A CACNA1C variant associated with cardiac arrhythmias provides mechanistic insights in the calmodulation of L-type Ca\textsuperscript{2+} channels

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We recently reported the identification of a \textit{de novo} single nucleotide variant in exon 9 of CACNA1C associated with prolonged repolarization interval. Recombinant expression of the glycine to arginine variant at position 419 produced a gain in the function of the L-type Ca\textsubscript{V}1.2 channel with increased peak current density and activation gating but without significant decrease in the inactivation kinetics. We herein reveal that these properties are replicated by overexpressing calmodulin (CaM) with Ca\textsubscript{V}1.2 WT and are reversed by exposure to the CaM antagonist W-13. Phosphomimetic (T79D or S81D), but not phosphosensitive (T79A or S81A), CaM surrogates reproduced the impact of CaM WT on the function of Ca\textsubscript{V}1.2 WT. The increased channel activity of Ca\textsubscript{V}1.2 WT following overexpression of CaM was found to arise in part from enhanced cell surface expression. In contrast, the properties of the variant remained unaffected by any of these treatments. Ca\textsubscript{V}1.2 substituted with the α-helix breaking proline residue were more reluctant to open than Ca\textsubscript{V}1.2 WT but were upregulated by phosphomimetic CaM surrogates. Our results indicate that (1) CaM and its phosphomimetic analogs promote a gain in the function of Ca\textsubscript{V}1.2 and (2) the structural properties of the first intracellular linker of Ca\textsubscript{V}1.2 contribute to its CaM-induced modulation. We conclude that the CACNA1C clinical variant mimics the increased activity associated with the upregulation of Ca\textsubscript{V}1.2 by Ca\textsuperscript{2+}–CaM, thus maintaining a majority of channels in a constitutively active mode that could ultimately promote ventricular arrhythmias.

Cardiac contraction during the systole is handled by the influx of Ca\textsuperscript{2+} into cardiomyocytes in response to depolarization during phase 2 of the cardiac action potential (1). Voltage-gated L-type calcium channel Ca\textsubscript{V}1.2 are expressed in the T-tubules such that localized Ca\textsuperscript{2+} entry triggers a sustained and more global Ca\textsuperscript{2+} release by the sarcoplasmic reticulum in the dyadic cleft (2). Cardiac L-type Ca\textsubscript{V}1.2 channels are heteromultimeric protein complexes formed by the pore-forming Ca\textsubscript{V}α1C subunit bound to the extracellular Ca\textsubscript{V}α2δ1 auxiliary subunits (3, 4) and to the cytoplasmic Ca\textsubscript{V}β (5) that binds with nanomolar affinity to the first intracellular linker (6). The Ca\textsubscript{V}α1 subunit is formed by a single polypeptide chain of 24 transmembrane helices grouped into four structural homologous domains (domains I, II, II, and IV) (Fig. 1). Although not a specific auxiliary subunit, calmodulin (CaM) contributes to Ca\textsuperscript{2+}-dependent facilitation and Ca\textsuperscript{2+}-dependent inactivation (CDI) of Ca\textsubscript{V}1.2 (7–9) through binding to the isoleucine–glutamine motif in the C-terminal tail of Ca\textsubscript{V}α1C (10–13).

First clinically described in 1957 (14), the long-QT syndrome (LQTS) is a major cause of sudden death in healthy infants and young adults (15–17). Congenital LQTS in the absence of structural defects (18) is often the result of inherited or \textit{de novo} genetic mutations in the DNA of a variety of ion channels (19). Gain-of-function mutations within the CACNA1C gene, coding for Ca\textsubscript{V}α1C, are associated with the LQTS type 8 also referred to as Timothy syndrome (TS) (20–22). Many TS variants were identified in a short region adjoining the sixth transmembrane segment of the Ca\textsubscript{V}α1C protein (Fig. 1). The canonical TS1 variant Gly406Arg results from a \textit{de novo} CACNA1C mutation in exon 8A (20). An atypical form of TS type 2 is associated with the Gly402Ser and Gly402Arg variants in the alternatively spliced exon 8 (21). More recent \textit{de novo} mutations have highlighted the importance of this region such as Glu407Gly/Ala (23, 24) and Arg518Cys/His (25). These missense variants are causing a gain of function in the Ca\textsubscript{V}1.2 channel as a result of slower inactivation kinetics that promote larger Ca\textsuperscript{2+} influx for the same depolarizing pulse (26). Nonetheless, functional outcomes of other TS mutations included marked loss of current density, a gain-of-function shift in activation, and increased window current (27). We have recently identified in the first intracellular region of Ca\textsubscript{V}α1C a missense variant, Gly419Arg, from a patient with prolonged QT interval (≈500 ms), syndactyly, left ventricular noncompaction, and slight delay in neurodevelopment (28). Unlike other TS variants located close to the high-affinity binding domain of Ca\textsubscript{V}β, Ca\textsubscript{V}1.2 Gly419Arg exhibited a gain-of-function shift in the activation gating and no decrease in the channel current decay (28).

Herein, we explored the regulation of the long QTS variant Gly419Arg (G449R in the rabbit clone numbering). Glycine
residues, inserted between the sixth transmembrane segment and the high-affinity binding site for CaVβ, have been shown to confer higher flexibility to this region (29–31), leading to reduced basal L-type CaV channel activity in cardiomyocytes (30). The reverse proposition, removing or substituting glycine residues in this locus, decreased the linker flexibility (31). Herein, we present evidence that the novel variant, whereby a conserved glycine is substituted by a larger arginine residue, promotes stronger activity (peak current density and activation gating) at physiological voltages akin to a constitutively hyperactive channel. This hyperactive mode was reconstituted in the WT channel by coexpression with CaM WT or pseudo-phosphorylated surrogates CaM T79D or CaM S81D and was abolished by the CaM antagonist W-13. In contrast, the inactivation kinetics of the gain-of-function TS CaV1.2 G419R variant classiﬁed as a pathogenic TS variant (35) were slightly faster than CaV1.2 WT (28). The faster inactivation kinetics were associated with increased peak current density and a leftward shift in the voltage of activation, leading to an increased probability of channel being open at physiological voltages without any significant change in the voltage dependence of inactivation (Table 1). Glycine residues close to the pore (e.g., Gly402 and Gly406) appear to be essential to convey the movement of the inactivation gate, whereas inserting glycine residues further away and closer to the high-affinity binding domain for CaVβ (Fig. 1) yielded opposite results (29, 30). Increased ﬂexibility within this stretch has been argued to loosen up the interaction between

**Results**

**Glycine substitution stimulates activation gating and peak current density of CaV1.2**

It is well known that gain-of-function mutations G402S and G406R (Fig. 1) decelerate inactivation kinetics (20, 21, 32–34). In contrast, the inactivation kinetics of the gain-of-function TS CaV1.2 G419R variant classified as a pathogenic TS variant (35) were slightly faster than CaV1.2 WT (28). The faster inactivation kinetics were associated with increased peak current density and a leftward shift in the voltage of activation, leading to an increased probability of channel being open at physiological voltages without any significant change in the voltage dependence of inactivation (Table 1). Glycine residues close to the pore (e.g., Gly402 and Gly406) appear to be essential to convey the movement of the inactivation gate, whereas inserting glycine residues further away and closer to the high-affinity binding domain for CaVβ (Fig. 1) yielded opposite results (29, 30). Increased ﬂexibility within this stretch has been argued to loosen up the interaction between

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**Figure 1. The CaV1.2 variant is located in the intracellular linker before the binding site for the CaVβ subunit.** The LQTS-related CaVα1C mutation G449 is located before the α-interacting domain (AID). A, the cryo-EM 3D structure of the rabbit CaV1.1 oligomeric complex at 3.6 Å for CaVα1S and at 3.9 Å for CaVβ (Protein Data Bank code: 5GJV). CaVα1C and CaVα1S share 81% homology in their primary protein sequence. L-type calcium channels share similar structure, being composed of the pore-forming subunit CaVα1 in red and CaVβ in blue and an intracellular subunit bound to CaVα1 through the intracellular helix linking domains I and II of CaVα1 (shown in dark green). The first transmembrane domain of CaVα1 (DI) is shown in yellow. The human LQTS-related CaVα1C G419R variant is similar to the rabbit CaVα1C G449R and is equivalent to Gly358 in CaVα1S. Image was produced by Discovery Studio 2020 (BIOVIA Pipeline Pilot 2020). B, cartoon of the corresponding secondary structure for the CaVα1C pore-forming subunit of the L-type CaV1.2 channel showing the four homologous domains (domains I to IV) with the N and C termini located into the cytoplasm. The CaVβ subunit–binding site on the CaVα1C subunit is referred to the “α-interacting domain” or AID. The AID is located within 20 residues of the sixth transmembrane segment in domain I (IS6). The primary sequence for the AID motif is shown below the primary sequence for the short region extending from the end of IS6 to the beginning of the AID. The relative position of three glycine variants reported in the Timothy syndrome (G402S, G406R, and G419R) is fully conserved across species and presented in red with the numbering in the rabbit clone used for this study. LQTS, long-QT syndrome.
Calmodulation of a Timothy syndrome variant

Table 1
Electrophysiological properties of CaV1.2 WT and G449R with W-13

| CaV1.2 | CaM | n/N | Peak I (pA/pF) | E0,act (mV) | R100 | n/N | E0,inact (mV) |
|--------|-----|-----|---------------|-------------|------|-----|--------------|
| WT     | Native | 30/7 | −15 ± 4      | −10 ± 3     | 0.65 ± 0.04 | 14/5 | −33 ± 3     |
|        | +W-13 | 17/4 | −8 ± 2       | −12 ± 4     | 0.57 ± 0.02 | 6/3  | −33 ± 3     |
| G449R  | Native | 31/4 | p < 0.002 versus control | −17 ± 3 | p < 0.001 versus WT | 13/4 | −35 ± 3 |
|        | +W-13 | 12/1 | −38 ± 8      | −17 ± 3     | 0.51 ± 0.03 | 5/2  | −34 ± 2     |

Effects of CaM inhibitor W-13 on the gating properties of CaV1.2 WT or G449R channels with native or endogenous CaM. CaV1.2 WT or G449R were coexpressed in HEKT cells with CaVβ2a and CaVα2δ1. Whole-cell currents were measured in the presence of 2 mM Ca²⁺ in the extracellular medium. E0,act values were estimated after a 5 s long depolarizing pulse to 0 mV. Fractional currents were fitted to Boltzmann equations as described in the Experimental procedures section. The R100 values report the relative current decay observed 100 ms after the peak current. n/N refers to the number of cells/transfections measured in each condition of study. Mean ± SD are shown. Statistical analysis was carried out by one-way ANOVA and a Bonferroni post hoc test.

CaVβ and CaVα2δ1 (30). We validated that the substitution of the glycine residue at position 449 (rabbit numbering) does not impair the interaction with the canonical CaVαβ and CaVα2δ1 subunits (Fig. 2). The latter observation is in line with the recent demonstration that interaction with CaVα2δ1 involves extracellular loops of CaV1.2 (4, 36, 37). We thus turned to investigate functional regulation by the ubiquitous CaM (38). Disease-causing mutations at CaM proteins lead to major cardiac dysfunction, and in turn, mutations at the CaM-binding site of ion channels have been associated with a host of diseases (39).

**CaM antagonist W-13 blocks CaV1.2 WT but not G449R whole-cell currents**

Functional regulation of CaV1.2 WT by endogenous CaM was examined with the membrane-permeable naphthalenesulfonamide derivative CaM antagonist W-13. Under our conditions, CaV1.2 WT currents activated at −35 mV and reached the peak inward current at +5 mV. As seen in Figure 3A, the peak current density of CaV1.2 WT was reduced by about 50% from −15 ± 4 pA/pF to −8 ± 2 pA/pF after adding 10 μM W-13 into the bath. Decay of the CaV1.2 WT current was accelerated in the presence of W-13, which
Calmodulation of a Timothy syndrome variant

Figure 3. CaV1.2 G449R is insensitive to W-13. A, representative CaV1.2 WT current traces recorded in the presence of native/endogenous CaM from HEKT cells before (left) and after (middle) the application of W-13. Peak current densities of CaV1.2 WT currents are plotted against the applied voltages and fitted by a Boltzmann equation (right). Incubation with 10 μM W-13 for 15 min reduced the CaV1.2 WT current density by approximately 50%, from −15 ± 4 pA/pF under control conditions versus −8 ± 2 pA/pF in the presence of W-13. B, representative CaV1.2 G449R current traces recorded in the presence of native/endogenous CaM from HEKT cells before (left) and after (middle) W-13 treatment. In contrast to CaV1.2 WT, CaV1.2 G449R currents were unaffected by the W-13 and did not display any inhibition in the peak current density (right). C, representative CaV1.2 WT current traces cotransfected with CaM WT recorded from HEKT cells before (left) and after (middle) W-13 treatment. Overexpression of CaM WT significantly enhanced the current density of CaV1.2 WT, whereas only approximately, 10% of peak CaV1.2 currents remained following W-13 treatment (right). Peak current densities of CaV1.2 WT coexpressed with CaM WT are plotted against the applied voltages and fitted by a Boltzmann-like equation. D, representative CaV1.2 G449R current traces cotransfected with CaM WT recorded from HEKT cells before (left) and after (middle) application of W-13. Unlike the CaV1.2 WT channels, overexpression of CaM WT did not alter the CaV1.2 G449R currents. Furthermore, inhibition of the CaV1.2 G449R peak currents by W-13 was undetectable (right). The vertical scale bars are 10 pA/pF, and the horizontal scale bars are 100 ms throughout. All biophysical values are reported in Tables 1–3. CaM, calmodulin; HEKT, human embryonic kidney 293T cell line.

Reduced the noninactivating component of CaV1.2 at the end of 100 ms depolarization (R100) from 0.65 ± 0.04 to 0.57 ± 0.02 (p < 0.001) (Table 1). Of note, W-13 did not impair Ca2+-dependent facilitation in cardiac cells (40). Under the same experimental conditions, the inhibitory effect of W-13 on the current amplitude and the acceleration of current decay were blunted in the G449R construct with −33 ± 12 versus −38 ± 8 pA/pF (Fig. 3B) suggesting that the glycine substitution prevents the channel modulation by endogenous CaM.

CaM promotes the activity of CaV1.2 WT

In a typical cellular environment, CaM targets could far exceed that of free endogenous CaM (41, 42). To further explore the regulation by CaM, CaM WT was overexpressed along with the complementary DNA (cDNA) coding for the channel subunits. Overexpressing CaM has been shown to compete with endogenous CaM WT and was successfully used to reveal the mechanistic actions of CaM on voltage-activated Ca2+ channels (43–46). Representative current traces from cells coexpressing CaV1.2 WT and CaM WT are shown in Figure 3C. As seen, under these conditions, the peak current density nearly doubled up from −15 ± 4 to −28 ± 8 pA/pF (p < 0.001 as compared with endogenous CaM) to reach values not significantly different than G449R under the same conditions (p > 0.05). CaM WT shifted the F0.5,act to hyperpolarized potentials (p < 0.05) and slightly accelerated the inactivation kinetics (Table 2). CaM enhanced the fraction of CaV1.2 WT currents that was inhibited by W-13, with about 90% reduction in peak current density, from 28 ± 8 pA/pF for control versus 3.3 ± 0.7 pA/pF for W-13 (p = 0.001). Overexpressing CaM WT caused undetectable changes in the peak current density, the voltage of activation, and the current decay of G449R that remained unaffected by the W-13 treatment (Fig. 3D) (Table 3). G449R functionally behaved like it intrinsically adopted a maximally active mode (47).

CaM was previously shown to bind to the I–II linker in addition to other intracellular sites within CaV1.2 (48). Pull-down assays demonstrated that CaM is tethered to the WT and the G449R channel complex (Fig. 4). In fact, the protein signal for G449R appeared to be more intense, hinting that it could maintain a stronger interaction with CaM. Enhanced channel activity could arise because of improved activation gating and/or increase in the relative cell surface protein expression/stability. Previous studies have reported that CaM enhances trafficking of CaV1.2 in human embryonic kidney (HEK) cells (49). CaM-induced increases in peak current density may reflect an improved surface expression of channel complexes. To sort this issue, we performed a series of cell fractionation assays. As seen in Figure 5 in the presence of endogenous CaM, the signal for CaV1.2 WT was stronger in the total membrane protein fraction (Fig. 5A, lane 3) than in the cell surface protein fraction (Fig. 5A, lane 4). Under the same conditions, the signal for CaV1.2 G449R was stronger in the cell surface protein fraction suggesting that G449R is better trafficked or more stable than channel complexes including the WT protein and endogenous CaM. Differences in the relative channel expression were obliterated when the channel complexes were overexpressed with CaM WT (Fig. 5B). Under these conditions, the WT and G449R channel complexes are similarly found in the cell surface fraction. Overexpression of CaM enhanced the cell surface trafficking of
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Effects of CaM inhibitor W-13 on the biophysical properties of CaV1.2 WT channels. CaV1.2 WT was coexpressed with CaV1.2, CaM WT, and phosphoresistant and phosphomimetic variants (T79A, T79D, S81A, or S81D). Activation properties ($E_{0.5, \text{act}}$) were estimated from the $I$–$V$ relationships and fitted as described in the Experimental procedures section. The R100 values report the relative current decay observed 100 ms after the peak current. n/N refers to the number of cells/transfections measured in each condition of study. Mean ± SD are shown. Statistical significance of observed differences was evaluated using one-way ANOVA and Bonferroni test ($p < 0.05$). As seen, all experimental conditions yielded whole-cell Ca$^{2+}$ currents that were not significantly different from one another ($p > 0.05$).

### Table 2
Electrophysiological properties of CaV1.2 WT with CaM WT and phosphorylation surrogates

| CaV1.2  | CaM       | n/N | Peak current density (pA/pF) | $E_{0.5, \text{act}}$ (mV) | R100 |
|---------|-----------|-----|-----------------------------|---------------------------|------|
| CaV1.2 WT | CaM WT    | 28/6| $-28 \pm 8$                 | $-14 \pm 3$               | 0.60 ± 0.03 |
| +W-13   |           | 9/2 | $-3.3 \pm 0.7$              | $-14 \pm 3$               | 0.65 ± 0.03 |
| CaM T79A | 18/2      |     | $-13 \pm 5$                | $-13 \pm 2$               | 0.69 ± 0.03 |
| +W-13   |           | 16/2| $-14 \pm 3$                | $-14 \pm 3$               | 0.56 ± 0.03 |
| CaM T79D | 15/4      |     | $-29 \pm 7$                | $-15 \pm 3$               | 0.55 ± 0.03 |
| +W-13   |           | 10/2| $-4 \pm 1$                 | $-12 \pm 2$               | 0.71 ± 0.02 |
| CaM S81A | 17/2      |     | $-13 \pm 4$                | $-13 \pm 2$               | 0.66 ± 0.03 |
| +W-13   |           | 8/1 | $-12 \pm 2$                | $-9 \pm 3$                | 0.61 ± 0.02 |
| CaM S81D | 14/1      |     | $-32 \pm 8$                | $-13 \pm 3$               | 0.62 ± 0.02 |
| +W-13   |           | 9/1 | $-5 \pm 1$                 | $-12 \pm 2$               | 0.70 ± 0.02 |

Effects of CaM inhibitor W-13 on the biophysical properties of CaV1.2 WT channels. CaV1.2 WT was coexpressed with CaV1.2, CaM WT, and phosphoresistant and phosphomimetic variants (T79A, T79D, S81A, or S81D). Activation properties ($E_{0.5, \text{act}}$) were estimated from the $I$–$V$ relationships and fitted as described in the Experimental procedures section. The R100 values report the relative current decay observed 100 ms after the peak current. n/N refers to the number of cells/transfections measured in each condition of study. Mean ± SD are shown. Statistical significance of observed differences was evaluated using one-way ANOVA and Bonferroni test ($p < 0.05$). As seen, all experimental conditions yielded whole-cell Ca$^{2+}$ currents that were not significantly different from one another ($p > 0.05$).

### Table 3
Electrophysiological properties of CaV1.2 G449R with CaM WT and phosphorylation surrogates

| CaV1.2  | CaM       | n/N | Peak current density (pA/pF) | $E_{0.5, \text{act}}$ (mV) | R100 |
|---------|-----------|-----|-----------------------------|---------------------------|------|
| CaV1.2 G449R | CaM WT   | 20/3| $-35 \pm 10$               | $-16 \pm 2$               | 0.50 ± 0.03 |
| +W-13   |           | 9/2 | $-35 \pm 9$                | $-17 \pm 3$               | 0.54 ± 0.01 |
| CaM T79A | 15/2      |     | $-32 \pm 10$              | $-16 \pm 2$               | 0.50 ± 0.02 |
| +W-13   |           | 14/2| $-33 \pm 10$              | $-17 \pm 3$               | 0.45 ± 0.03 |
| CaM T79D | 24/3      |     | $-35 \pm 9$               | $-18 \pm 4$               | 0.50 ± 0.02 |
| +W-13   |           | 8/1 | $-32 \pm 9$               | $-16 \pm 2$               | 0.50 ± 0.02 |
| CaM S81A | 13/1      |     | $-34 \pm 9$               | $-18 \pm 2$               | 0.48 ± 0.02 |
| +W-13   |           | 9/1 | $-33 \pm 7$               | $-19 \pm 2$               | 0.51 ± 0.02 |
| CaM S81D | 9/1       |     | $-35 \pm 8$               | $-17 \pm 3$               | 0.52 ± 0.02 |
| +W-13   |           | 10/1| $-33 \pm 10$              | $-15 \pm 3$               | 0.50 ± 0.02 |
CaV1.2 WT and G449R were successfully pulled indicating that CaV1.2 G449R interacts with CaV1.2. Roughly 10 to 45% of endogenous CaM is constitutively phosphorylated in vivo by casein kinase II (CK2) (52, 56, 57), and in vitro studies confirmed that CaM Thr79 and Ser81 are the most likely targets (58, 59). Experiments were thus performed in the presence of 4,5,6,7-tetrabromobenzotriazole (TBB; Tocris, Bio-Techne), a specific inhibitor of CK2. As shown in Table 4, TBB significantly decreased the peak current density by ≈70% and right shifted the activation gating of whole-cell currents recorded in the presence of CaV1.2 WT with native CaM. Furthermore, TBB annihilated the impact of overexpressing CaM WT on the peak current density of CaV1.2 WT. The impact of TBB was comparable to the disrupting effect of W-13 and much greater than the coexpression with either CaM T79A or S81A.

Ca2+ binding to CaM remains a prerequisite step for driving the channel complex into its higher functioning mode. Overexpression of the Ca2+-free form of CaM (CaM1234 or CaM D20A/D56A/D93A/D129A) decelerated, as expected, the CDI kinetics (Table 5). It also abrogated the increased peak current density and restored its activation gating to the level observed in the presence of endogenous CaM.

**The gain of function in CaV1.2 G449R requires the Ca2+-bound CaM form**

Unlike CaV1.2 WT, coexpressing either phosphoresistant CaM T79A (Fig. 7A, left) and S81A (Fig. 7C, left) or phosphomimetic CaM T79D (Fig. 7B, left) and S81D (Fig. 7D, left) with Cav1.2 G449R did not appreciably affect the peak current density, activation gating kinetics (E0.5,act), and current decay (R100) of CaV1.2 G449R (Fig. 7, E and F and Table 3). As observed in the presence of CaM WT, the peak current densities (Fig. 7, A−D, middle, right; Fig. 7E), the E0.5,act (Fig. 7F) were not altered by the application of W-13. This sharply contrasts with the results obtained with the CaV1.2 WT channel complex. Nonetheless, preventing the phosphorylation of all CaM molecules with TBB reduced by 50% the peak current measured under all other conditions (Table 4) save for CaM1234 (Table 5). Indeed, limiting Ca2+ binding to CaM with the CaM1234 variant not only impaired the CDI of CaV1.2 G449R but also prevented the leftward shift in activation gating and the increase in peak current density (Table 5).
Calmodulation of a Timothy syndrome variant

Alanine substitutions in the hinge region of CaM are not disrupting interaction with CaV1.2

We next evaluated whether CaM variants T79A and S81A alter the interaction of CaM with the pore-forming CaVα1C subunit (Fig. 8). Whether for CaV1.2 WT or CaV1.2 G449R, the pull-down assays failed to reveal a correlation between the signal intensity and any of the tested CaM-substituted proteins indicating that phosphomimetic analogs of CaM were not bound to CaV1.2 and the membrane-anchored CaVβ2a. This result was successfully obtained from two independent transfections carried out over the course of 2 months. CaM, calmodulin; HEKT, human embryonic kidney 293T cell line.

Substitution with an alpha-helix breaker in CaV1.2 antagonizes channel function

The structural properties of the I–II linker near the high-affinity binding site for CaM have been consistently shown to modulate the gating properties of CaV1 and CaV2 channels (31). In CaV1.2, most, if not all substitutions, tested at position 449 altered the channel properties. Stronger activation gating and faster inactivation kinetics characterized CaV1.2 G449A, G449D, and G449K in the presence of endogenous CaM (Fig. S1 and Table 6). All these substituted channels activated inward Ca2+ currents measured in nontransfected cells. The activation gating of G449P was right shifted when compared with CaV1.2 WT. In contrast to G449R and G449K, CaV1.2 G449P was modulated by CaM phosphomimetic
Figure 6. Phosphomimetic CaM T79D and S81D upregulate CaV1.2 WT channels. A and C, middle, CaV1.2 WT was coexpressed with the “phosphoresistant” CaM mutations T79A or S81A. Overexpression of CaM T79A or S81A failed to enhance the currents and was insensitive to W-13. A and C, right, average I-V curves of CaV1.2 WT coexpressed with CaM T79A or S81A. The peak current densities were not different between control and W-13 treatment. B and D, left, middle, CaV1.2 WT current traces recorded from HEKT cells after coexpression with phosphomimetic CaM T79D or S81D. Overexpression of CaM T79D or CaM S81D boosted CaV1.2 peak currents that were sharply abolished by the extracellular application of W-13. The vertical scale bars are 10 pA/pF, and the horizontal scale bars are 100 ms throughout. B and D, right, average I-V curves of CaV1.2 WT coexpressed with CaM T79D or S81D for control and W-13 treatment. E and F, the distribution of the peak current densities and E_{0.5,act} for control and W-13 are summarized as filled circles for CaV1.2 WT coexpressed with either CaM WT (black), T79A (red), T79D (blue), S81A (green), or S81D (light purple). The mean data ± SD are shown as gray hyphens. The values of the average peak current densities and E_{0.5,act} are listed in Table 2. CaM, calmodulin; HEKT, human embryonic kidney 293T cell line.
Calmodulation of a Timothy syndrome variant

Table 4
Electrophysiological properties of CaV1.2 WT and G449R with TBB

| CaV1.2     | CaM       | n/N | Peak current density (pA/pF) | E_{0.5,act} (mV) | R100  |
|------------|-----------|-----|-------------------------------|------------------|-------|
| CaV1.2 WT  | Native CaM| 30/7| −15 ± 4                      | −10 ± 3          | 0.65 ± 0.04 |
|            | +TBB      | 6/2 | −4 ± 1                       | −5 ± 1           | 0.80 ± 0.01 |
| CaM WT     | +TBB      | 28/6| p = 0.001 versus control     | p = 0.001 versus control | p < 0.001 versus control |
| CaV1.2 G449R| Native CaM| 31/4| −33 ± 12                     | −17 ± 3          | 0.52 ± 0.03 |
|            | +TBB      | 7/2 | −15 ± 6                      | −15 ± 3          | 0.55 ± 0.03 |
| CaM WT     | +TBB      | 20/3| p = 0.001 versus control     | p = 0.001 versus control | p < 0.001 versus control |
| CaV1.2 G449R| Native CaM| 10/1| −35 ± 12                     | −16 ± 2          | 0.50 ± 0.03 |
|            | +TBB      | 7/2 | −13 ± 5                      | −13 ± 4          | 0.57 ± 0.03 |
|            |           |    | p < 0.001 versus control     | p = 0.06 versus control | p < 0.01 versus control |

Effects of TBB, the cell-permeable inhibitor of CK2 on the biophysical properties of CaV1.2 WT and CaV1.2 G449R channels. CaV1.2 WT or G449R was coexpressed with CaVβ2a, CaVα2δ1, and CaM WT as indicated. Two days after transfection, experiments were performed in the presence of 2.5 μM TBB, usually regarded as a membrane-permeable specific inhibitor of CK2. Activation properties (E_{0.5,act}) were estimated from the I–V relationships and fitted as described in the Experimental procedures section. The R100 values report the relative current decay observed 100 ms after the peak current. n/N refers to the number of cells/transfections measured in each condition of study. Mean ± SD are shown. Statistical analysis was evaluated using one-way ANOVA and Bonferroni post hoc test. As seen, TBB significantly decreased the channel peak current density under all conditions. It also significantly right shifted the activation gating of CaV1.2 WT but not of CaV1.2 G449R.

variants (Fig. 9 and Table 6). Peak currents of G449P nearly tripled in the presence of CaM WT, CaM T79D, or CaM S81D and were not significantly altered by coexpressing CaM T79A or CaM S81A (Fig. 9, A and B). Remarkably, the activation of the G449P channel was left shifted in the presence of the phosphor-silenced CaM variants (Fig. 9D), the only occurrence where the larger peak currents were not associated with stronger activation gating. Altogether, these observations support a strong mechanistic link between the structural properties of the I–II linker near the binding site for CaVβ and the modulation of the channel activation gating by CaM. In particular, the channel propensity to adopt a longer α-helix in this region appears to improve the activation gating of the channel and to supersede the modulation by the phosphor-lated forms of CaM.

Discussion

Ca^{2+}–CaM modulates the activity of L-type CaV1.2 through multifaceted mechanisms

The ubiquitous multifunctional Ca^{2+}-binding protein CaM is a two-lobe protein with each of two hydrophilic pockets for Ca^{2+} sensing separated by a flexible central linker. It is regulating the function of many voltage-gated ion channels, such as Kv7.2 (61), Na_{v}1.4 (7), and in particular, voltage-gated Ca_{v} channels (7, 62) (for review, see Ref. (62)). At least two CaM molecules can simultaneously bind to the C-terminal region of CaV1.2 (63, 64), but additional binding sites in the N-terminal region and the first intracellular linker of CaV_{α}1C have been identified (48, 63, 65–67). The overall structural organization of CaM within the CaV1.2 channel complex remains to be established. CaM-binding sites were not resolved in the cryo-electron microscopy structure of the homologous CaV_{α}1.1 channel (3).

In CaV1.2 channels, Ca^{2+} binding to CaM contributes to CDI and Ca^{2+}-dependent facilitation (7–9). Either process requires the binding of incoming Ca^{2+} ions to CaM pre-associated to the isoleucine–glutamine motif in the C-terminal region of the pore-forming CaV_{α}1C subunit (10–13). The potentiating form of CaM-dependent facilitation or upregulation is observed in native cardiac L-type channels during trains of depolarization (68, 69) but usually not reported in recombinant systems with the intact CaV1.2 WT channel (8, 9, 29). We herein report that phosphomimetic analogs of CaM stimulate Ca^{2+} influx and promotes the activation gating of CaV1.2. CaM promotes the cell surface trafficking of CaV_{α}1.2 and stimulates function through an increase in peak current density and a leftward shift in the activation gating. In our

Table 5
Effect of CaM1234 on electrophysiological properties of CaV1.2 WT and CaV1.2 G449R

| CaV1.2     | CaM       | n/N | Peak current density (pA/pF) | E_{0.5,act} (mV) | R100  |
|------------|-----------|-----|-------------------------------|------------------|-------|
| CaV1.2 WT  | CaM1234   | 10/2| −9 ± 2                        | −6 ± 2           | 0.76 ± 0.02 |
|            | +W-13     | 7/1 | p < 0.001 versus CaM WT       | p < 0.001 versus CaM WT | p < 0.001 versus CaM WT |
| CaV1.2 G449R| CaM1234   | 4/2 | −17 ± 4                       | −10 ± 2          | 0.73 ± 0.01 |
|            | +W-13     | 6/1 | p < 0.001 versus CaM WT       | p < 0.001 versus CaM WT | p < 0.001 versus CaM WT |

Whole-cell currents were recorded from HEK cells transiently transfected with CaV1.2 WT or variants coexpressed with CaVβ2a, CaVα2δ1, and CaM1234. Activation properties (E_{0.5,act}) were estimated from the I–V relationships and fitted to a BoltzIV equation as described in the Experimental procedures section. The R100 values report the relative current decay observed 100 ms after the peak current. n/N refers to the number of cells/transfections measured in each condition of study. Mean ± SD are shown. Statistical analysis was carried out against the values measured for CaM WT. Herein “control” refers to the data collected in the presence of CaM1234 in the absence of W-13.
Figure 7. CaV1.2 G449R is not modulated by CaM or CaM inhibitor W-13. Representative CaV1.2 G449R current traces were recorded from HEKT cells in the presence of 2 mM Ca\(^{2+}\). A–D, left, CaV1.2 G449R was coexpressed with CaM WT, with the phosphoresistant CaM (T79A or S81A) or with phosphomimetic CaM (T79D or S81D) as shown. A–D, middle, CaV1.2 G449R channels coexpressed with either CaM WT, T79A, T79D, S81A, or S81D are resistant to block by W-13. The vertical scale bars are 10 pA/pF, and the horizontal scale bars are 100 ms throughout. A–D, right, average I–V curves of CaV1.2 G449R coexpressed with CaM T79A, T79D, S81A, or S81D. The peak current densities were not different between control and W-13 treatment. E and F, the distribution of the peak current densities and E\(_{0.5,\text{act}}\) for control conditions and after W-13 treatment are summarized individually as filled circles for CaV1.2 G449R coexpressed with either CaM WT (black), T79A (red), T79D (blue), S81A (green), or S81D (light purple). The mean data ± SD are shown as gray hyphens. The complete set of values is found in Table 3. CaM, calmodulin; HEKT, human embryonic kidney 293T cell line.
hands, the latter actions of CaM require Ca\(^{2+}\) as it was impaired in the presence of CaM1234 where the four Ca\(^{2+}\)-binding sites are invalidated. This observation is compatible with data from Kim et al. (70), who reported that the interaction between the CaM-bound C-terminal peptide and the I–II linker is disrupted in the complete absence of Ca\(^{2+}\). CaM1234 prevented the increase in peak current density, failed to promote channel activation gating, and as expected, slowed down the CDI kinetics by 30%. Nonetheless, Ca\(^{2+}\) binding is not sufficient to account for the wide-ranging impact of CaM on channel function. The structural properties of the flexible linker region of CaM contribute to the channel response to CaM. Coexpression with CaM T79A or CaM S81A averted the boost in peak current density (although it did not alter the activation gating). In contrast, coexpression with either CaM WT or phosphomimetic CaM T79D or CaM S81D yielded similar results suggesting that phosphorylation of either site participates to the modulation of Ca\(_{\text{V}}\)1.2 by CaM. Indeed, preventing the phosphorylation of native and overexpressed CaM by incubating the cells with TBB, a membrane-permeable inhibitor of CK2, nearly abrogated channel function. Hence, Ca\(^{2+}\)-bound CaM modulates the function of the Ca\(_{\text{V}}\)1.2 channel complex in a fashion reminiscent of the ancillary subunits Ca\(_{\text{V}}\)\(_{\beta}\) and Ca\(_{\text{V}}\)\(_{\alpha}\)2\(_{\delta}\), which like CaM may also modulate other ion channels (71).

### Multiple mechanisms converge toward Ca\(_{\text{V}}\)1.2 G449R

The missense variant, glycine to arginine, was identified from a patient with prolonged QT interval (∼500 ms) and features associated with the TS, but its heterologous expression revealed a novel phenotype where the gain of function resulted from increased peak current density, a negative shift in the activation potential, and no decrease in the channel current decay (28). The hyperactive mode of the variant expressed in HEK293T (thereafter referred to as HEKT) cells was mimicked by the coexpression of Ca\(_{\text{V}}\)1.2 WT with CaM WT or phosphorylated surrogates CaM T79D or CaM S81D. The functional properties of the clinical glycine to arginine variant remained remarkably insensitive to pharmacological inhibition by W-13 and by overexpression with phosphorylation-resistant CaM analogs (T79A and S81A). The impact of the phosphorylation of CaM appears to be limited to function. Ca\(_{\text{V}}\)1.2 G449R was pulled down equally by CaM WT, T79A, and T79D. Preventing the phosphorylation of CaM with TBB, an inhibitor of CK2, significantly reduced the peak current density of Ca\(_{\text{V}}\)1.2 G449R by ∼50% without a significant

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**Figure 8. CaM T79A and T79D coimmunoprecipitate Ca\(_{\text{V}}\)1.2 WT and G449R.** HEKT cells were transiently transfected with Ca\(_{\text{V}}\)\(_{\beta}\)2a in the presence of Ca\(_{\text{V}}\)1.2 WT or Ca\(_{\text{V}}\)1.2 G449R and either CaM WT, CaM T79A, or CaM T79D. A, total proteins are shown. B, coimmunoprecipitation was carried out with anti-His magnetic beads. Immunoblotting was carried out after elution of the bound proteins using the antibodies described in the legend of Figure 4. As seen, Ca\(_{\text{V}}\)1.2 WT and G449R, Ca\(_{\text{V}}\)\(_{\beta}\)2a, and CaM proteins were translated at the expected molecular masses of 250, 70, and 18 to 24 kDa, respectively. There was no significant difference between the signals measured in the presence of either CaM WT, CaM T79A, or CaM T79D. The signals were nonetheless systematically stronger for Ca\(_{\text{V}}\)1.2 G449R than for Ca\(_{\text{V}}\)1.2 WT despite equivalent loading and similar signals for the total proteins. Similar data were obtained from three independent transfections carried out over the course of 2 months with protein extraction carried out with digitonin or CHAPS. CaM, calmodulin; HEKT, human embryonic kidney 293T cell line.
Calmodulation of a Timothy syndrome variant

### Table 6
Electrophysiological properties of Ca\textsubscript{v}1.2 Gly449 variants with CaM phosphorylation surrogates

| Cav1.2     | CaM       | n/N | Peak current density \(\text{pA/pF}\) | \(E_{\text{0.5,act}}\) [mV] | \(R_{100}\) |
|------------|-----------|-----|--------------------------------------|-----------------------------|----------|
| Ca\textsubscript{v}1.2 G449A Native | 12/2 | −35 ± 8 | −18 ± 3 | 0.55 ± 0.03 |
| CaM WT     | 22/4 | −31 ± 8 | −15 ± 2 | 0.57 ± 0.03 |
| CaM T79A 7/1 |      | −11 ± 2 | −13 ± 2 | 0.70 ± 0.01 |
| CaM T79D 6/1 |      | −28 ± 8 | −18 ± 2 | 0.55 ± 0.02 |
| CaM S81A 13/2 |     | −14 ± 3 | −13 ± 2 | 0.70 ± 0.03 |
| CaM S81D 19/2 |     | −30 ± 9 | −14 ± 3 | 0.58 ± 0.03 |
| Ca\textsubscript{v}1.2 G449D Native | 17/2 | −14 ± 4 | −15 ± 2 | 0.62 ± 0.03 |
| CaM WT     | 17/2 | −27 ± 8 | −17 ± 2 | 0.56 ± 0.02 |
| CaM T79A 16/1 |      | −15 ± 4 | −16 ± 3 | 0.59 ± 0.02 |
| CaM T79D 14/1 |      | −29 ± 6 | −18 ± 2 | 0.55 ± 0.02 |
| CaM S81A 21/2 |     | −16 ± 4 | −15 ± 2 | 0.60 ± 0.02 |
| CaM S81D 10/1 |     | −33 ± 7 | −18 ± 2 | 0.52 ± 0.02 |
| Ca\textsubscript{v}1.2 G449P Native | 10/2 | −2.5 ± 0.8 | −4 ± 1 | 0.72 ± 0.02 |
| CaM WT     | 8/2  | −6 ± 1 | 1.0 ± 2 | 0.65 ± 0.02 |
| CaM T79A 3/1 |      | −1.6 ± 0.3 | 0.76 ± 0.01 | p < 0.001 versus native CaM |
| CaM T79D 4/1 |      | −11 ± 3 | 0.5 ± 1.5 | 0.57 ± 0.01 |
| CaM S81A 4/1 |      | −1.8 ± 0.5 | −10 ± 2 | 0.73 ± 0.02 |
| CaM S81D 3/1 |      | −9.7 ± 0.7 | 3.5 ± 0.7 | 0.69 ± 0.03 |
| Ca\textsubscript{v}1.2 G449K Native | 18/2 | −23 ± 5 | −15 ± 3 | 0.60 ± 0.02 |
| CaM WT     | 26/6 | −26 ± 6 | −15 ± 3 | 0.61 ± 0.03 |
| CaM T79A 8/1 |      | −28 ± 7 | −17 ± 2 | 0.57 ± 0.02 |
| CaM T79D 12/1 |     | −30 ± 6 | −17 ± 2 | 0.54 ± 0.02 |
| CaM S81A 15/2 |     | −27 ± 7 | −16 ± 3 | 0.56 ± 0.03 |
| CaM S81D 15/2 |     | −29 ± 5 | −17 ± 3 | 0.56 ± 0.03 |

Ca\textsubscript{v}1.2 Gly449 variants were coexpressed with Ca\textsubscript{v}1.2, Ca\textsubscript{v}2.1, and CaM WT or CaM T79A, CaM T79D, CaM S81A, or CaM S81D. Activation properties \(E_{\text{0.5,act}}\) were estimated from the \(I-V\) relationships as described in the Experimental procedures section. n/N refers to the number of cells/transfections measured in each condition of study. Mean ± SD are shown. Statistical analysis was carried out against CaM WT or against endogenous/native CaM. As seen, Ca\textsubscript{v}1.2 WT, G449P, and G449K were modulated by CaM phosphorylation surrogates, whereas the properties of G449K remained unaffected. G449A was not upregulated by CaM WT but was downregulated by phosphoresistant CaM variants.

alteration in the channel activation voltage as compared with the control conditions. The rate-limiting factor appears to be \(Ca^{2+}\) binding to CaM. Coexpression of G449R with the CaM1234 variant not only impaired the CDI and the increased peak current density but also prevented the leftward shift in activation gating. Overexpression of the CaM1234 variant obliterated the gain in the function of Ca\textsubscript{v}1.2 G449R, yielding an activity profile akin to Ca\textsubscript{v}1.2 WT in the presence of endogenous/native CaM. The stronger activity of Ca\textsubscript{v}1.2 G449 thus minimally requires the direct or indirect action of the \(Ca^{2+}\)-bound CaM form.

These observations suggest that the higher channel activity of G449R could result from a stronger affinity for native CaM. Though not measured in this article, the affinity between the two full-length proteins can be roughly approximated by the relative intensity of the signal measured in coimmunoprecipitation assays. Within all the limitations of this exercise, the protein signal obtained for G449R in coimmunoprecipitation assays was indeed systematically stronger than the signal measured for the WT channel complex when measured under the same experimental conditions and this over the course of 12 months. This interpretation is compatible with the cell surface fractionation assays showing that G449R was more likely to be found in the cell surface fraction than the WT channel complex in the presence of endogenous CaM, whereas this differential localization was not discernable when the cells
were saturated with overexpressed CaM. CaM bound to the C-terminal region of CaV1.2 has been previously reported to interact in a Ca\textsuperscript{2+}-dependent manner with the cytosolic I–II loop, where is located the glycine to arginine variant (70). It is thus conceivable that the higher “intrinsic” activity of G449R results from a stronger interaction with endogenous CaM. In this model, the cellular availability of CaM could modulate the operating window of CaV1.2.

CaV1.2 G449R is located in a structural region involved in activation gating (72), inactivation kinetics (73), protein stability, ubiquitination (74), and cell surface trafficking (75). The proximal segment of the first intracellular linker hosts the high-affinity binding site for CaV\textsubscript{β} (76) and plays a role in networking with direct partners such as galec tin (74) or Ras/ Rad proteins through CaV\textsubscript{β} (30, 77, 78). Glycine residues are unique in their lack of side-chain steric interference, permitting a higher flexibility to protein structures. Increasing flexibility by inserting glycine residues (29, 30) decreases channel function. In contrast, decreasing flexibility of this region by removing glycine residues promoted channel function (28, 31, 79, 80). The presence of a glycine residue proximal to the α-interacting domain in CaV1.2 WT could thus explain the requirement of a stronger depolarization in CaV1.2 WT versus G449R channels. The same position is already occupied by an arginine residue in CaV2.2 (31) and CaV2.3 channels whose activation is left shifted when compared with CaV1.2 under the same expression conditions (81).

The high-affinity binding site of CaV\textsubscript{β} adopts an α-helical structure in vitro (82). The relative rigidity α-helix could promote a strong van der Waals interaction between the guanylate domain of CaV\textsubscript{β} and hydrophobic residues of CaV1.2 (29, 83, 84). In the native protein, this α-helix breaks at the glycine located at position 449 (79). Crystallographic and circular dichroism spectroscopic studies demonstrated that the arginine substitution prolongs the α-helix (31). We also report that substitution with other α-helix-promoting residues, such as alanine (85), produced channels with strong activation properties, and from the contrary, substitution with proline, regarded as a α-helix breaker, was found to curb channel activation. The substituted channels however manifested distinct electrophysiological signatures in the presence of the phosphomimetic and phosphoresistant CaM proteins, from a complete indifference (G449K) to impaired peak current density in the presence of phosphoresistant CaM variants (G449A, G449D, G449P, and G449Q). Our data are compatible with the proposal that the longer α-helix enhances the coupling of the I–II linker with the inner pore responsible for channel activation. The intracellular linker would contribute to electromechanical coupling in CaV1.2 either through its intrinsic structural properties or following interaction with CaM.

The structural properties of the clinical variant could be envisioned to facilitate the interplay between accessory CaM proteins bound onto the C terminus of CaV1.2 and channel function as it was postulated for AKAP150 (86). In this context, the LQTS phenotype associated with the glycine to arginine substitution in the I–II linker could result from either process: an intrinsically stronger activation of CaV1.2 that renders the channel insensitive to cellular variations in phosphorylated CaM or else a higher affinity to CaM that causes the channel to be maximally activated at near endogenous concentration of CaM.

**Experimental procedures**

**Recombiant DNA techniques**

The CaV\textsubscript{α1C} subunit of CaV1.2 (GenBank accession number: X15539), CaV\textsubscript{β2a} (GenBank accession number: NM_001398773), and CaV\textsubscript{α2δ1} (GenBank accession number: NM_001398773) were used in this study.

**Figure 9. CaV1.2 G449R is modulated by CaM phosphorylation surrogates.** A, whole-cell currents were recorded from HEKT cells transiently transfected with CaV1.2 G449R coexpressed with CaV\textsubscript{α2δ1} and either CaM WT, with the phosphoresistant CaM (T79A or S81A), or with phosphomimetic CaM (T79D or S81D) as indicated. Exemplar traces are shown (from left to right) for CaV1.2 G449R + CaM WT, G449R + CaM T79A, G449R + CaM T79D, G449R + CaM S81A, and G449R + CaM S81D. The vertical scale bars are 10 pA/pF, and the horizontal scale bars are 0.1 mV throughout.

**B:** the corresponding peak current densities are plotted as a function of applied voltage. C and D, the summarized distribution of the peak current densities and the midpotential of activation E\textsubscript{0.5,act}. Peak whole-cell currents and E\textsubscript{0.5,act} are reported individually as black circles. The mean data ± SD are shown as red hyphens. Values of peak current densities and E\textsubscript{0.5,act} are reported in Table 6. CaM, calmodulin; HEKT, human embryonic kidney 293T cell line.
calmodulation of a timothy syndrome variant

NM_000722 was subcloned in commercial vectors under the control of the cytomegalovirus (CMV) promoter as described elsewhere (36, 37, 75). The cDNA sequence of the rabbit clone is near identical to the human clone save for an additional 30 amino acids in its N terminus, accounting for the +30 residue shift in residue numbering. The human CaM (GenBank accession number: M27319), subcloned in pcDNA3.1 (Thermo Fisher Scientific) vector with consecutive histidine (His-His-His-His-His) and cMyc (Glu-Gln-Lys-Leu-Iso-Ser-Glu-Glu-Asp-Leu) tags in C-terminal region, was a gift from Dr Rémy Sauvé, Université de Montréal. The cDNA mutations of CaM were introduced in this vector. CaM is numbered as reported (88) to take into account that the mature protein lacks N-terminal Met residue. All cDNA mutations in Caα1C of CaV1.2 and CaM were produced with the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Inc) according to the manufacturer’s instructions. Briefly, substitutions of nucleotides were created by incorporating the desired mutation in the center of the forward primer, and the reverse primer is designed so that the 5’ ends of the two primers anneal back to back. Following the PCR, the amplified DNA is circularized, and the template is removed with a kinase–ligase–DpnI enzyme mixture, before transformation into high-efficiency NEB DH5α competent Escherichia coli. All constructs were verified by automated double-stranded sequence analysis (“Centre d’expertise et de services Génome Québec”). The protein expression at the expected molecular weight was confirmed by standard Western blot analysis for each construct.

Gene transfection and cell culture

HEKT cells were grown using standard tissue culture conditions (5% CO2, 37 °C) in high-glucose Dulbecco’s modified Eagle’s medium supplemented with fetal bovine serum (10%), l-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (10 mg/ml) as described before (36, 37, 75). Using Lipofectamine 2000 (Invitrogen), as per the manufacturer’s instructions, HEKT cells (80% confluence, 35 mm petri dish) were transiently transfected with cDNA plasmids, namely pCMV-CaVα1.2 WT or variants (4 μg), pCMV-CaVα2δ1 (4 μg), and in some experiments, pcDNA3-HisB-cMyc-CaM WT or variants (2 μg), with a weight ratio of 1:1:0.5 for a total of 12 to 14 μg cDNAs. The molar ratio was 7:1 for CaM and CaVα1.2. Unless otherwise noted, the plasmids pCMV-CaVα2δ2a, pCMV-CaVα2δ1, and pcDNA3-HisB-cMyc-CaM WT are simply referred to as CaVα2δ2a, CaVα2δ1, and CaM WT in the text and figures. The cDNA coding for pEGFP (0.2 μg) was included in the cDNA mixture as a marker of successful transfection for patch-clamp experiments (4, 81). The culture medium was changed, and cells were detached with 0.05% trypsin before being replated on 35 mm petri dishes 6 h post-transfection. Whole-cell patch clamp experiments were performed 24 to 32 h after transfection.

Coimmunoprecipitation

HEKT cells were transiently transfected with the appropriate constructs (as indicated later), and protein extraction proceeded 2 days after transfection. Experiments described in Figure 2 were carried out as follows. HEKT cells were transiently transfected with CaVα1.2 WT or CaVα1.2 G449R with pCMV-CaVα2δ1 and cMyc-tagged versions of CaVβ3 or CaVβ2a using, respectively, the pCMV-Tag5-CaVαβ3 or the pCMV-Tag5-CaVβ2a plasmids. CaVβ acted as the bait. Cell lysates were immunoprecipitated overnight with anti-cMyc magnetic beads (Pierce Anti-c-Myc Magnetic Beads; catalog no.: 88842, Thermo Fisher Scientific) to capture the given CaVβ. In the experiments shown in Figures 4 and 5, the constructs were pCMV-CaVβ2a with pCMV-CaVα1.2 WT or G449R and pcDNA3-HisB-cMyc-CaM WT and used CaM as the bait. Cell lysates were immunoprecipitated overnight with anti-His magnetic beads (code no.: MBL-D29111). The procedure was otherwise similar for the three experimental groups. Two different detergents have been used to compare extraction efficiency between digitonin (a nonionic saponin detergent) and CHAPS–Na (zwitterionic detergent). Both extraction conditions have produced the same results and were thus combined, for three independent experiments over the course of 2 months. Two days after transfection, cells were homogenized in 20 mM Na–Mops (pH 7.4), 300 mM NaCl, and 1% digitonin or 0.5% CHAPS–Na, supplemented with protease inhibitors without EDTA (Thermo Fisher Scientific). Homogenates were sonicated, incubated for 1 h at 4 °C, and centrifuged at 13,000 rpm for 30 min. A fraction (20 μg) of the homogenates or starting material was set aside as representative of total proteins and was immunoblotted to confirm normal protein expression. Coimmunoprecipitation was carried out using 200 μg homogenates diluted in 150 μl of 20 mM Na–Mops (pH 7.4) and 300 mM NaCl. The 200 ± 20 μl protein solution was incubated overnight with the appropriate antibody-coated magnetic beads that were collected using a PureProteome magnetic rack (Millipore). The magnetic beads were washed three times with a buffer containing 20 mM Na–Mops (pH 7.4), 300 mM NaCl, and 0.2% digitonin or alternatively 20 mM Na–Mops (pH 7.4), 300 mM NaCl, without additional detergent for the extraction under the “CHAPS conditions.” The bound proteins were eluted with Laemmli buffer (20 μl) at 95 °C for 5 min, electrophoresed on a 6% or 10% SDS-polyacrylamide gel, and transferred onto a nitrocellulose membrane for Western blotting. Antibodies are described in the figure legends. Signals were detected with the enhanced chemiluminescence substrate. Blots were visualized with the ChemiDoc Touch system (Bio-Rad). Molecular weights were estimated using Image Lab software, version 5.2 (Bio-Rad) by linear regression of standard molecular weight markers.

Cell surface fractionation assay

Four different protein fractions (total cell lysates, cytosolic, total membrane, and plasma membrane fractions) were prepared as explained before (4). Briefly, transfected HEKT cells cultured in 100 mm dishes were homogenized at 4 °C in a Tris-based solution containing a mixture of protease inhibitors (Sigma) at pH 7.4. The cell homogenate was aliquoted into
three tubes. After a 2 h incubation period at 4 °C with 1% (v/v) Triton X-100, the first tube was centrifuged at 10,000g for 10 min to remove cell debris, nuclei, and mitochondria. The supernatant was kept as the total protein fraction (whole-cell lysates). The second tube was centrifuged at 200,000g and 4 °C for 20 min. The supernatant is referred to as the cytosolic fraction. The pellet was resuspended in homogenizing buffer containing 1% (v/v) Triton X-100. After 30 min of incubation on ice, a second centrifugation was performed at 200,000g. The resulting supernatant is referred to as the total membrane protein fraction. The third tube was centrifuged at 10,000g for 10 min. The supernatant obtained was centrifuged at 200,000g and 4 °C for 20 min. The pellet was resuspended in homogenizing buffer containing 0.6 M KCl. Subsequent centrifugations were performed at 200,000g and 4 °C for 20 min to wash out the KCl. The final pellet was resuspended in the homogenizing buffer and is considered to be enriched in plasma membrane proteins. Proteins (20 μg) were electrophoresed on a 10% SDS-polyacrylamide gel.

**Whole-cell patch-clamp recordings and data analysis**

Whole-cell Ca$^{2+}$ currents from transfected HEKT cells were recorded using pCLAMP software 11.2 (Molecular Devices) and an Axopatch 200B amplifier (Molecular Devices). Patch electrodes were pulled from borosilicate glass (Corning; code: 8161) and heat-polished to a final resistance about 3.0 to 3.5 MΩ when filled with the intracellular solution. Whole-cell currents were low-pass filtered at 2 kHz, digitized at a sampling rate of 100 μs during acquisition, and stored on a microcomputer equipped with an AD converter (Axon Digitdata 1440A; Molecular Devices). Electrodes were filled with a solution containing (in millimolar) 140 CsCl, 0.6 NaGTP, 3 MgATP, 10 EGTA, 10 Hepes, titrated to pH 7.4 with NaOH. HEKT cells were bathed in a modified Earle’s saline solution (in millimolar) as follows: 135 NaCl, 20 tetraethylammonium chloride, 2 CaCl$_2$, 1 MgCl$_2$, 10 Hepes, titrated to pH 7.4 with potassium hydroxide. Stock solution of the cell-permeable CaM antagonists W-13 (Tocris, Bio-Technne) was prepared in distilled water, diluted to its final concentration just before use, and added directly in the bath solution. Cells were incubated for 15 min prior to whole-cell recordings. A few experiments were performed in the presence of 2.5 μM TBB, a cell-permeable inhibitor of CK2. Stock solution of TBB (5 mM) was prepared in dimethylsulfoxide, diluted to its final concentration just before use, and added directly in the bath solution. Whole-cell currents were recorded 15 min after drug equilibration. All experiments were carried out at room temperature (23–25 °C). Cellular capacitance was estimated by measuring the time constant of current decay evoked by a depolarizing pulse of 10 mV applied to the cell from a holding potential of −100 mV.

Whole-cell Ca$^{2+}$ currents were elicited from a holding potential of −100 mV and depolarized to potentials ranging from −80 to 65 mV in 5 mV increments lasting 450 ms for each step. Ca$^{2+}$ current densities (pA/pF) were obtained by dividing the peak current by the cell capacitance. Average $I$–$V$ curves were obtained by plotting the peak current densities versus the voltage and fitted to a Boltzmann function, which is a transformed Boltzmann function for $I$–$V$ data of the following form:

$$I = \frac{(V_m - V_{rev}) \cdot G_{max}}{1 + e^{(V - E_{0.5,act})/dx}}$$

where $I$ is the current, $V_m$ is the applied voltage, $E_{0.5,act}$ is the voltage at which channels are half-maximally activated, $dx$ is the steepness of the slope, $G_{max}$ is the maximal conductance, and $V_{rev}$ is the reversal potential. Steady-state activation curves were constructed by dividing the peak $I$–$V$ data by the driving force. The R100 ratio of CaV1.2 current was defined as the peak current remaining after a 100 ms depolarizing pulse ($I_{100ms}/I_{peak}$) and was used as an indicator of the inactivation kinetics. n/N refers to the number of cells/transfections measured in each condition of study.

The steady-state inactivation was determined using a two-step protocol in which conditioning prepulses were applied from a holding potential of −100 mV to a range of potentials from −100 to 40 mV in 10 mV increments for 5 s, immediately followed by a test pulse to 5 mV for 100 ms. For the construction of inactivation curves, the peak current amplitudes during the test pulses were normalized to the maximum peak current amplitude measured at −100 mV and plotted against the conditioning pulse. Steady-state inactivation curves were fitted to a modified Boltzmann equation:

$$\frac{I}{I_{max}} = \frac{A1 - A2}{1 + e^{(V - E_{0.5,inact})/dx}} + A2$$

where $I/I_{max}$ is the relative current measured at the test pulse, $A1$ and $A2$ represent, respectively, the maximum relative current value and the fraction of the noninactivated current, $V_m$ is the voltage applied during the conditioning pulse, $E_{0.5,inact}$ is the voltage at which channels are half-maximally inactivated, and $dx$ is the steepness of the slope.

**Data analysis and statistics**

Data were analyzed using a combination of pCLAMP software 11.2, Microsoft Excel, and OriginPro 2020 (OriginLab Corporation). Data in the tables are expressed as mean ± SD. Statistical significance was determined by one-way ANOVA and Bonferroni post hoc test in OriginPro 2020. The level of statistical significance was set at $p < 0.05$.

**Data availability**

All data are contained within the article.

**Supporting information**—This article contains supporting information.

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Abbreviations—The abbreviations used are: CaM, calmodulin; CDI, Ca2+-dependent inactivation; cDNA, complementary DNA; CK2, casein kinase II; CMV, cytomegalovirus; HEK, human embryonic kidney cell line; LQTS, long-QT syndrome; TBB, 4,5,6,7-tetrabromobenzotriazole; TS, Timothy syndrome.

References

1. Grant, A. O. (2009) Cardiac ion channels. Circ. Arrhythm. Electrophysiol. 2, 185–194
2. Wang, S. Q., Song, L. S., Lakatta, E. G., and Cheng, H. (2001) Ca2+ signalling between single L-type Ca2+ channels and ryanodine receptors in heart cells. Nature 410, 592–596
3. Wu, J., Yan, Z., Li, Z., Qian, X., Lu, S., Dong, M., et al. (2016) Structure of the voltage-gated calcium channel Cav1.1 at 3.6 Å resolution. Nature 537, 191–196
4. Segura, E., Bourdin, B., Tétreau, M. P., Briot, J., Allen, B. G., Mayer, G., et al. (2017) Proteolytic cleavage of the hydrophobic domain in the Ca(V)1.2a1 subunit improves assembly and activity of cardiac Ca(V)1.2 channels. J. Biol. Chem. 292, 11109–11124
5. Colecraft, H. M., Alseikhan, B., Takahashi, S. X., Chaudhuri, D., Mittman, S., Yegnasubramanian, V., et al. (2002) Novel functional properties of Ca(2+) channel beta subunits revealed by their expression in adult rat heart cells. J. Physiol. 541, 435–452
6. Van Petegem, F., Duderstadt, K. E., Clark, K. A., Wang, M., and Minor, D. L., Jr. (2008) Alaneanine-sensing mutagenesis defines a conserved energetic hotspot in the CaV alpha1 AID-CaVeta interaction site that is critical for channel modulation. Structure 16, 280–294
7. Adams, P. J., Ben-Johney, M., Dick, I. E., Inoue, T., and Yue, D. T. (2014) Apocam inulin itself promotes ion channel opening and Ca(2+) regulation. Cell 159, 608–622
8. Zühlke, R. D., Pitt, G. S., Deisseroth, K., Tsien, R. W., and Reuter, H. (1999) Calmodulin supports both inactivation and facilitation of L-type calcium channels. Nature 399, 159–164
9. Zühlke, R. D., Pitt, G. S., Tsien, R. W., and Reuter, H. (2000) Ca2+-sensitive inactivation and facilitation of L-type Ca2+ channels both depend on specific amino acid residues in a consensus calmodulin-binding motif in the(alpha)1C subunit. J. Biol. Chem. 275, 21121–21129
10. Pate, P., Mochca-Morales, J., Wu, Y., Zhang, J. Z., Rodney, G. G., Serysheva, I. I., et al. (2000) Determinants for calmodulin binding on voltage-dependent Ca2+ channels. J. Biol. Chem. 275, 39786–39792
11. Romanini, C., Gamsjaeger, R., Kahr, H., Schaufler, D., Carlson, O., Abernethy, D. R., et al. (2000) Ca(2+)-sensors of L-type Ca(2+)-channel. FEBS Lett. 487, 301–306
12. Pitt, G. S., Zühlke, R. D., Hudmon, A., Schulman, H., Reuter, H., and Tsien, R. W. (2001) Molecular basis of calmodulin tethering and Ca2+-dependent inactivation of L-type Ca2+ channels. J. Biol. Chem. 276, 30794–30802
13. Mouton, J., Feltz, A., and Maulet, Y. (2001) Interactions of calmodulin with two peptides derived from the C-terminal cytoplasmic domain of the Ca(V)1.2 Ca2+ channel provide evidence for a molecular switch involved in Ca2+-induced inactivation. J. Biol. Chem. 276, 22359–22367
14. Jervell, A., and Lange-Nielsen, F. (1957) Congenital deaf-mutism, functional heart disease with prolongation of the Q-T interval and sudden death. Am. Heart J. 54, 59–68
15. Libethsorn, R. R. (1996) Sudden death from cardiac causes in children and young adults. N. Engl. J. Med. 334, 1039–1044
16. Chugh, S. S., Reinier, K., Teodorescu, C., Evanado, A., Kehr, E., Al Samara, M., et al. (2008) Epidemiology of sudden cardiac death: clinical and research implications. Prog. Cardiovasc. Dis. 51, 213–228
17. Schwartz, P. J., Stramba-Badiale, M., Crotti, L., Pedrazzini, M., Besana, A., Bosi, G., et al. (2009) Prevalence of the congenital long-QT syndrome. Circulation 120, 1761–1767
18. Tester, D. J., and Ackerman, M. J. (2007) Postmortem long QT syndrome genetic testing for sudden unexplained death in the young. J. Am. Coll. Cardiol. 49, 240–246
19. Tester, D. J., Will, M. L., Haglund, C. M., and Ackerman, M. J. (2005) Compendium of cardiac channel mutations in 541 consecutive unrelated patients referred for long QT syndrome genetic testing. Heart Rhythm 2, 507–517
20. Splavski, I., Timothy, K. W., Sharpe, L. M., Decher, N., Kumar, P., Bloise, R., et al. (2004) Ca(V)1.2 calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. Cell 119, 19–31
21. Splavski, I., Timothy, K. W., Decher, N., Kumar, P., Sachse, F. B., Begg, A. H., et al. (2005) Severe arrhythmia disorder caused by cardiac L-type calcium channel mutations. Proc. Natl. Acad. Sci. U. S. A. 102, 8089–8096. discussion 8086–8088
22. Gillis, J., Burashnikov, E., Antzelevitch, C., Blaser, S., Gross, G., Turner, L., et al. (2012) Long QT, syndactyly, joint contractures, stroke and novel CACNA1C mutation: Extending the spectrum of Timothy syndrome. Am. J. Med. Genet. A 158A, 182–187
23. Po, C., Zordan, R., Vecchi, M., Cerutti, A., Sartori, S., Trevisson, E., et al. (2019) Photosensitive epilepsy and long QT: Expanding Timothy syndrome phenotype. Clin. Neurophysiol. 130, 2134–2136
24. Colson, C., Mittle, H., Busson, A., Leenhardt, A., Denjov, I., Fressard, V., et al. (2019) Unusual clinical description of adult with Timothy syndrome, carrier of a new heterozygote mutation of CACNA1C. Eur. J. Med. Genet. 62, 103648
25. Boczek, N. J., Ye, D., Jin, F., Tester, D. J., Huseby, A., Bos, J. M., et al. (2015) Identification and functional characterization of a novel CACNA1C-mediated cardiac disorder characterized by prolonged QT intervals with hypertrophic cardiomyopathy, congenital heart defects, and sudden cardiac death. Circ. Arrhythm. Electrophysiol. 8, 1122–1132
26. Han, D., Xue, X., Yan, Y., and Li, G. (2019) Highlight article: dysfunctional Cav1.2 channel in Timothy syndrome, from cell to bedside. Exp. Biol. Med. (Maywood) 244, 960–971
27. Hennessey, J. A., Boczek, N. J., Jiang, Y. H., Miller, J. D., Patrick, W., Pfeiffer, R., et al. (2014) A CACNA1C variant associated with reduced voltage-dependent inactivation, increased Cav1.2 channel window current, and arrhythmogenesis. PLoS One 9, e106982
28. Kolu Bisabu, K., Zhao, J., Mokrane, A. E., Segura, É., Marsolais, M., Grondin, S., et al. (2020) Novel gain-of-function variant in CACNA1C associated with Timothy syndrome, multiple accessory pathways, and noncompaction cardiomyopathy. Circ. Genom Precis Med. 13, e003123
29. Findeisen, F., and Minor, D. L., Jr. (2009) Disruption of the IS6-AID linker affects voltage-gated calcium channel inactivation and facilitation. J. Gen. Physiol. 133, 327–343
30. Papa, A., Kushner, J., Hennessey, J. A., Katchman, A. N., Zakharov, S. I., Chen, B. X., et al. (2021) ADrenergic Ca(V)1.2 activation via Rad phosphorylation converges at (alpha)I-II loop. Circ. Res. 128, 76–88
31. Almagor, L., Chomskey-Hecht, O., Ben-Mocha, A., Hendin-Barak, D., Dascal, N., and Hirsch, J. A. (2012) The role of a voltage-dependent Ca2+ channel intracellular linker: A structure-function analysis. J. Neurosci. 32, 7602–7613
32. Barrett, C. F., and Tsien, R. W. (2008) The Timothy syndrome mutation differentially affects voltage- and calcium-dependent inactivation of
33. Asmara, H., Joshi-Mukherjee, R., Yang, W., and Yue, D. T. (2016) Arrhythmogenesis in Timothy Syndrome is associated with defects in Ca(2+)-dependent inactivation. Nat. Commun. 7, 10370
34. Raybaud, A., Dodier, Y., Bissonnette, P., Simoes, M., Bichet, D. G., Sauvé, R., et al. (2006) The role of the G3XG3X3G motif in the gating of high voltage-activated Ca2+ channels. J. Biol. Chem. 281, 39424–39436
35. Tarnovskaya, S. I., Kostareva, A. A., and Zhorov, B. S. (2021) L-type calcium channel: predicting pathogenic/likely pathogenic status for variants of uncertain clinical significance. Membranes 11, 599
36. Bourdin, B., Briot, J., Tétreault, M. P., Sauvé, R., and Parent, L. (2017) Negatively charged residues in the first extracellular loop of the L-type Ca(V)1.2 channel anchor the interaction with the Ca(V)zY281 auxiliary subunit. J. Biol. Chem. 292, 17236–17249
37. Briot, J., Mailhot, O., Bourdin, B., Tétreault, M. P., Najmanovich, R., and Parent, L. (2018) A three-way inter-molecular network accounts for the Ca(V)xV281-induced functional modulation of the pore-forming Ca(V)1.2 subunit. J. Biol. Chem. 293, 7176–7188
38. Saimi, Y., and Kung, C. (2002) Calmodulin as an ion channel subunit. Annu. Rev. Physiol. 64, 289–311
39. Urrutia, I., Aguado, A., Muguruza-Montero, A., Núñez, E., Malo, C., Casis, O., et al. (2019) The crossroad of ion channels and calmodulin in disease. Int. J. Mol. Sci. 20, 400
40. Tiaho, F., Piot, C., Nargeot, J., and Richard, S. (1994) Regulation of the frequency-dependent facilitation of L-type Ca2+ currents in rat ventricular myocytes. J. Physiol. 477, 237–251
41. Villarroel, A., Taglialetela, M., Bernardo-Seisdedos, G., Alaimo, A., Agirre, J., Alberdi, A., et al. (2014) The ever changing moods of calmodulin: how structural plasticity entails transductional adaptability. J. Mol. Biol. 426, 2717–2735
42. Pereschi, A., and stemmer, P. M. (2002) Calmodulin is a limiting factor in the cell. Trends Cardiovasc. Med. 12, 32–37
43. DeMaria, C. D., Soong, T. W., Alseikhan, B. A., Alvania, R. S., and Yue, D. T. (2001) Calmodulin bifurcates the local Ca2+ signal that modulates P/Q-type Ca2+ channels. Nature 411, 484–489
44. Simms, B. A., Souza, I. A., and Zamponi, G. W. (2014) Effect of the Brugada syndrome mutation A39V on calmodulin regulation of Cav1.2 channels. Mol. Brain 7, 34
45. Ravindran, A., Lao, Q. Z., Harry, J. B., Abrahimi, P., Kobrinsky, E., and Soldatov, N. M. (2008) Calmodulin-dependent gating of Ca(v)1.2 calcium channels in the absence of Ca(v)beta subunits. Proc. Natl. Acad. Sci. U. S. A. 105, 8154–8159
46. Limpitskul, W. B., Dick, I. E., Joshi-Mukherjee, R., Overgaard, M. T., George, A. L., Jr., and Yue, D. T. (2014) Calmodulin mutations associated with long QT syndrome prevent inactivation of cardiac L-type Ca2+ currents and promote proarrhythmic behavior in ventricular myocytes. J. Mol. Cell Cardiol. 74, 115–124
47. Noweycky, M. C., Fox, A. P., and Tsien, R. W. (1985) Long-opening mode gating of neuronal calcium channels and its promotion by the dihydropyridine calcium agonist Bay K 8644. Proc. Natl. Acad. Sci. U. S. A. 82, 2178–2182
48. Asmara, H., Minobe, E., Saud, Z. A., and Kameyama, M. (2010) Interactions of calmodulin with the multiple binding sites of Cav1.2 Ca2+ channels. J. Pharmacol. Sci. 112, 397–404
49. Wang, H. G., George, M. S., Kim, J., Wang, C., and Pitt, G. S. (2007) Cal/ Calmodulin regulations trafficking of Cav1.2 Ca2 channels in cultured hippocampal neurons. J. Neurosci. 27, 9086–9093
50. Osawa, M., Swindells, M. B., Tanikawa, J., Tanaka, T., Mase, T., Furuya, T., et al. (1998) Solution structure of calmodulin-W-7 complex: the basis of diversity in molecular recognition. J. Mol. Biol. 276, 165–176
51. Osawa, M., Kuwamoto, S., Izumi, Y., Yap, K. L., Ikura, M., Shibanuma, T., et al. (1999) Evidence for calmodulin inter-domain compaction in solution induced by W-7 binding. FEBS Lett. 442, 173–177
52. Quadrini, M., James, P., and Carafoli, E. (1994) Isolation of phosphorylated calmodulin from rat liver and identification of the in vivo phosphorylation sites. J. Biol. Chem. 269, 16116–16122
53. Villalobo, A. (2018) The multifunctional role of phospho-calmodulin in pathophysiological processes. Biochem. J. 475, 4011–4023
54. Tabernero, L., Taylor, D. A., Chandross, R. J., VanBerkum, M. F., Means, A. R., Quiocco, F. A., et al. (1997) The structure of a calmodulin mutant with a deletion in the central helix: implications for molecular recognition and protein binding. Structure 5, 613–622
55. Bildl, W., Strassmaier, T., Thurm, H., Andersen, J., Eble, S., Oliver, D., et al. (2004) Protein kinase CK2 is coassembled with small conductance Ca(2+)-activated K+ channels and regulates channel gating. Neuron 43, 847–858
56. Plancke, Y. D., and Lazarides, E. (1983) Evidence for a phosphorylated form of calmodulin in chicken brain and muscle. Mol. Cell Biol. 3, 1412–1420
57. Quadroni, M., L’Hostis, E. L., Corti, C., Miyagkikh, I., Duruoso, I., Cox, J., et al. (1998) Phosphorylation of calmodulin alters its potency as an activator of target enzymes. Biochemistry 37, 6523–6532
58. Sacks, D. B., Davis, H. W., Crimmins, D. L., and McDonald, J. M. (1992) Insulin-stimulated phosphorylation of calmodulin. Biochem. J. 286, 211–216
59. Sacks, D. B., Mazus, B., and Joyal, J. L. (1995) The activity of calmodulin is altered by phosphorylation: modulation of calmodulin function by the site of phosphate incorporation. Biochem. J. 312, 197–204
60. Li, S. C., Goto, N. K., Williams, K. A., and Deber, C. M. (1996) Alpha-helical, but not beta-sheet, propensity of proline is determined by peptide environment. Proc. Natl. Acad. Sci. U. S. A. 93, 6676–6681
61. Ambrosino, P., Alaimo, A., Bartollino, S., Manocchio, L., De Maria, M., Mosca, I., et al. (2015) Epilepsy-causing mutations in Kv7.2 C-terminus affect binding and functional modulation by calmodulin. Biochim. Biophys. Acta 1852, 1856–1866
62. Ben-Johny, M., and Yue, D. T. (2014) Calmodulin regulation (calmodulation) of voltage-gated calcium channels. J. Gen. Physiol. 143, 679
63. Kim, E. Y., Rumpf, C. H., Fujivara, Y., Cooley, E. S., Van Petegem, F., and Minor, J. (2008) Structures of CaV2.2 Ca2+/CaM-IQ domain complex reveal binding modes that underlie calcium-dependent inactivation and facilitation. Structure 16, 1455–1467
64. Van Petegem, F., Chatelain, F., C., Barzilai, R., and Dascal, N. (2000) Modulation of L-type Ca2+ channels by gbeta gamma and calmodulin via interactions with N and C termini of alpha 1D. J. Biol. Chem. 275, 39846–39854
65. Noble, S., and Shimon, Y. (1981) The calcium and frequency dependence of the slow inward current ‘staircase’ in frog atrium. J. Physiol. 310, 57–75
66. Barbasi, L. A., and Shimoni, Y. (1982) Enhancement of calcium current during digitalis inotropy in mammalian heart: positive feedback regulation by intracellular calcium? J. Physiol. 329, 589–614
67. Kim, J., Ghosh, S., Nunziato, D. A., and Pitt, G. S. (2004) Identification of the components controlling inactivation of voltage-gated Ca(2+) channels. Neuron 41, 745–754
68. Campiglio, M., and Flucher, B. E. (2015) The role of auxiliary subunits for the functional diversity of voltage-gated calcium channels. J. Cell Physiol. 230, 2019–2031
69. Beyl, S., Depil, K., Hohaus, A., Stary-Weinzinger, A., Shabbir, W., et al. (2011) Physicochemical properties of pore residues predict activation gating of CaV1.2: a correlation mutation analysis. Pflugers Arch. 461, 53–63
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73. Daﬁ, O., Berrou, L., Dodier, Y., Raybaud, A., Sauvé, R., and Parent, L. (2004) Negatively charged residues in the N-terminal of the AID helix confer slow voltage dependent inactivation gating to CaV1.2. *Biophys. J.* 87, 3181–3192

74. Hu, Z., Li, G., Wang, J.-W., Chong, S. Y., Yu, D., Wang, X., et al. (2018) Regulation of blood pressure by targeting CaV1.2-galectin-1 protein interaction. *Circulation* 138, 1431–1445

75. Bourdin, B., Marger, F., Wall-Lacelle, S., Schneider, T., Klein, H., Sauvé, R., et al. (2010) Molecular determinants of the CaVbeta-induced plasma membrane targeting of the CaV1.2 channel. *J. Biol. Chem.* 285, 22853–22863

76. Buraei, Z., and Yang, J. (2010) The β subunit of voltage-gated Ca2+ channels. *Physiol. Rev.* 90, 1461–1506

77. Finlin, B. S., Crump, S. M., Satin, J., and Andres, D. A. (2003) Regulation of voltage-gated calcium channel activity by the Rem and Rad GTPases. *Proc. Natl. Acad. Sci. U. S. A.* 100, 14469–14474

78. Liu, G., Papa, A., Katchman, A. N., Zakharov, S. I., Roybal, D., Hennessey, J. A., et al. (2020) Mechanism of adrenergic CaV1.2 stimulation revealed by proximity proteomics. *Nature* 577, 695–700

79. Almagor, L., Avinery, R., Hirsch, J. A., and Beck, R. (2013) Structural flexibility of CaV1.2 and CaV2.2 I-II proximal linker fragments in solution. *Biophys. J.* 104, 2392–2400

80. Almagor, L., Chomsky-Hecht, O., Ben-Mocha, A., Hendin-Barak, D., Dascal, N., and Hirsch, J. A. (2012) CaV1.2 I-II linker structure and Timothy syndrome. *Channels (Austin)* 6, 468–472

81. Shakeri, B., Bourdin, B., Demers-Giroux, P. O., Sauvé, R., and Parent, L. (2012) A quartet of leucine residues in the guanylate kinase domain of CaVβ determines the plasma membrane density of the CaV2.3 channel. *J. Biol. Chem.* 287, 32835–32847

82. Opatowsky, Y., Chen, C. C., Campbell, K. P., and Hirsch, J. A. (2004) Structural analysis of the voltage-dependent calcium channel beta subunit functional core and its complex with the alpha 1 interaction domain. *Neuron* 42, 387–399

83. Van Petegem, F., Clark, K. A., Chatelain, F. C., and Minor, D. L., Jr. (2004) Structure of a complex between a voltage-gated calcium channel beta-subunit and an alpha-subunit domain. *Nature* 429, 671–675

84. Chen, Y. H., Li, M. H., Zhang, Y., He, L. L., Yamada, Y., Fitzmaurice, A., et al. (2004) Structural basis of the alpha1-beta subunit interaction of voltage-gated Ca2+ channels. *Nature* 429, 675–680

85. Pace, N. C., and Scholtz, M. I. (1998) A helix propensity scale based on experimental studies of peptides and proteins. *Biophys. J.* 75, 422–427

86. Dixon, R. E., Cheng, E. P., Mercado, J. L., and Santana, L. F. (2012) L-type Ca2+ channel function during Timothy syndrome. *Trends Cardiovasc. Med.* 22, 72–76

87. Bourdin, B., Shakeri, B., Tétreault, M. P., Sauvé, R., Lesage, S., and Parent, L. (2015) Functional characterization of CaVα2β mutations associated with sudden cardiac death. *J. Biol. Chem.* 290, 2854–2869

88. Lee, C. H., and MacKinnon, R. (2018) Activation mechanism of a human SK-calmodulin channel complex elucidated by cryo-EM structures. *Science* 360, 508–513