Splicing of an autoregulatory domain in Cav1.4 Ca2+ channels confers distinct regulation by calmodulin

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Ca2+ influx through Cav1.4 L-type Ca2+ channels supports the sustained release of glutamate from photoreceptor synaptic terminals in darkness, a process that is critical for vision. Consistent with this role, Cav1.4 exhibits weak Ca2+-dependent inactivation (CDI)—a negative feedback regulation mediated by Ca2+-bound calmodulin (CaM). CaM binds to a conserved IQ domain in the proximal C-terminal domain of Cav channels, but in Cav1.4, a C-terminal modulatory domain (CTM) disrupts interactions with CaM. Exon 47 encodes a portion of the CTM and is deleted in a Cav1.4 splice variant (Cav1.4Δex47) that is highly expressed in the human retina. Cav1.4Δex47 exhibits CDI and enhanced voltage-dependent activation, similar to that caused by a mutation that is associated with congenital stationary night blindness type 2, in which the CTM is deleted (K1591X). The presence of CDI and very negative activation thresholds in a naturally occurring variant of Cav1.4 are perplexing considering that these properties are expected to be maladaptive for visual signaling and result in night blindness in the case of K1591X. Here we show that Cav1.4Δex47 and K1591X exhibit fundamental differences in their regulation by CaM. In Cav1.4Δex47, CDI requires both the N-terminal (N lobe) and C-terminal (C lobe) lobes of CaM to bind Ca2+, whereas CDI in K1591X is driven mainly by Ca2+ binding to the C lobe. Moreover, the CaM N lobe causes a Ca2+-dependent enhancement of activation of Cav1.4Δex47 but not K1591X. We conclude that the residual CTM in Cav1.4Δex47 enables a form of CaM N lobe regulation of activation and CDI that is absent in K1591X. Interaction with the N lobe of CaM, which is more sensitive to global elevations in cytosolic Ca2+ than the C lobe, may allow Cav1.4Δex47 to be modulated by a wider range of synaptic Ca2+ concentrations than K1591X; this may distinguish the normal physiological function of Cav1.4Δex47 from the pathological consequences of K1591X.

Introduction

Voltage-gated Cav Ca2+ channels are essential regulators of Ca2+ signaling and are composed of a pore-forming α1 subunit and auxiliary β and α2δ subunits. The pharmacological and biophysical properties of Cav α1 subunits are largely determined by the α1 subunit (Simms and Zamponi, 2014). In contrast to the tremendous diversity of genes encoding voltage-gated K+ channels, the Cav α1 subunit in mammals is encoded by only 10 different genes (Yu et al., 2005). However, each α1 gene can give rise to thousands of splice variants, the exact complement of which depends on the types of splicing factors that are expressed (Lipscombe et al., 2013). Combined with the splice variation affecting β and α2δ subunits (Burac and Yang, 2013; Dolphin, 2013), the vast molecular diversity of α1 splice isoforms helps tailor Cav channel properties according to a defined cellular and/or developmental context.

Among the cytoplasmic regions of the Cav α1 subunit, the C-terminal domain (CTD) is the largest and subject to significant splice variation that can alter channel function. For example, the inclusion of either of two mutually exclusive exons (37a and 37b) in the proximal C-terminal domain (pCTD) of Cav2.2 affects the voltage dependence of channel inhibition by G protein–coupled receptors (Raingo et al., 2007), and the role of Cav2.2 in mediating the analgesic effects of morphine in spinal nociceptors (Andrade et al., 2010). Alternative splicing of the analogous exons in Cav2.1 (P/Q-type) channels as well as an exon in the distal CTD transforms the Ca2+-sensitivity of Ca2+/calmodulin (CaM)-dependent facilitation of these channels (Chaudhuri et al., 2004). The expression of these Cav2.1 splice variants is developmentally regulated in ways that may support the maturation of the firing properties of cerebellar Purkinje neurons (Chaudhuri et al., 2005).

In the retina, Cav1.4 L-type channels are localized in the synaptic terminals of rod and cone photoreceptors, where they mediate
the tonic release of glutamate necessary for the encoding of light responses (McRory et al., 2004; Mansergh et al., 2005; Specht et al., 2009; Liu et al., 2013). Unlike Cav1.2 and Cav1.3 channels, Cav1.4 exhibits little Ca2+-dependent inactivation (CDI) in heterologous expression systems (Baumann et al., 2004; McRory et al., 2004; Singh et al., 2006; Wahl-Schott et al., 2006). CDI is a hallmark feature of most Ca v1 and Ca v2 channels and depends on CaM binding to a consensus IQ domain in the pCTD of the channel (Ben-Johny and Yue, 2014). CDI of Ca v1.4 is blunted by a CTM that competes with CaM interactions with the channel (Singh et al., 2006; Liu et al., 2010; but see Wahl-Schott et al., 2006; Griessmeier et al., 2009).

Numerous Ca v1.4 splice variants have been identified in human retina with variations affecting the CTM. For example, Cav1.4 variants containing the alternately spliced exon 43 (Cav1.4 + ex43) are prematurely truncated, resulting in removal of the CTM. Like channels in which the entire CTM is deleted (Singh et al., 2006; Wahl-Schott et al., 2006), Cav1.4 + ex43 exhibits robust CDI as well as a negative shift in the half-maximal voltage ($V_{h}$; Tan et al., 2012) of channel activation. Strong CDI and a hyperpolarized $V_{h}$ are also caused by a mutation (K1591X) associated with congenital stationary night blindness type 2 (CSNB2; Strom et al., 1998), which deletes the CTD distal to the IQ domain, including the CTM (Singh et al., 2006). The physiological significance of naturally occurring splice variants with properties similar to disease-causing mutant channels is unclear.

We previously characterized a splice variant of Cav1.4 L-type channels lacking exon 47 (Cav1.4Δex47), which encodes part of the CTM. Cav1.4Δex47 is highly expressed in human retina and exhibits prominent CDI as well as a negative shift in $V_{h}$ (Haeseleer et al., 2016). These properties suggest that deletion of exon 47 might enable CaM regulation by altering or preventing the CTM interaction with the pCTD, but mechanistic details are lacking. Moreover, it is not known how deletion of exon 47 promotes voltage-dependent activation of Cav1.4Δex47. Considering that alternative splicing of Ca v channels can affect the functional impact of disease-causing

![Figure 1. Deletion of exon 47 promotes CDEA of Ca v1.4Δex47. (A) Schematic showing the proposed function of the CTM modulating CaM interactions with Ca v1.4 + ex47 and Ca v1.4Δex47. (B and C) Representative family of traces (left panels) and I–V plots for $I_{Ca}$ (B) and $I_{Ba}$ (C) in cells transfected with Ca v1.4 + ex47 or Ca v1.4Δex47. Currents were evoked by 50-ms depolarizations to various voltages. Current amplitudes were normalized to cell capacitance and plotted against test voltage (middle panels). Tail current amplitudes were normalized to −60 mV for $I_{Ca}$ (Norm. $I_{Ca}$) and −20 mV for $I_{Ba}$ (Norm. $I_{Ba}$) and plotted against test voltage (right panels). Here and in all figures, parentheses indicate numbers of cells. Error bars represent mean ± SEM.](image-url)
mutations (Adams et al., 2009), a thorough analysis of the properties of Cav1.4Δex47 is important for understanding how CACNA1F mutations may cause vision impairment in humans.

Here, we report a dual role for CaM in regulating both CDI and voltage-dependent activation of Cav1.4Δex47. In addition, we demonstrate distinct routes whereby the individual Ca2+ binding lobes of CaM regulate K1591X and Cav1.4Δex47. Our findings highlight the versatility of CaM as a regulator of Ca v channels, and how modulation by CaM can be affected by naturally and pathologically occurring variations in the channel protein.

Materials and methods

Complementary DNAs (cDNAs) and molecular biology
The following cDNAs were used: Cav1.4 (GenBank no. AF201304), β2×13 (GenBank no. AF465485), and α 2δ4 (GenBank no. NM_017326.3) in pcDNA3.1 (Lee et al., 2018); CaM deficient in Ca2+ binding to the N-terminal lobe (N lobe; CaM12), CaM deficient in Ca2+ binding to the C-terminal lobe (C lobe; CaM1234), CaM deficient in Ca2+ binding to both the N lobe and C lobe (CaM1234; GenBank no. NM_017326.3) in pcDNA6V5-His (Lee et al., 2003); β2a (GenBank no. M80545.1). In Cav1.4Δex47, alanine substitutions at residues I1588A, Q1589A, D1590A, Y1591A, and F1592A (5A), and F1582A, Y1583A, and F1586A (3A), were generated with primers incorporating the mutations (5′-CTACGCCACATTTCGGCGCGGCCTGTCGCCGAATTCCGGC-3′ for 5A, 5′-GTACCGCGCGCCACAGCTCTGTACCGAGGTATCTCCCGC-3′ for 3A, respectively) using the QuikChange Lightning Multi Site-directed Mutagenesis kit (Agilent) according to the manufacturer’s protocol. The 3A mutations were generated in K1591X-3A with the HiFi DNA Assembly Cloning System (New England Biolabs) protocol. The 3A mutations were generated in K1591X-3A with the HiFi DNA Assembly Cloning System (New England Biolabs) protocol. For some experiments, cells were cotransfected with cDNAs encoding CaM12, CaM1234, or CaM1234 (1 µg each) or pcDNA3.1 (1 µg) as a control. Cells treated with the transfection mixture were incubated at 37°C for 24 h. After 24 h, cells were incubated at 30°C for at least 24 h before whole-cell patch clamp recordings.

Electrophysiology

Whole-cell patch clamp recordings were performed at room temperature between 48 and 72 h after transfection with an EPC-9 patch clamp amplifier operated by either Patchmaster software (HEKA Elektronik). External recording solutions consisted of (in mM) Tris (140), CaCl2 or BaCl2 (20), and MgCl2 (1). Internal recording solution consisted of (in mM) NMDG (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 MΩ in the bath solution, and series resistance compensated up to 70%. Leak subtraction was conducted using a P/−4 protocol.

To measure current density, Ca2+ and Ba2+ currents (ICa, IBa) were evoked by 50-ms pulses from −80 mV to various voltages and normalized to the cell capacitance. To characterize voltage-dependent activation, currents were evoked by 10-ms steps from −80 mV to various voltages. Tail currents were measured upon repolarization to −60 mV for 2 ms. For ICa, tail current amplitudes were normalized to that at +60 mV. Because of a decline in the amplitude of IBa tail currents at positive voltages, tail currents for IBa were normalized to that at +20 mV. I–V data were fitted with single or double Boltzmann equations: $I = I_{\text{max}} / [1 + \exp (V - V_h) / k_1] + I_{\text{max}} / [1 + \exp (V - V_h) / k_2]$, where $I_{\text{max}}$ is the maximal current, $V$ is the test voltage, $V_h$ is the half-maximal activation, and $k$ is the slope of the Boltzmann function.

Cell culture and transfection

Human embryonic kidney 293 T cells (CRL-3216, Research Resource Identifier; CVCL_0063, American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies) with 10% FBS (Atlantic Biologicals) at 37°C in 5% CO2. At 70–80% confluence, the cells were cotransfected with cDNAs encoding human Cav1.4 α1 (1.8 µg; Cav1.4 + ex47, Cav1.4Δex47, K1591X, or 5A or 3A mutants), β2×13 (0.6 µg), α 2δ4 (0.6 µg), and enhanced GFP in pEGFP-C1 (0.1 µg) using FuGENE 6 transfection reagent (Promega) according to the manufacturer’s protocol. For some experiments, cells were cotransfected with cDNAs encoding CaM12, CaM1234, or CaM1234 (1 µg each) or pcDNA3.1 (1 µg) as a control. Cells treated with the transfection mixture were incubated at 37°C for 24 h. After 24 h, cells were incubated at 30°C for at least 24 h before whole-cell patch clamp recordings.

| Construct        | Peak current density (pA/pF) | P value | Vh       | P value versus Cav1.4Δex47 | k        | P value |
|------------------|------------------------------|---------|----------|---------------------------|----------|---------|
| Cav1.4 + ex47    | −8 ± 2.3                     | 0.958   | −6 ± 1.0 | <0.001                    | −8 ± 0.3 | 0.333   |
| Cav1.4Δex47      | −7 ± 1.3                     | 0.915   | −6 ± 1.1 | <0.001                    | −7 ± 0.2 | 0.448   |
| Cav1.4Δex47 + CaM12 | −11 ± 1.5                   | 0.908   | −6 ± 1.0 | <0.001                    | −7 ± 0.2 | 0.448   |
| Cav1.4Δex47 + CaM14 | −10 ± 1.0                   | 0.915   | −6 ± 1.0 | <0.001                    | −7 ± 0.2 | 0.448   |

Vh and k values (mean ± SEM) were determined from Boltzmann fits of I–V data. Peak current density was defined as the maximum current amplitude normalized to the cell capacitance. P values indicate comparisons with Cav1.4Δex47 and were determined by Student’s t test.

Table 1. Parameters for voltage-dependent activation and current density from I–V data

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factor. To measure CDI, currents were evoked by 1-s depolarizations from −80 mV to various voltages. Fractional ICa or IBa was defined as the residual current amplitude at the end of the pulse normalized to the peak current amplitude. Fractional CDI was the difference in fractional ICa at −20 mV and mean fractional IBa at −20 mV, except where indicated. Kinetic parameters for ICa inactivation were obtained by fitting with a double exponential function \(y_0 + A_{\text{fast}} \exp(-t/\tau_{\text{fast}}) + A_{\text{slow}} \exp(-t/\tau_{\text{slow}})\), where \(y_0\) is the offset (asymptote), \(\tau_{\text{fast}}\) and \(\tau_{\text{slow}}\) are the time constants, and \(A_{\text{fast}}\) and \(A_{\text{slow}}\) are the relative amplitudes of fast and slow components of inactivation.

Data were analyzed offline with Igor Pro software (WaveMetrics). Statistical analysis was performed using SigmaPlot (Systat Software Inc.) or GraphPad Prism software. The data were initially analyzed for normality using the Shapiro–Wilk or D’Agostino–Pearson omnibus test. For parametric data, significant differences were determined by Student’s t test or ANOVA with post hoc Dunnett or Tukey test. For nonparametric data, Kruskal–Wallis and post hoc Dunn’s test were used. Data were incorporated into figures using GraphPad, SigmaPlot, and Adobe Illustrator software. All averaged data represent the mean ± SEM from at least three independent transfections.

Online supplemental material

Fig. S1 shows that CaM mutants do not impact ICa density or voltage-dependent activation of Caβ1.4 + ex47. Fig. S2 shows the lack of CDI in a subset of cells transfected with Caβ1.4Δex47-3A in which very slow activation was observed.

Results

Deletion of exon 47 causes a Ca2+-dependent enhancement in activation of Caβ1.4

Because Caβ1.4Δex47 is expressed in human but not mouse retina (Lee et al., 2015), we focused on Caβ1.4 channels containing auxiliary Caβ and α2δ subunits that predominate in the human retina—\(\beta_{2-13}\) (a splice variant of Caβ2) and \(\alpha_2\delta_1\) (Lee et al., 2015). We compared the properties of Caβ1.4 + ex47 and Caβ1.4Δex47 (Fig. 1A) cotransfected with \(\beta_{2-13}\) and \(\alpha_2\delta_1\) in whole-cell patch clamp recordings of human embryonic kidney 293 T cells. Due to our interests in comparing CDI, voltage protocols were performed with either Ca2+ or Ba2+ as the permeant ion.

Table 2. Parameters for voltage-dependent activation from tail I–V data

| Construct         | \(V_h\) (mV) | P value versus Caβ1.4 + ex47 | P value versus Caβ1.4Δex47 | P value versus K1591X | \(k\) P value versus Caβ1.4 + ex47 | P value versus Caβ1.4Δex47 | P value versus K1591X |
|-------------------|--------------|-----------------------------|-----------------------------|------------------------|-----------------------------------|-----------------------------|------------------------|
| ICa               |              |                             |                             |                        |                                   |                             |                        |
| Caβ1.4 + ex47     | −3 ± 1.5     | −                           | −                           | −11 ± 1.0              | −                                 | −                           | −                      |
| Caβ1.4Δex47      | −18 ± 1.0    | <0.001\(^a\)                | −                           | −7 ± 1.2               | <0.001\(^b\)                    | −                           | −                      |
| Caβ1.4Δex47 + CaM34 | −13 ± 2.2  | <0.001\(^c\)                | 0.144\(^d\)                | −7 ± 1.1               | 0.011\(^e\)                     | >0.999\(^f\)                | −                      |
| Caβ1.4Δex47-5A   | −7 ± 0.2.4   | 0.165\(^g\)                 | 0.005\(^h\)                | −11 ± 0.4              | >0.999\(^i\)                    | 0.004\(^j\)                 | −                      |
| K1591X           | −22 ± 1.0    | 0.001\(^k\)                 | 0.933\(^l\)                | −9 ± 0.6               | 0.264\(^m\)                     | 0.369\(^n\)                 | −                      |
| K1591X + CaM12   | −20 ± 0.7    | 0.001\(^p\)                 | >0.999\(^q\)               | −8 ± 0.5               | 0.038\(^r\)                     | −                           | >0.999\(^s\)           |
| K1591X-3A        | −16 ± 1.2    | <0.001\(^t\)                | −0.064\(^u\)               | −10 ± 0.6              | >0.999\(^v\)                    | −                           | >0.999\(^w\)           |

\(V_h\) and \(k\) values (mean ± SEM) were determined from single Boltzmann fits of the tail I–V data as indicated in the “Methods” section.

\(^a\)Student’s t test.
\(^b\)Mann–Whitney test.
\(^c\)One-way ANOVA with Tukey’s multiple comparison test.
\(^d\)Kruskal–Wallis test and post hoc Dunn’s multiple comparison test.

V, and \(k\) values (mean ± SEM) were determined from single Boltzmann fits of the tail I–V data as indicated in the “Methods” section.

Because Caβ1.4Δex47 is expressed in human but not mouse retina (Lee et al., 2015), we focused on Caβ1.4 channels containing auxiliary Caβ and α2δ subunits that predominate in the human retina—\(\beta_{2-13}\) (a splice variant of Caβ2) and \(\alpha_2\delta_1\) (Lee et al., 2015). We compared the properties of Caβ1.4 + ex47 and Caβ1.4Δex47 (Fig. 1A) cotransfected with \(\beta_{2-13}\) and \(\alpha_2\delta_1\) in whole-cell patch clamp recordings of human embryonic kidney 293 T cells. Due to our interests in comparing CDI, voltage protocols were performed with either Ca2+ or Ba2+ as the permeant ion. In agreement with our previous study (Haeseleer et al., 2016), ICa mediated by Caβ1.4Δex47 activated at more negative voltages than Caβ1.4 + ex47. While this was also seen for Ba2+ currents (IBa), the doubling in current density for ICa in cells transfected with Caβ1.4Δex47 was not observed for IBa.
of the tail current reflects the number of channels open during the test pulse and rises sigmoidally with the test voltage. Boltzmann fits of these data indicated a significant hyperpolarizing shift in the activation curve, and steepening of the slope (k), for Cav1.4Δex47 compared with Cav1.4 + ex47. While these alterations in tail I–V curves were found for both ICa and IBa (Fig. 1, B and C; and Table 2), the difference in Vh and k values between Cav1.4Δex47 and Cav1.4 + ex47 was significantly larger for ICa than for IBa (ΔVh = −15 ± 1.0 mV for ICa versus ΔVh = −10 ± 1.1 mV for IBa; P = 0.003 by t test; Δk = −3 ± 0.5 for ICa versus Δk = −1 ± 0.2 for IBa; P < 0.001 by t test). Therefore, in addition to enhancing voltage-dependent activation of IBa, deletion of exon 47 causes a Ca2+-dependent enhancement of activation (CDEA) of ICa.

CDEA is mediated by the N lobe of CaM
To investigate the mechanism underlying CDEA of Cav1.4Δex47, we considered CaM because of its role in Ca2+-dependent modulation of Ca v channels (Ben-Johny and Yue, 2014). For Ca1.2, Ca2+ binding to the pairs of EF-hand motifs in the C lobe and N lobe of CaM is required for CDI (Peterson et al., 1999) and CDF (Van Petegem et al., 2005), respectively. CaM mutants deficient in Ca2+ binding to the N lobe (CaM12) or C lobe (CaM34) have been instrumental in revealing CaM lobe-specific modulation of Ca1 and Ca2 channels (Ben-Johny and Yue, 2014). To determine if CaM is involved in CDEA, we cotransfected Cav1.4Δex47 with either CaM12 or CaM34. While CaM34 did not affect Cav1.4Δex47 current density, or cause alterations in I–V or tail I–V parameters that were specific for ICa (Fig. 2; and Tables 1 and 2), there was a
significant decrease in current density and positive shift in $V_h$ in the $I−V$ curve in cells cotransfected with Cav1.4Δex47 and CaM12 for $I_{Ca}$, and these alterations were not observed for $I_{Ba}$ (Fig. 3, A and B; and Table 1). Coexpression of CaM12 also caused a positive shift in the tail $I−V$ relationship only for $I_{Ca}$ and not $I_{Ba}$ (Fig. 3 C). Unlike for Cav1.4Δex47 transfected alone or with CaM34 (Table 2), the tail $I−V$ for $I_{Ca}$ in cells cotransfected with CaM12 was best fit with a double Boltzmann function (Fig. 3 D and Table 3) with a decline in channel availability over voltages that paralleled the enhanced activation of $I_{Ca}$ mediated by Cav1.4Δex47 compared with Cav1.4 + ex47 (Fig. 1 B). There was also no impact of either CaM12 or CaM34 on the $I−V$ or tail $I−V$ relationship for Cav1.4 + ex47 (Fig. S1), demonstrating that this effect of CaM12 is specific for channels lacking exon 47. Collectively, our results show that deletion of exon 47 not only enhances voltage-dependent activation of $I_{Ba}$ but also enables CDEA that involves the N lobe of CaM.

In Cav1.4, CaM binds to a consensus IQ domain in the pCTD of Cav1 channels (see Fig. 5 A). Mutation of the initial 5 amino acids within this domain (IQDYF) to alanine abolishes the binding of CaM to Cav1.4 (Griessmeier et al., 2009). Assuming that the 5A mutations would also disrupt CaM modulation of Cav1.4Δex47, we introduced these mutations (II588A, Q1589A, D1590A, Y1591A, and F1592A) into Cav1.4Δex47 (Cav1.4Δex47-5A; Fig. 4 A) and tested their involvement in CDEA. In Boltzmann fits of the tail $I−V$ curves for Cav1.4Δex47-5A, $V_h$ was significantly more positive, and $k$ shallower, than that for Cav1.4Δex47. These alterations were seen for $I_{Ca}$ and not for $I_{Ba}$ mediated by Cav1.4Δex47-5A (Fig. 4, B and C; and Table 2), indicating the importance of the IQ domain for CDEA of $I_{Ca}$ but not voltage-dependent activation of $I_{Ba}$. To further probe the molecular determinants underlying CDEA, we focused on three aromatic residues that were shown to mediate CaM N lobe interactions with Cav1.2 (F1618A, Y1619A, F1622A; Van Petegem et al., 2005). Although there are differences in the residues surrounding the IQ domain of Cav1.4 and Cav1.2, these residues are conserved in Cav1.4 (F1582A, Y1583A, and F1586A; F1622A; Van Petegem et al., 2005). Therefore, we reasoned that mutations of these residues might disrupt CaM N lobe regulation of CDEA. Consistent with this possibility, the 3A mutations in Cav1.4Δex47 (Cav1.4Δex47-5A) reversed the negative shift in $V_h$ for $I_{Ca}$ (Fig. 5 B; and Tables 2 and 3). Moreover, the tail $I−V$ relationship of $I_{Ca}$ for Cav1.4Δex47-5A was best fit by a double Boltzmann equation (Fig. 5 C) with no significant differences in fit parameters compared with those obtained for Cav1.4Δex47 cotransfected with CaM12 (Table 3). Compared with Cav1.4Δex47, the positive shift in the tail $I−V$ curve for $I_{Ca}$ mediated by Cav1.4Δex47-5A (Fig. 4 B)
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was weaker than that for Cav1.4Δex47-3A (Fig. 5 B), perhaps due to residual CaM N lobe modulation of Cav1.4Δex47-5A. There was no effect of either 5A or 3A mutations on \( I_{\text{Ba}} \), which further suggested that the enhanced voltage-dependent activation of \( I_{\text{Ba}} \) relies on distinct molecular determinants as compared with CDEA. Collectively, our results demonstrate that CaM N lobe mediates CDEA of Cav1.4Δex47, which requires F1582A, Y1583A, and F1586A.

Cav1.4Δex47 undergoes CDI that is mediated by both the N lobe and C lobe of CaM

In contrast to Cav1.4 + ex47, Cav1.4Δex47 undergoes CDI that is evident as stronger inactivation of \( I_{\text{Ca}} \) as compared with \( I_{\text{Ba}} \) (Haeseleer et al., 2016). Previous analyses of Cav1.3 channels revealed the importance of the C lobe of CaM in mediating a rapid phase of CDI, whereas the N lobe mediates a slower component (Yang et al., 2006). To determine if this was also true for Cav1.4Δex47, we tested the impact of CaM mutants on CDI of Cav1.4Δex47. Inactivation of \( I_{\text{Ca}} \) and \( I_{\text{Ba}} \) was measured as the current amplitude at the end of a 1-s pulse normalized to the peak current amplitude (fractional I). Fractional \( Ca^{2+} \)-dependent inactivation was defined as the difference in fractional \( I_{\text{Ca}} \) and fractional \( I_{\text{Ba}} \) (FCDI). In cells transfected with Cav1.4Δex47 alone, \( I_{\text{Ca}} \) inactivation was greatest (smallest fractional I) at voltages on the rising phase of the I–V relationship (from −20 to 0 mV; Fig. 1 C), consistent with a role for \( Ca^{2+} \) influx during the test.

Table 4. Fractional CDI

| Construct                  | FCDI   | P value versus +ex47 | P value versus Δex47 | P value versus Δex47 + CaM12 | P value versus Δex47 + 3A |
|----------------------------|--------|-----------------------|-----------------------|-----------------------------|--------------------------|
| Cav1.4 + ex47              | -0.19 ± 0.03 | —                     | <0.001 \(^a\)         | —                           | —                        |
| Cav1.4Δex47                | 0.57 ± 0.03 | —                     | —                     | —                           | —                        |
| Cav1.4Δex47 + CaM12        | 0.37 ± 0.07 | <0.001 \(^a\)         | 0.008 \(^a\)           | —                           | —                        |
| Cav1.4Δex47 + CaM34        | 0.22 ± 0.05 | 0.012 \(^a\)          | <0.001 \(^a\)          | 0.171 \(^a\)                | —                        |
| Cav1.4Δex47 + CaM1234      | 0.09 ± 0.05 | 0.530 \(^a\)          | <0.001 \(^a\)          | 0.002 \(^a\)                | —                        |
| Cav1.4Δex47-5A             | -0.12 ± 0.07 | 0.840 \(^a\)          | <0.001 \(^a\)          | —                           | —                        |
| Cav1.4Δex47-3A             | 0.37 ± 0.13 | 0.004 \(^a\)          | 0.056 \(^a\)           | —                           | —                        |
| Cav1.4Δex47-3A + CaM34     | 0.06 ± 0.01 | —                     | —                     | —                           | 0.044 \(^b\)            |

\(^a\)One-way ANOVA with Tukey’s multiple comparison test.

\(^b\)Student’s t test.

FCDI values (mean ± SEM) were determined as indicated in the "Methods" section with the exception that for Cav1.4Δex47 + CaM12, FCDI was measured at −10 mV to accommodate the positive shift in the tail-current voltage curves.
pulse in promoting CDI. In contrast, $I_{Ba}$ showed little inactivation across all voltages tested (Fig. 6 A). In cells cotransfected with CaM12 or CaM34, Fractional $I_{Ca}$ was still smaller than that for $I_{Ba}$, but to a lesser extent than in cells transfected with Ca1.4Δex47 alone, such that $F_{CDI}$ was significantly weaker with CaM12 and CaM34 than with Ca1.4Δex47 alone (Fig. 6, B and C; and Table 4). To verify the importance of both lobes of CaM in regulating CDI, we analyzed the effects of a CaM mutant with both N and C lobes disabled (CaM1234). There was no difference in fractional $I_{Ca}$ and $I_{Ba}$ in cells cotransfected with CaM1234, indicating that CDI was abolished (Fig. 6 D and Table 4). These results show that CDI of Ca1.4Δex47 is mediated by both the N and C lobe of CaM.

To investigate how molecular determinants in the IQ domain may influence CDI, we compared CDI in cells transfected with Ca1.4Δex47-5A and Ca1.4Δex47-3A. For Ca1.4Δex47-5A, there was no CDI in that there was no difference in inactivation of $I_{Ca}$ and $I_{Ba}$, similar to Ca1.4 + ex47 (Fig. 7, A and B; and Table 4). For Ca1.4Δex47-3A, the results were more complex. In a subset of cells, there was no CDI, and $I_{Ca}$ activated very slowly, likely reflecting the loss of CaM N lobe regulation of CDEA (Fig. S2).
The lack of inactivation of both ICa and IBa in these cells suggests that the 3A mutations weaken CaM C lobe as well as CaM N lobe contributions to CDI. However, in some cells, there was residual CDI (Fig. 7 C), perhaps due to higher concentrations of CaM (Liu et al., 2010). In these cells, the fast phase of ICa inactivation was similar to that in cells cotransfected with Cav1.4Δex47 and CaM12 (Fig. 6 B), suggesting that it was mediated by the CaM C lobe. Consistent with this possibility, cotransfection of Cav1.4Δex47-3A with CaM34 eliminated CDI in all cells analyzed (Fig. 7 D and Table 4). We interpret these findings to mean that the 3A mutations in Cav1.4Δex47 disrupt CaM N lobe–mediated CDI but also weaken CaM C lobe–mediated CDI under some conditions.

CaM N lobe regulation of CDI is diminished with a CSNB2 mutation in Cav1.4

Although it responds to more global elevations in cytosolic Ca2+ with respect to CDI of Ca2.2 channels (DeMaria et al., 2001; Lee et al., 2003), the N lobe of CaM is a local Ca2+ sensor in the context of Ca2.1.2 and Ca2.1.3 CDI due to CaM binding to an N-terminal spatial Ca2+ transforming element (NSCaTE) in these channels. Thus, CaM N lobe–triggered CDI can be measured even with strong Ca2+ buffering (i.e., 5–10 mM EGTA) when Ca2.1.2 and Ca2.1.3 are coexpressed with CaM34 (Dick et al., 2008). The NSCaTE is not conserved in Ca2.1.4, and yet CaM N lobe–mediated CDI of Ca2.1.4Δex47 (i.e., +CaM34; Fig. 6 C) was still evident under our experimental conditions with 5 mM EGTA in the intracellular recording solution. We hypothesized that while deletion of exon 47 might disrupt the CTM’s ability to blunt CaM interactions with the channel, the C TM and/or the rest of the C-terminal domain might enable CaM N lobe–mediated CDI even in the presence of high intracellular Ca2+ buffering. If so, then deletion of these C-terminal regions should prevent CaM N lobe–driven CDI.

To test this, we turned to the K1591X mutation associated with CSNB2 (Strom et al., 1998). This mutation truncates the entire CTD of Ca2.1.4 distal to the IQ domain (Fig. 8 A); the loss of the CTM enables CaM–dependent CDI (Singh et al., 2006). In contrast to the suppression of CDI of Cav1.4Δex47 caused by both CaM12 and CaM34 (Fig. 6, B and C), ICa inactivation in K1591X mutant channels was suppressed by CaM34 but unaffected by CaM12. Moreover, CaM34 completely abolished inactivation of ICa mediated by K1591X channels (Fig. 8 A), whereas CaM34 had a more modest impact in this respect for Ca2.1.4Δex47 (Fig. 6 C). ICa in cells transfected with K1591X alone or together with CaM34 showed a rapid initial phase of inactivation that was abolished in cells cotransfected with K1591X and CaM34 (Fig. 8 A), similar to the CaM C lobe–mediated CDI of Ca2.1.4Δex47 (i.e., +CaM34; Fig. 6 B). For both K1591X and Ca2.1.4Δex47, ICa could be fit with a double exponential function. However, the time constant (τ) for fast inactivation was significantly shorter for K1591X than for Ca2.1.4Δex47 (Fig. 8 B). There was no significant difference in the amplitude of slow (Aslow) and fast (Afast) inactivation for Ca2.1.4Δex47, but Afast was generally larger than that for Aslow for K1591X, although the difference did not reach statistical significance. These results show that K1591X is incapable of CaM N lobe–mediated CDI, and that the CaM C lobe mediates a fast phase of CDI that is more prominent in K1591X than in Ca2.1.4Δex47.

We also analyzed the impact of the 3A mutations in K1591X (K1591X-3A). In contrast to the mixed effects of these mutations in weakening CDI of Ca2.1.4Δex47 (Fig. 7, C and D; and Fig. S2), CDI
was completely abolished in all recordings of cells transfected with K1591X-3A (Fig. 9, A and B; FCDI = 0.47 ± 0.04 for K1591X versus −0.04 ± 0.03 for K1591X-3A; P = 0.001 by t test). These results reveal a more prominent role for F1582A, Y1583A, and F1586A in CaM C lobe–mediated CDI of K1591X as compared with Cav1.4Δex47.

Given that K1591X is deficient in CaM N lobe–dependent CDI, we tested if this mutant was also lacking in CaM N lobe regulation of CDEA. If so, then the tail current I–V relationship for ICa, mediated by K1591X transfected alone or with CaM12, or for K1591X and K1591X-3A (Table 2). Thus, although F1582A, Y1583A, and F1586A regulate CaM C lobe–dependent CDI, these residues are dispensable for regulating activation of K1591X.

Discussion
Our study provides new insights into the regulation of Cav1.4 channels by CaM. First, we identify an unexpected role for CaM in CDEA—a Ca2+-dependent enhancement of Cav1.4 activation mediated by the N lobe of CaM. CDEA requires the deletion of exon 47 as well as the inclusion of other regions of the CTD in Cav1.4Δex47; the absence of these regions in K1591X may account for its lack of CaM N lobe–mediated CDEA. Second, we show that when CDI of Cav1.4 is unmasked by partial disruption of the CTM, CaM N lobe mediates a slow component of CDI that is significantly weakened upon full deletion of CTD distal to the IQ domain. This contribution of CaM N lobe is evident in CDI of Cav1.4Δex47 but not that of K1591X, which is dominated by the CaM C lobe. Our findings support a model in which variations in the CTD adjust how the N and C lobe of CaM modulate Cav1.4 (Fig. 11), providing a context for understanding the functional consequences of naturally occurring and pathological variants of Cav1.4 in the retina.

A novel role for CaM in enhancing activation of Cav1.4
CaM is a dynamic regulator of Cav1.4 channels and can vary its modulatory impact upon binding Ca2+. When bound to the pCTD, Ca2+-free (apo) CaM boosts channel open probability (Po), which then diminishes as Ca2+ binding to CaM triggers CDI (Adams et al., 2014). In the context of Cav1.4 + ex47 and the long variant of Cav1.3 (+exon 42A; Singh et al., 2008), the CTM competes with apoCaM binding (Liu et al., 2010; Adams et al., 2014). Deletion of exon 47 may allow for stronger binding of apoCaM to the pCTD.
which may explain the negative shifts in the tail I-V curves for $I_{ca}$ mediated by Ca$_{1,4}$Δex47 (Fig. 1C). While these shifts were not affected by CaM mutants (Figs. 2C and 3C) or by the 3A or 5A mutations (Figs. 4C and 5C), it is possible that these experimental manipulations incompletely blunted apoCaM interactions with the channel, as some determinants for apoCaM binding may reside outside the IQ domain (Ben Johny et al., 2013). As has been proposed for Ca$^{2+}$/CaM-dependent facilitation of Ca$_{2,1}$ channels (Chaudhuri et al., 2007), Ca$^{2+}$/CaM may promote transitions to a gating mode characterized by even higher $P_c$ than with apoCaM, and this may be disrupted by the 3A and 5A mutations. Finer resolution of the mechanism by which the CTM modulates activation of $I_{ca}$ and $I_{Ba}$ awaits detailed alanine scanning mutagenesis to unearth specific contacts for CaM in the pCTD in combination with single-channel analyses of $P_c$.

**Differences in the CaM lobe dependence of Ca$_{1,4}$Δex47 and K1591X**

Unlike Ca$_{1,4}$Δex47, K1591X does not undergo CDEA in that neither CaM N lobe nor the 3A mutations had any effects on the tail I-V curves (Fig. 10). The absence of CaM N lobe regulation of K1591X is further suggested by our kinetic analyses of $I_{ca}$ inactivation, as well as the blockade of CDI by CaM$_{34}$ (Fig. 8A). These experiments implicated the CaM C lobe in mediating CDI of K1591X, in stark contrast to the contributions of both CaM lobes to CDI of Ca$_{1,4}$Δex47 (Fig. 6). Deletion of exon 47 removes the initial 47 amino acids of the CTM (Singh et al., 2006), 26 residues according to the CTM region defined by Wahl-Schott et al. (2006). However, Ca$_{1,4}$Δex47 still possesses a 34- amino acid stretch within the CTM shown to be critical for CDI suppression (Singh et al., 2006; Wahl-Schott et al., 2006). We propose that the residual CTM in concert with the post-IQ region unmask$\overline{s}$ and/or stabilizes CaM N lobe contacts within the IQ domain that support the slow component of CDI in Ca$_{1,4}$Δex47 (Fig. 11), which is blocked by CaM$_{34}$ (and by 3A mutations; Figs. 6B and 7C). In the context of Ca$_{1,2}$, the threonylalanine, tyrosine, and phenylalanine residues affected by the 3A mutations form extensive contacts with CaM N lobe (Van Petegem et al., 2005). Since these residues are conserved in Ca$_{1,4}$, as well as in all Ca$_{1}$ channels (Van Petegem et al., 2005), we propose that they may similarly serve as anchoring points for CaM N lobe in Ca$_{1,4}$Δex47. Although these residues may be free to interact with CaM in K1591X, the absence of both CTM and post-IQ regions in K1591X may prevent the CaM N lobe interaction, or its functional consequences in promoting CDI (Fig. 11). However, our findings also reveal a key role for F1582A, Y1583A, and F1586A in weakening CaM C lobe-mediated CDI in both Ca$_{1,4}$Δex47 and K1591X, which is not wholly unexpected given that CaM C lobe contacts may be intermingled among these residues (Van Petegem et al., 2005). Additional structural studies are needed to resolve how the CTM with and without exon 47 affects CaM interactions with the pCTD.

**Physiological relevance of Ca$_{1,4}$ CTM modifications**

Although our results suggest differences in CaM regulation of Ca$_{1,4}$Δex47 and K1591X, the impact of CaM on these channels in photoreceptors is likely influenced by CaBP4 (Haeseeler et al., 2004; Lee et al., 2015)—a member of a family of Ca$^{2+}$ binding proteins (CaBPs) that suppress CDI of Ca$_{1}$ channels in part by competing with CaM binding to the IQ domain (Hardie and Lee, 2016). CaBP4 binds to the same sites as CaM within the pCTD of Ca$_{1,4}$ (Shaltiel et al., 2012; Haeseeler et al., 2016), and suppresses but does not completely abolish CDI of Ca$_{1,4}$Δex47 (Haeseeler et al., 2016). This suggests that CaM may still interact with and modulate Ca$_{1,4}$Δex47 despite the presence of CaBP4 in photoreceptor synaptic terminals (Haeseeler et al., 2004; Lee et al., 2015). When bound to Ca$^{2+}$, CaM is expected to compete more effectively for binding to the Ca$_{1}$ IQ domain than CaBPs (Findeisen et al., 2013), and CaBPs can bind to sites other than the IQ domain in regulating CDI (Zhou et al., 2005; Yang et al., 2014). Fluctuations in presynaptic Ca$^{2+}$ levels may promote dynamic regulation of Ca$_{1,4}$Δex47 by CaM and CaBP4, allowing for CDEA and CDI to shape presynaptic Ca$^{2+}$ signals supporting glutamate release from photoreceptor terminals. If K1591X is similarly regulated by CaM, the lack of CaM N lobe regulation of CDEA and CDI may lead to aberrant regulation of Ca$^{2+}$ signals that could degrade the transmission of visual information. Considering that the CTD of Ca$_{1}$ channels is a hotspot for protein interactions (Calin-Jageman and Lee, 2008), it is also conceivable that K1591X is unable to assemble with key synaptic proteins needed for the proper localization and/or function of the channel in photoreceptors.

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