptor (RXR) and ecdysone receptor (VgEcR). After 24 h, transfected cells were treated with an ecdysone analog, Ponasterone A (10 μM; Thermo-Fisher Scientific) or 1% ethanol (control) for 8–10 h to induce CaBP4 expression.

Whole cell patch clamp recordings were performed at room temperature between 48 and 72 h after transfection. Data were obtained under voltage-clamp with an EPC-9 patch clamp amplifier operated by either Patchmaster or PULSE software (HEKA Elektronik) and analyzed with Igor Pro software (Wavembetics). External recording solutions consisted of (in mM): Tris (140), CaCl2 or BaCl2 (20), and MgCl2 (1). Internal pipette solution consisted of (in mM): N-methyl-D-glucamine (140), HEPES (10), MgCl2 (2), Mg-ATP (2), and EGTA (5). The pH of external and internal recording solutions was adjusted to 7.3 with methanesulfonic acid. Pipette resistances were typically 2–4 megohms, and series resistance was compensated up to 70%. Leak subtraction was conducted using a P/4 protocol. Statistical analysis (Student’s t test, Mann-Whitney rank sum test, or by a one-way ANOVA) was done and graphs were made with SigmaPlot (Systat Software). All averaged data
represent mean ± S.E., and result from at least 5 independent transfections.

Author Contributions—F. H. and B. W. performed experiments and analyzed data. A. L., F. H., and B. W. contributed to experimental design and writing and approval of the final manuscript.

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