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

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Running title: Calmodulation of a Timothy syndrome variant

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Abstract

Voltage-gated L-type calcium channel Cav1.2, containing the Cavα1C subunit encoded by CACNA1C, triggers sustained Ca\(^{2+}\) release to maintain normal cardiac contraction. We recently reported the identification of a single nucleotide variant of CACNA1C associated with prolonged repolarization interval, and showed recombinant expression of this variant produced a gain-in-function L-type Cav1.2 channel with increased peak current density and activation gating. However, the mechanism by which this variant adopts this gain of function was unclear. We herein reveal that these properties are replicated by overexpressing calmodulin (CaM) with wild-type Cav1.2 (WT) and are reversed by exposure to CaM antagonist W-13. Phospho-mimetic, but not phoso-resistant CaM surrogates reproduced the impact of CaM on the function of Cav1.2 WT. The increased channel activity of Cav1.2 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. Furthermore, Cav1.2 substituted with the \(\alpha\)-helix breaking proline residue were more reluctant to open than Cav1.2 WT, but were upregulated by phospho-mimetic CaM surrogates. Our results indicate that: 1) CaM and its phospho-mimetic analogues promote a gain in the function of Cav1.2; and 2) the structural properties of the first intracellular linker of Cav1.2 contribute to its CaM-induced modulation. We conclude that the CACNA1C clinical variant mimics the increased activity associated with the upregulation of Cav1.2 by Ca\(^{2+}\)-CaM, thus maintaining a majority of channels in a constitutively active mode that could ultimately promote ventricular arrhythmias.

Introduction

Cardiac contraction during the systole is handled by the influx of Ca\(^{2+}\) into cardiomyocytes in response to depolarisation during the phase 2 of the cardiac action potential (1). Voltage-gated L-type calcium channel Cav1.2 are expressed in the T-tubules such that localized Ca\(^{2+}\) entry triggers a sustained and more global Ca\(^{2+}\) release by the sarcoplasmic reticulum in the dyadic cleft (2). Cardiac L-type Cav1.2 channels are heteromultimeric protein complexes formed by the pore-forming Cavα1C subunit bound to the extracellular Cavα2δ1 auxiliary subunits (3,4) and to the cytoplasmic Cavβ (5) that binds with nanomolar affinity to the first intracellular linker (6). The Cavα1 subunit is formed by a polypeptide chain of 24 transmembrane helices grouped into 4 structural homologous domains (domain I, II, II, and IV) (Fig 1). Although not a specific auxiliary subunit, calmodulin (CaM) contributes to Ca\(^{2+}\)-dependent facilitation and Ca\(^{2+}\)-dependent inactivation of Cav1.2 (7-9) through binding to the Isoleucine-Glutamine (IQ) motif in the C-terminal tail of Cavα1C (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) are often the results of inherited or de novo genetic mutations in the DNA of a variety of ion channels (19). Gain-of-function mutations within the CACNA1C gene, coding for Cavα1C, 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 6\(^{th}\) transmembrane segment of the Cavα1C protein (Fig 1). The canonical TS1 variant Gly406Arg results from a de novo CACNA1C mutation in exon 8A (20). An atypical form of Timothy syndrome type 2 (TS2) is associated with the Gly402Ser and Gly402Arg variants in the alternatively
spliced exon 8 (21). More recent 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 Cav1.2 channel as a result of slower inactivation kinetics that promote larger Ca\(^{2+}\) 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 Cav\(\alpha\)1C a missense variant, Gly419Arg, from a patient with prolonged QT interval (≈ 500 ms), syndactyly, left ventricular non-compaction and slight delay in neurodevelopment (28). Unlike other TS-variants located close to the high-affinity binding domain of Cav\(\beta\), Cav1.2 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 6th transmembrane segment and the high-affinity binding site for Cav\(\beta\), 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 co-expression with CaM WT or pseudo-phosphorylated surrogates CaM T79D or CaM S81D and was abolished by the CaM antagonist W-13. In contrast, the functional parameters of the clinical Glycine to Arginine variant remained remarkably insensitive to these treatments. Substitution with the \(\alpha\)-helix breaker proline residue yielded opposite results with channels more reluctant to open at physiological voltages but more likely to respond to the modulation by CaM. Altogether, the functional characterization of the Glycine to Arginine variant provides mechanistic insight on the regulation of Cav1.2 by CaM (sometimes referred to as calmodulation) and specifically the role played by the I-II linker as relaying the signal to the channel activation gate.

**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 Cav\(\beta\) (Fig 1) yielded opposite results (29,30). Increased flexibility within this stretch has been argued to loosen up the interaction between Cav\(\beta\) and Cav1.2 (30). We validated that the substitution of the Glycine residue at position 449 (rabbit numbering) does not impair the interaction with the canonical Cav\(\beta\) and Cava2\(\delta\)1.
subunits (Fig 2). The latter observation is in line with the recent demonstration that interaction with Cavα261 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 in 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 Fig 3A, the peak current density of Cav1.2 WT was reduced by about 50% from -15 ± 4 pA/pF to -8 ± 2 pA/pF (p < 0.05) 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 reduced the non-inactivating 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 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 co-expressing Cav1.2 WT and CaM WT are shown in Fig 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 to endogenous CaM) to reach values not significantly different than G449R under the same conditions (p > 0.05). CaM WT shifted the E0.5,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 v. 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 due to 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 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 Fig 5 in the presence of endogenous CaM, the signal for Cav1.2 WT was stronger in the total membrane protein fraction (Fig 5, panel A, lane 3) than in the cell surface protein fraction (Fig 5, panel A, 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 5, panel B). 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 Cav1.2 WT, which can account in part for the increased peak current density and possibly the increase in the activation gating.

**Phospho-mimetic surrogates of CaM are associated with increased activity of Cav1.2 WT**

The precise mechanism though which W-13 inhibits CaM is not currently known but it has been shown that W-13 bends the flexible linker of CaM between Met78 and Glu82 (50,51), a region that harbors 2 important phosphorylation sites Thr79 and Ser81 (52). Phosphorylation at these two sites causes structural changes in the relative orientation of the C- and N-lobes which in turn modulate the interaction of CaM with its protein targets (53,54). To evaluate the structural properties of the flexible linker, we introduced phospho-mimetic and phospho-resistant mutations on CaM by individually changing phosphorylation sites Thr79 and Ser81 to alanine (A) or aspartate (D), respectively, the latter mimicking the negative charge change induced by post-translational modification.

Overexpression of phospho-resistant CaM T79A (Fig 6A left) or CaM S81A (Fig 6C left) abrogated the upregulation Cav1.2 WT currents by CaM WT. The peak current densities of Cav1.2 WT were -13 ± 5 pA/pF for CaM T79A (p < 0.001) and -13 ± 4 pA/pF for CaM S81A (p < 0.001) as compared with -28 ± 8 pA/pF when co-expressed with CaM WT (Table 2). The two phospho-resistant mutations failed to increase the peak current density and activation gating. Overexpression with phospho-mimetic CaM variants T79D (Fig 6B, left) or S81D (Fig 6D, left) produced peak current densities and activation potentials comparable to those obtained with Cav1.2 WT + CaM WT (Fig 6E-F). The structural properties of the flexible linker were also shown to regulate the activity of Ca²⁺- activated SK2 channels although in this latter case CaM T79D reduced channel activity (55).

CaM inhibitor W-13 substantially diminished Cav1.2 + CaM T79D or Cav1.2 + CaM S81D currents by around 85% (Fig 6B and 6D middle) with values of -4 ± 1 pA/pF (Fig 6B right) and -5 ± 1 pA/pF respectively (Fig 6D right). Treatment with W-13 did not however further inhibit Cav1.2 channels co-expressed with phospho-resistant CaM T79A and S81A (Fig 6A, C middle and right; Fig 6E) and had little impact on the activation gating under any of these conditions (Fig 6F, Table 2).

The modulation of CaM WT on the function of Cav1.2 WT was equivalent to the action of phospho-mimetic surrogates CaM T79D and S81D, suggesting that the phosphorylated form of CaM is responsible for the functional upregulation of Cav1.2. Roughly 10-45% of endogenous CaM is constitutively phosphorylated in vivo by casein kinase II (CK2) (56-58) and in vitro studies confirmed that CaM Thr79 and Ser81 are the most likely targets (59,60). Experiments were thus performed in the presence of 4,5,6,7-
Tetramethylammonium (TBB), 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 co-expression with either CaM T79A or S81A.

Ca\textsuperscript{2+} binding to CaM remains a pre-requisite step for driving the channel complex into its higher functioning mode. Overexpression of the Ca\textsuperscript{2+}-free form of CaM (CaM1234 or CaM D20A/D56A/D93A/D129A) decelerated, as expected, the Ca\textsuperscript{2+}-dependent inactivation (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 Cav\textsubscript{1.2} G449R requires the Ca\textsuperscript{2+}-bound CaM form**

Unlike Cav\textsubscript{1.2} WT, co-expressing either phospho-resistant CaM T79A (Fig 7A left) and S81A (Fig 7C left) or phospho-mimetic CaM T79D (Fig 7B left) and S81D (Fig 7D left) with Cav\textsubscript{1.2} G449R did not appreciably affect the peak current density, activation gating kinetics (E\textsubscript{0.5_job}, and current decay (R100) of Cav\textsubscript{1.2} G449R (Fig 7E, F and Table 3). As observed in the presence of CaM WT, the peak current densities (Fig 7A-D middle, right; Fig 7E), the E\textsubscript{0.5_job} (Fig 7F) were not altered by the application of W-13. This sharply contrasts with the results obtained with the Cav\textsubscript{1.2} WT channel complex. Nonetheless, preventing the phosphorylation of all CaM molecules with TBB reduced by 50% the peak current density measured under all other conditions (Table 4) save for CaM1234 (Table 5). Indeed, limiting Ca\textsuperscript{2+}-binding to CaM with the CaM1234 variant not only impaired the Ca\textsuperscript{2+}-dependent inactivation of Cav\textsubscript{1.2} G449R but prevented the leftward shift in activation gating and the increase in peak current density (Table 5).

**Alanine substitutions in the hinge region of CaM are not disrupting interaction with Cav\textsubscript{1.2}**

We next evaluated whether CaM variants T79A and S81A alters the interaction of CaM with the pore-forming Cav\textsubscript{1.2} subunit (Fig 8). Whether for Cav\textsubscript{1.2} WT or Cav\textsubscript{1.2} G449R, the pull-down assays failed to reveal a correlation between the signal intensity and any of the tested CaM substituted proteins indicating that phospho-mimetic analogues of Ca\textsuperscript{2+}-bound CaM impact channel function rather than protein interaction. Nonetheless, co-immunoprecipitation assays performed over a one-year period consistently revealed a stronger signal for G449R proteins than for Cav\textsubscript{1.2} WT proteins suggesting that the Glycine to Arginine substitution at position 449 could increase the affinity of CaM for Cav\textsubscript{1.2}.

**Substitution with an alpha-helix breaker in Cav\textsubscript{1.2} antagonizes channel function**

The structural properties of the I-II linker near the high-affinity binding site for Cav\textsubscript{β} have been consistently shown to modulate the gating properties of Cav1 and Cav2 channels (31). In Cav\textsubscript{1.2}, most if not all substitutions tested at position 449 altered the channel properties. Stronger activation gating and faster inactivation kinetics characterized Cav\textsubscript{1.2} G449A, G449D, and G449K in the presence of endogenous CaM (Suppl Fig, Table 6). All these substituted channels activated at more hyperpolarized voltages than Cav\textsubscript{1.2} WT with a threshold at -40 mV and currents peaking between 0 and -5 mV. These data could suggest that α-helix enhancing residues and/or positively charged residues increase the channel affinity for CaM. Hence the CaM-channel complex would be very stable in the presence of endogenous CaM as to avert the impact of CaM mutants. To test the role of the secondary structure, position 449 in
CaV1.2 was substituted with Proline, recognized as α-helix breaker (61). G449P produced whole-cell peak currents (-2.5 ± 0.4 pA/pF, n = 10, N = 2, p < 0.001 vs. CaV1.2 WT) that were 5-times smaller than CaV1.2 WT but significantly different than voltage-activated Ca2+ currents measured in non-transfected 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 phospho-mimetic variants (Fig 9, 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 co-expressing CaM T79A or CaM S81A (Fig 9A, 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 phosphorylated forms of CaM.

Discussion

Ca2+-calmodulin modulates the activity of L-type Cav1.2 through multifaceted mechanisms

The ubiquitous multifunctional Ca2+-binding protein CaM is a two-lobe protein with each two hydrophilic pockets for Ca2+ sensing separated by a flexible central linker. It is regulating the function of many voltage-gated ion channels such as Kv7.2 (62), Nav1.4 (7) and in particular, voltage-gated Cav channels (7,63) (for review (63)). At least two CaM molecules can simultaneously bind to the C-terminal region of Cav1.2 (67,68) but additional binding sites in the N-terminal and the first intracellular linker of Cavα1C have been identified (48,67,69-71). 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 Cav1.1 channel (3).

In Cav1.2 channels, Ca2+ binding to CaM contributes to Ca2+-dependent inactivation and Ca2+-dependent facilitation (7-9). Either process requires the binding of incoming Ca2+ ions to CaM preassociated to the Ile-Gln (IQ) motif in the C-terminal of the pore-forming Cavα1C subunit (10-13). The potentiating form of CaM-dependent facilitation or upregulation is observed in native cardiac L-type channels during trains of depolarization (64,65) but usually not reported in recombinant systems with the intact Cav1.2 WT channel (8,9,29). We herein report that phospho-mimetic analogues of CaM stimulate Ca2+ influx and promotes the activation gating of Cav1.2. CaM promotes the cell surface trafficking of Cav1.2 and stimulates function through an increase in peak current density and a leftward shift in the activation gating. In our hands, the latter actions of CaM requires Ca2+ as it was impaired in the presence of the constitutively Ca2+-free form CaM1234 where the four Ca2+ binding sites are invalidated. This observation is compatible with data from Kim and collaborators (66) who reported that the interaction between the CaM-bound C-terminal peptide and the I-II linker is disrupted in the complete absence of Ca2+. CaM1234 prevented the increase in peak current density, failed to promote channel activation gating and as expected, slowed down the Ca2+-dependent inactivation kinetics by 30%. Nonetheless Ca2+-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. Co-expression with CaM T79A or CaM S81A
averted the boost in peak current density (although it did not alter the activation gating). In contrast, co-expression with either CaM WT or phospho-mimetic CaM T79D or CaM S81A yielded similar results suggesting that phosphorylation of either site participates to the modulation of Cav1.2 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\textsuperscript{2+}-bound CaM modulates the function of the Cav1.2 channel complex in a fashion reminiscent of the ancillary subunits Cav\beta and Cav\alpha2\delta, which like CaM may also modulate other ion channels (80).

**Multiple mechanisms converge toward Cav1.2 G449R**

The missense variant, Gly to Arg was identified from a patient with prolonged QT interval (≈ 500 ms) ans features associated with the Timothy syndrome 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 hyper-active mode of the variant expressed in HEKT cells was mimicked by the co-expression of Cav1.2 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 over-expression with phospho-resistant CaM analogues (T79A and S81A). The impact of the phosphorylation of CaM appear to be limited to function. Cav1.2 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 Cav1.2 G449R by ≈ 50% without a significant alteration in the channel activation voltage as compared with the control conditions. The rate limiting factor appears to be Ca\textsuperscript{2+}-binding to CaM. Co-expression of G449R with the CaM1234 variant not only impaired the Ca\textsuperscript{2+}-dependent inactivation and the increased peak current density but prevented the leftward shift in activation gating. Overexpression of the CaM1234 variant obliterated the gain in the function of Cav1.2 G449R yielding an activity profile akin to Cav1.2 WT in the presence of endogenous/native CaM. The stronger activity of Cav1.2 G449 thus minimally requires the direct or indirect action of the Ca\textsuperscript{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 manuscript, the affinity between the two full-length proteins can be roughly approximated by the relative intensity of the signal measured in co-immunoprecipitation assays. Within all the limitations of this exercise, the protein signal obtained for G449R in co-immunoprecipitation 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 twelve 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 cell 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 (66). 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 (81), inactivation kinetics (82), protein stability, ubiquitination (83), and cell surface trafficking (84). The proximal segment of the first intracellular linker hosts the high-affinity binding site for Cavβ (85) and plays a role in networking with direct partners such as galectin (83) or Ras/Rad proteins through Cavβ (30, 86, 87). 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, 88, 89). 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 (90).

The high-affinity binding site of Cavβ adopts an α-helical structure in vitro (91). The relative rigidity α-helix could promote a strong van der waals interaction between the guanylate domain of Cavβ and hydrophobic residues of Cav1.2 (29, 92, 93). In the native protein, this α-helix breaks at the glycine located at position 449 (88). 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, (94), produced channels with strong activation properties and a contrario, 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 phospho-mimetic and the phospho-resistant CaM proteins, from a complete indifference (G449K) to impaired peak current density in the presence of phospho-resistant CaM variants (G449A, G449D, G449P, and G449Q). Our data are compatible with the proposition 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 (95). In this context the LQTS phenotype associated with the Glycine to Arginine substitution in the I-II linker could result from either process: a 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 cause the channel to be maximally activated at near endogenous concentration of CaM.

**Experimental procedures**

**Recombinant DNA techniques**

The Cavα1C subunit of Cav1.2 (GenBank™ accession number X15539), Cavβ2a (GenBank™ accession number NM_001398773), and Cavα2δ1 (GenBank™ accession number NM_000722) were subcloned in commercial vectors under the control of the CMV promoter as described elsewhere (36, 37, 84, 96). 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 calmodulin (CaM) (GenBank™ accession number M27319), subcloned in pcDNA3.1 (Thermofisher) 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, 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 in (97) to take into account that the mature protein lacks N-terminal Met residue. All cDNA mutations in Cavα1C of Cav1.2 and CaM were produced with the Q5 Site-Directed Mutagenesis Kit (New England Biolabs Inc., Whitby, Ontario, Canada) 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 DH5-α competent E. coli. All constructs were verified by automated double-stranded sequence analysis (“Centre d’expertise et de services Génome Québec”, 3175 Chemin de la Côte-Sainte-Catherine, Montréal, QC, H3T 1C5, Canada). The protein expression at the expected molecular weight was confirmed by standard western-blot analysis for each construct.

**Gene transfection and cell culture**

HEK293T (thereafter referred to as HEKT) cells were grown using standard tissue culture conditions (5% CO2, 37°C) in high glucose DMEM supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (10 mg/ml) as described before (36,37,84). 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-Cav1.2 WT or variants (4 µg), pCMV-Cavβ2a (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: 1: 0.5 for a total of 12 to 14 µg cDNAs. The molar ratio was 7:1 for CaM and Cav1.2. Unless otherwise noted, the plasmids pCMV-Cavβ2a, pCMV-Cavα2δ1, and pcDNA3-HisB-cMyc-CaM WT are simply referred to as Cavβ2a, Cavα2δ1 and CaM WT in the text and in the figures. cDNA coding for peGFP (0.2 µg) was included in the cDNA mixture as a marker of successful transfection for patch-clamp experiments (4,90). 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 24-32 h after transfection.

**Co-immunoprecipitation**

HEKT cells were transiently transfected with the appropriate constructs (as indicated below) and protein extraction proceeded two days after transfection. Experiments described in Fig 2 were carried out as follows. HEKT cells were transiently transfected with Cav1.2 WT or Cav1.2 G449R with pCMV-Cavα2δ1 and cMyc-tagged versions of Cavβ3 or Cavβ2a using respectively the pCMV-Tag5-Cavβ3 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, 88842, ThermoFisher Scientific) to capture the given Cavβ. In the experiments shown in Figs 4 and 5, the constructs were pCMV-Cavβ2a with pCMV-Cav1.2 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 (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 non ionic 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 two months. Two days after transfection, cells were
homogenized in 20 mM NaMOPS (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. Co-immunoprecipitation was carried out using 200 μg homogenates diluted in 150 μL of 20 mM NaMOPS (pH 7.4), 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 NaMOPS (pH 7.4), 300 mM NaCl, and 0.2% digitonin or alternatively 20 mM NaMOPS (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 ECL 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 fraction) 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 the 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 Ca²⁺ currents from transfected HEKT cells were recorded using pCLAMP software 11.2 and an Axopatch 200B amplifier (Molecular Devices, Union City, CA). Patch electrodes were pulled from borosilicate glass (Corning 8161) and heat-polished to a final resistance about 3.0-3.5 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 Digidata 1440A, Molecular Devices, Sunnyvale, CA, USA). Electrodes were filled with a solution containing (in mM) 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 mM) as follows: 135 NaCl, 20
tetraethylammonium chloride, 2 CaCl₂, 1 MgCl₂, 10 HEPES, titrated to pH 7.4 with KOH. Stock solution of the cell-permeable calmodulin antagonists W-13 N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide, monohydrochloride (Tocris, Biotechnne Canada, 21 Canmotor Ave, Toronto, ON M8Z 4E6, Canada) and in some instances, W-7 (Sigma-Aldrich, Canada, 2149 Winston Park Drive, Oakville, ON L6H 6J8, Canada) were 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 (4,5,6,7-Tetrabromobenzotriazole, Tocris, Biotechnne Canada), a cell-permeable inhibitor of casein kinase-2. Stock solution of TBB (5 mM) were prepared in dimethylsulfoxide or DMSO, 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 10-mV depolarizing pulse applied to the cell from a holding potential of -100 mV.

Whole-cell Ca²⁺ currents were elicited from a holding potential of -100 mV and were depolarized to potentials ranging from -80 to 65 mV in 5 mV increments lasting 450 ms for each step. Ca²⁺ 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 BoltzIV equation, which is a transformed Boltzmann function for I-V data of the following form:

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

Where I is the current, Vm is the applied voltage, E₀.5,act is the voltage at which channels are half-maximal activated, dx is the steepness of the slope, Gmax is the maximal conductance, and Vrev is the reversal potential. Steady-state activation curves were constructed 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 BoltzIV equation, which is a transformed Boltzmann function for I-V data of the following form:

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\[ I = \frac{(V_m - V_{rev}) \cdot G_{max}}{1 + e^{(V - E_{0.5, act})/d_x}} \]
Data analysis and statistics

Data was analyzed using a combination of pCLAMP software 11.2 (Molecular Devices), Microsoft Excel, and OriginPro 2020 (OriginLab Corporation, One Roundhouse Plaza, Suite 303, Northampton, MA 01060, USA). Data in the Tables were expressed as mean ± S.D. 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 manuscript.

ACKNOWLEDGMENTS

We are grateful for the ongoing collaboration with Dr Rafik Tadros from the Cardiovascular Genetics Center at the Montreal Heart Institute and we thank Dr Rémy Sauvé for stimulating discussions and critical reading of the manuscript. This work was completed with the operating grant 159556 from the Canadian Institutes of Health Research to L.P. E.S. is the recipient of a Ph.D. award from “Fonds de la recherche du Québec en nature et technologies”.

CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest with the contents of this manuscript.

REFERENCES


FIGURE LEGENDS

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 (PDB: 5GJV). Cavα1C and Cavα1S share 81% homology in their primary protein sequence. LTCCs share similar structure, being composed of the pore-forming subunit Cavα in red and Cavβ in blue, 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 G449R variant is similar to the rabbit Cavα1C G449R and is assessed in Cavα1S by Gly358. 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 the 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 6th transmembrane segment in Domain I (IS6). The primary sequence for the AID motif is shown below the primary sequence for the transmembrane segment in Domain I extending from the end of S6 to the beginning of the AID. The relative position of three Glycine variants reported in the Timothy syndrome (G402S, G406R, and G419R) are fully conserved across species and are presented in red with the numbering in the rabbit clone used for this study.

FIGURE 2. G449R interacts with Cavβ proteins. HEKT cells were transiently transfected with Cav1.2 WT (WT) or Cav1.2 G449R (GR) with cMyc-tagged Cavβ3 or cMyc-tagged Cavβ2a. Cavα2δ1 was present throughout. Cell lysates were immunoprecipitated overnight with anti-cMyc magnetic beads (Pierce Anti-c-Myc Magnetic Beads, 88842, ThermoFisher Scientific) to capture the Cavβ, eluted in a Laemmli buffer 2X, and fractionated by 8% SDS-PAGE gels. A. Immunoblotting was carried out on total proteins (20 µg) collected from the cell lysates before the immunoprecipitation assay (Total proteins). The signal for the housekeeping protein GAPDH is shown below each blot. B. Immunoblotting of “IP-proteins” was carried out after eluting the protein complexes from the beads. All immunoblots were carried out in parallel under the same transfection and extraction conditions. Western blotting was carried out with either anti-Cavβ3 (Alomone ACC008, 1:10000), anti-Cavβ2a (Alomone ACC105, 1:1000), anti-Cav1.2 directed against Cavα1C (Alomone ACC018, 1:250), anti-Cavα2δ1 (Alomone ACC015, 1:1000), and GAPDH (Sigma, 1:10000) with an anti-rabbit as secondary antibody (Jackson ImmunoResearch, 1:10,000). Signals were detected with the ECL substrate. Blots were visualized with the ChemiDoc Touch system (Bio-Rad). Molecular weights were estimated using Image LabTM software version 5.2 (Bio-Rad) by linear regression of standard molecular weight markers. GAPDH, Cavβ3, Cavβ2a, Cavα2δ1 and Cavα1.2 proteins migrated (in kDa) at 35, 60, 80, 175, and 250 kDa respectively. From left to right in A and in B: Lane 1: Cav1.2 WT + Cavα2δ1 + Cavβ2a; Lane 2: Cav1.2 G449R + Cavα2δ1 + Cavβ2a; Lane 3: Cav1.2 WT + Cavα2δ1 + Cavβ3; and Lane 4: Cav1.2 G449R + Cavα2δ1 + Cavβ3.

FIGURE 3. Cav1.2 G449R is insensitive to W-13. Panel 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 v. -8 ± 2
pA/pF in the presence of W-13. **Panel 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). **Panel C.** Representative Cav1.2 WT current traces co-transfected 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 co-expressed with CaM WT are plotted against the applied voltages and fitted by a Boltzmann-like equation. **Panel D.** Representative Cav1.2 G449R current traces co-transfected 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.

**FIGURE 4. Calmodulin (CaM) pulls down the L-type calcium channel.** HEKT cells were transiently transfected with Cavβ2a, CaM WT and either Cav1.2 WT (WT) or G449R (GR). CaM was captured by the anti-His coated beads. **Panel A.** Proteins were homogenized and a fraction of this solution (referred to as Total) was set aside to validate protein expression. **Panel B.** Co-immunoprecipitation was carried out with anti-His magnetic beads. The bound proteins were eluted (referred to as Pull-down), electrophoresed on a 6% SDS-polyacrylamide gel or a 10% SDS-polyacrylamide gel for CaM and GAPDH before being transferred onto a nitrocellulose membrane. Western blotting was carried out with anti-Cavβ2 (Alomone ACC105, 1:1000), anti-Cav1.2 (Alomone ACC003, 1:250) or an anti-rabbit as secondary antibody (Jackson Immuno Research, 1:10000) and anti-CaM (Millipore 05-193, 1:1000) with an anti-mouse as secondary antibody (Jackson ImmunoResearch, 1:10,000). Molecular weights were estimated using Image LabTM software version 5.2 (Bio-Rad) by linear regression of standard molecular weight markers. Cav1.2 WT and G449R, Cavβ2a, and CaM proteins were translated at the expected molecular masses of 250, 70, and 18-24 kDa, respectively. Cav1.2 WT and G449R were successfully pulled indicating that Cav1.2 G449R interacts with Cavβ2a and CaM. This result was successfully obtained from 4 independent transfections carried out over the course of 3 months.

**FIGURE 5. CaM promotes the cell surface localization of Cav1.2 WT but not Cav1.2 G449R.** **Panel A.** HEKT cells were transiently transfected with Cav1.2 WT + Cavα2δ1 + Cavβ2a (left) and Cav1.2 G449R + Cavα2δ1 + Cavβ2a (right) in the presence of native / endogenous CaM. **Panel B.** HEKT cells were transiently transfected with Cav1.2 WT + Cavα2δ1 + Cavβ2a + CaM WT (left) and Cav1.2 G449R + Cavα2δ1 + Cavβ2a + CaM WT (right). Two days after transfection, the cells were lysed, and cell fractions were obtained through preparative ultracentrifugation as described in Experimental Procedures. Western blotting was carried out for the four protein fractions found in lanes 1-4; Lane 1: Total proteins; Lane 2: Cytoplasmic proteins; Lane 3: Total membrane proteins; and Lane 4: Plasma membrane proteins. The proteins were probed with the following antibodies: Cav1.2: Alomone ACC003 1:250 with anti-rabbit 1:10000; Cavα2δ1: Alomone ACC015 1:1000 with anti-rabbit 1:10000; Cavβ2a: Alomone ACC105 1:1000 with anti-rabbit 1:10000; CaM: Millipore 05-193, 1:1000 with anti-mouse 1:10000; His: Invitrogen 71700, 1:1000 with anti-mouse 1:10000 and cadherin: Thermofisher Pan-cadherin 71-7100, 1:1000 with anti-rabbit 1:10000. Cadherin was used as a marker for the plasma membrane. The membrane
was cut at 115 kDa and at 28 kDa to probe first Cav1.2, Cavβ2a, and CaM. Membranes were then stripped and reprobed with antibodies against the proteins: Cavα2δ1, cadherin, and housekeeping GAPDH (Sigma G9545, 1:10000 with anti-rabbit 1:10000). Each lane was loaded with 20 μg proteins. The lines to the left of the blots indicate the position of the molecular markers and the value is provided in kDa. The molecular masses were estimated by linear regression and interpolation from the molecular mass markers using the Image LabTM Software 5.2 (Bio-Rad). As seen in panel A in the presence of endogenous CaM, the signal for Cav1.2 WT was stronger in the total membrane protein fraction (lane 3) than in the cell surface protein fraction (lane 4). Under the same conditions, the signal for Cav1.2 G449R was stronger in the cell surface protein fraction. Panel B demonstrates that under conditions where CaM was overexpressed the signal for Cav1.2 WT and Cav1.2 G449R is stronger in the cell surface protein fraction. Along with Cav1.2, Cavα2δ1 (as previously reported (4)) and Cavβ2a are found in the cell surface fraction but not CaM and GAPDH. This observation suggests that the interaction of CaM with the pore-forming subunit is very robust during the cell surface export and is compatible with the binding and unbinding kinetics of CaM in other cell types (42). No significant signal was found in the cytoplasmic fraction for Cavα2δ1 and the membrane-anchored Cavβ2a. This result was successfully obtained from 2 independent transfections carried out over the course of 2 months.

**FIGURE 6. Phospho-mimetic CaM T79D and S81D upregulate Cav1.2 WT channels.** Panels A and C, middle. Cav1.2 WT was co-expressed with the “phospho-resistant” CaM mutations T79A or S81A. Overexpression of CaM T79A or S81A failed to enhance the currents and were insensitive to W-13. Panels A and C, right. Average I-V curves of Cav1.2 WT co-expressed with CaM T79A or S81A. The peak current densities were not different between control and W-13 treatment. Panels B and D, left, middle. Cav1.2 WT current traces recorded from HEKT cells after co-expression with phospho-mimetic 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. Panels B and D, right. Average I-V curves of Cav1.2 WT co-expressed 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 co-expressed with either CaM WT (black), T79A (red), T79D (blue), S81A (green), or S81D (light purple). The mean data ± S.D. are shown as gray hyphens. The values of the average peak current densities and E_{0.5, act} are listed in Table 2.

**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+}. Panels A, B, C, D, left. Cav1.2 G449R was co-expressed with CaM WT, with the phospho-resistant CaM (T79A or S81A), or with phospho-mimetic CaM (T79D or S81D) as shown. Panels A, B, C, D, middle. Cav1.2 G449R channels co-expressed 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. Panels A, B, C, D, right. Average I-V curves of Cav1.2 G449R co-expressed with CaM T79A, T79D, S81A, or S81D. The peak current densities were not different between control and W-13 treatment. Panel E and Panel F. The distribution of the peak current densities and E_{0.5,act} for control conditions and after W-13 treatment are summarized individually as filled circles for Cav1.2 G449R co-expressed with either CaM WT (black), T79A (red), T79D (blue), S81A (green), or S81D (light purple). The mean data ± S.D. are shown as gray hyphens. The complete set of values is found in Table 3.
FIGURE 8. CaM T79A and T79D co-immunoprecipitate Cav1.2 WT and G449R. HEKT cells were transiently transfected with Cavβ2a in the presence of Cav1.2 WT or Cav1.2 G449R and either CaM WT, CaM T79A, or CaM T79D. Panel A. Total proteins are shown. Panel B. Co-immunoprecipitation 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 Fig 4. As seen, Cav1.2 WT and G449R, Cavβ2a, and CaM proteins were translated at the expected molecular masses of 250, 70, and 18-24 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 Cav1.2 G449R than for Cav1.2 WT despite equivalent loading and similar signals for the total proteins. Similar data were obtained from 3 independent transfections carried out over the course of 2 months with protein extraction carried out with digitonin or CHAPS.

FIGURE 9. Cav1.2 G449P is modulated by CaM phosphorylation surrogates. Panel A. Whole-cell currents were recorded from HEKT cells transiently transfected with Cav1.2 G449P co-expressed with Cavβ2a and Cavα2δ1 and either CaM WT, with the phospho-resistant CaM (T79A or S81A), or with phospho-mimetic CaM (T79D or S81D) as indicated. Exemplar traces are shown (from left to right) for Cav1.2 G449P + CaM WT, G449P + CaM T79A, G449P + CaM T79D, G449P + CaM S81A, and G449P + CaM S81D. The vertical scale bars are 10 pA/pF and the horizontal scale bars are 100 ms throughout. Panel B. The corresponding peak current densities are plotted as a function of applied voltage. Panels C and D show the summarized distribution of the peak current densities and the mid-potential of activation $E_{0.5, \text{act}}$. Peak whole-cell currents and $E_{0.5, \text{act}}$ are reported individually as black circles. The mean data ± S.D are shown as red hyphens. Values of peak current densities and $E_{0.5, \text{act}}$ are reported in Table 6.

SUPPL FIGURE. Cav1.2 channel activity is modulated by substitutions at position Gly449. Panel A. Whole-cell currents were recorded from HEKT cells transiently transfected with Cav1.2 WT or variants co-expressed with Cavβ2a and Cavα2δ1. Exemplar traces are shown (from left to right) for Cav1.2 WT, G449A, G449D, G449P, and G449R. Cav1.2 currents were elicited from a holding potential of −100 mV and were depolarized to potentials ranging from −80 to 65 mV in 5 mV increments lasting 450 ms for each step (depicted above each series of recordings). The vertical scale bars are 10 pA/pF and the horizontal scale bars are 100 ms throughout. Panel B. Peak current densities of Cav1.2 WT, G449A, G449D, G449P, and G449R currents are plotted as a function of applied voltage and fitted by a Boltzman equation described in Experimental Procedures. The activation curves were generated using the same protocol as in Panel A. Panels C and D show the summarized distribution of the peak current densities and the mid-potential of activation $E_{0.5, \text{act}}$. Peak whole-cell currents and $E_{0.5, \text{act}}$ are reported individually as black circles. The mean data ± S.D. are shown as red hyphens. Values of peak current densities and $E_{0.5, \text{act}}$ are reported in Table 6.
Table 1. Electrophysiological properties of Cav1.2 WT and G449R with W-13

<table>
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<th>Cav1.2</th>
<th>CaM</th>
<th>n / N</th>
<th>Peak I (pA/pF)</th>
<th>E_{0.5, act} (mV)</th>
<th>R100</th>
<th>n / N</th>
<th>E_{0.5, inact} (mV)</th>
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<tr>
<td>WT</td>
<td>Native</td>
<td>30/7</td>
<td>-15 ± 4</td>
<td>-10 ± 3</td>
<td>0.65 ± 0.04</td>
<td>14/5</td>
<td>-33 ± 3</td>
</tr>
<tr>
<td></td>
<td>+W-13</td>
<td>17/4</td>
<td>-8 ± 2</td>
<td>-12 ± 4</td>
<td>0.57 ± 0.02</td>
<td>6/3</td>
<td>-33 ± 3</td>
</tr>
<tr>
<td>G449R</td>
<td>Native</td>
<td>31/4</td>
<td>-33 ± 12</td>
<td>-17 ± 3</td>
<td>0.52 ± 0.03</td>
<td>13/4</td>
<td>-35 ± 3</td>
</tr>
<tr>
<td></td>
<td>+ W-13</td>
<td>12/1</td>
<td>-38 ± 8</td>
<td>-17 ± 3</td>
<td>0.51 ± 0.03</td>
<td>5/2</td>
<td>-34 ± 2</td>
</tr>
</tbody>
</table>

Table 1. Effects of calmodulin (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 co-expressed in HEK cells with Cavβ2a, Cavα2δ1. Whole-cell currents were measured in the presence of 2 mM Ca^{2+} in the extracellular medium. E_{0.5, inact} values were estimated after a 5-s long depolarizing pulse to 0 mV. Fractional currents were fitted to Boltzmann equations as described in Experimental Procedures. 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.
Table 2. Electrophysiological properties of Cav1.2 WT with CaM WT and phosphorylation surrogates

<table>
<thead>
<tr>
<th>Cav1.2</th>
<th>CaM</th>
<th>n / N</th>
<th>Peak Current density (pA/pF)</th>
<th>E0.5, act (mV)</th>
<th>R100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cav1.2 WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaM WT</td>
<td>28/6</td>
<td>-28 ± 8</td>
<td>p &lt; 0.001 v. native CaM</td>
<td>-14 ± 3</td>
<td>0.60 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>+ W-13</td>
<td>9/2</td>
<td>-3.3 ± 0.7</td>
<td>p = 0.001 v. control</td>
<td>0.65 ± 0.03</td>
</tr>
<tr>
<td>CaM T79A</td>
<td>18/2</td>
<td>-13 ± 5</td>
<td>p &lt; 0.001 v. CaM WT, T79A, S81A</td>
<td>-14 ± 3</td>
<td>0.69 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>+ W-13</td>
<td>16/2</td>
<td>-14 ± 3</td>
<td>p = 0.001 v. control</td>
<td>0.56 ± 0.03</td>
</tr>
<tr>
<td>CaM T79D</td>
<td>15/4</td>
<td>-29 ± 7</td>
<td>p &lt; 0.001 v. native CaM</td>
<td>-14.7 ± 2.7</td>
<td>0.55 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>+ W-13</td>
<td>10/2</td>
<td>-4 ± 1</td>
<td>p = 0.001 v. control</td>
<td>0.71 ± 0.02</td>
</tr>
<tr>
<td>CaM S81A</td>
<td>17/2</td>
<td>-13 ± 4</td>
<td>p &lt; 0.001 v. CaM WT, T79D, S81D</td>
<td>-13 ± 2</td>
<td>0.66 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>+ W-13</td>
<td>8/1</td>
<td>-12 ± 2</td>
<td>p = 0.02 v. control</td>
<td>0.61 ± 0.02</td>
</tr>
<tr>
<td>CaM S81D</td>
<td>14/1</td>
<td>-32 ± 8</td>
<td>p = 0.001 v. native CaM,</td>
<td>-13 ± 3</td>
<td>0.62 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>+ W-13</td>
<td>9/1</td>
<td>-5 ± 1</td>
<td>p &lt; 0.001 v. control</td>
<td>0.70 ± 0.02</td>
</tr>
</tbody>
</table>

Table 2. Effects of calmodulin inhibitor W-13 on the biophysical properties of Cav1.2 WT channels. Cav1.2 WT was co-expressed with Cavβ2a, Cavα2δ1, and CaM WT or phospho-resistant and phospho-mimetic variants (T79A, T79D, S81A, or S81D). Activation properties (E0.5,act) were estimated from the I-V relationships and fitted as described in Experimental Procedures. 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, Bonferroni test (P < 0.05). As seen, co-expression with CaM WT, CaM T79D, or T81D potentiated Ca2+ currents that were sensitive to CaM antagonists and sped up current decay. In contrast, co-expression with CaM T79A or CaM S81A (phospho-resistant analogues) produced Ca2+ currents similar to the ones measured in the presence of endogenous / native CaM in terms of peak current density and current decay. Nonetheless, CaM T79A and CaM S81A promoted robust activation and rendered Ca2+ currents insensitive to inhibition by W-13.
Table 3. Electrophysiological properties of Cav1.2 G449R with CaM WT and phosphorylation surrogates

<table>
<thead>
<tr>
<th>Cav1.2 G449R</th>
<th>CaM</th>
<th>n / N</th>
<th>Peak Current density (pA/pF)</th>
<th>E₀.₅, act (mV)</th>
<th>R100</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaM WT</td>
<td>20/3</td>
<td>-35 ± 10</td>
<td>-16 ± 2</td>
<td>0.50 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>+ W-13</td>
<td>9/2</td>
<td>-35 ± 9</td>
<td>-17 ± 3</td>
<td>0.54 ± 0.01</td>
<td>p = 0.04 v. Control</td>
</tr>
<tr>
<td>CaM T79A</td>
<td>15/2</td>
<td>-32 ± 10</td>
<td>-16 ± 2</td>
<td>0.50 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>+ W-13</td>
<td>14/2</td>
<td>-33 ± 10</td>
<td>-17 ± 3</td>
<td>0.45 ± 0.03</td>
<td>p &lt; 0.001 v Control</td>
</tr>
<tr>
<td>CaM T79D</td>
<td>24/3</td>
<td>-35 ± 9</td>
<td>-18 ± 4</td>
<td>0.50 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>+ W-13</td>
<td>8/1</td>
<td>-32 ± 9</td>
<td>-16 ± 2</td>
<td>0.50 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>CaM S81A</td>
<td>13/1</td>
<td>-34 ± 9</td>
<td>-18 ± 2</td>
<td>0.48 ± 0.02</td>
<td>p = 0.002 v. native CaM</td>
</tr>
<tr>
<td>+ W-13</td>
<td>9/1</td>
<td>-33 ± 7</td>
<td>-19 ± 2</td>
<td>0.51 ± 0.02</td>
<td>p = 0.02 v. CaM S81D</td>
</tr>
<tr>
<td>CaM S81D</td>
<td>9/1</td>
<td>-35 ± 8</td>
<td>-17 ± 3</td>
<td>0.52 ± 0.02</td>
<td>p = 0.02 v. CaM S81A</td>
</tr>
<tr>
<td>+ W-13</td>
<td>10/1</td>
<td>-33 ± 10</td>
<td>-15 ± 3</td>
<td>0.50 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Effects of calmodulin inhibitor W-13 on the biophysical properties of Cav1.2 G449R channels. Cav1.2 G449R was co-expressed with Cavβ2a, Cavα2δ1, and CaM WT or phospho-resistant and phospho-mimetic variants (T79A, T79D, S81A, or S81D). Activation properties (E₀.₅,act) were estimated from the I-V relationships and fitted as described in Experimental Procedures. 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, all experimental conditions yielded whole-cell Ca²⁺ currents that were not significantly different from one another (p > 0.05).
Table 4. Electrophysiological properties of CaV1.2 WT and G449R with 4,5,6,7-Tetrabromobenzotriazole (TBB)

<table>
<thead>
<tr>
<th>CaV1.2</th>
<th>CaM</th>
<th>n / N</th>
<th>Electrophysiological properties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Peak Current density (pA/pF)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Cav1.2 WT</td>
<td>Native CaM</td>
<td>30/7</td>
<td>-15 ± 4</td>
</tr>
<tr>
<td></td>
<td>+ TBB</td>
<td>6/2</td>
<td>-4 ± 1 p = 0.001 v. control</td>
</tr>
<tr>
<td>Cav1.2 WT</td>
<td>CaM WT</td>
<td>28/6</td>
<td>-28 ± 8</td>
</tr>
<tr>
<td></td>
<td>+ TBB</td>
<td>5/1</td>
<td>-4.9 ± 0.7 p &lt; 0.001 v. control</td>
</tr>
<tr>
<td>Cav1.2 G449R</td>
<td>Native CaM</td>
<td>31/4</td>
<td>-33 ± 12</td>
</tr>
<tr>
<td></td>
<td>+ TBB</td>
<td>7/2</td>
<td>-15 ± 6 p = 0.001 v. control</td>
</tr>
<tr>
<td>Cav1.2 G449R</td>
<td>CaM WT</td>
<td>20/3</td>
<td>-35 ± 10</td>
</tr>
<tr>
<td></td>
<td>+ TBB</td>
<td>7/2</td>
<td>-13 ± 5 p &lt; 0.001 v. control</td>
</tr>
</tbody>
</table>

Table 4. Effects of TBB, the cell-permeable inhibitor of casein kinase 2 on the biophysical properties of Cav1.2 WT and Cav1.2 G449R channels. Cav1.2 (WT or G449R) was co-expressed 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 (E0.5,act) were estimated from the I-V relationships and fitted as described in Experimental Procedures. 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.
Table 5. Effect of CaM1234 on electrophysiological properties of Cav1.2 WT and Cav1.2 G449R

<table>
<thead>
<tr>
<th>CaM1.2 WT</th>
<th>CaM</th>
<th>n / N</th>
<th>Peak Current density (pA/pF)</th>
<th>Electrophysiological properties</th>
<th>R100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CaM1234</td>
<td>10/2</td>
<td>-9 ± 2</td>
<td>-6 ± 2</td>
<td>0.76 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>+ W-13</td>
<td>7/1</td>
<td>-4 ± 2</td>
<td>p =0.04 v. Control</td>
<td>0.71 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>CaM1234</td>
<td>4/2</td>
<td>-17 ± 4</td>
<td>-10 ± 2</td>
<td>0.73 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>+ W-13</td>
<td>6/1</td>
<td>-19 ± 2</td>
<td>-10 ± 2</td>
<td>0.63 ± 0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CaM1.2 G449R</th>
<th>CaM</th>
<th>n / N</th>
<th>Peak Current density (pA/pF)</th>
<th>Electrophysiological properties</th>
<th>R100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CaM1234</td>
<td>10/2</td>
<td>-6 ± 2</td>
<td>p &lt; 0.001 v. CaM WT</td>
<td>p &lt; 0.001 v. CaM WT</td>
</tr>
<tr>
<td></td>
<td>+ W-13</td>
<td>7/1</td>
<td>-5 ± 2</td>
<td>p =0.04 v. Control</td>
<td>p &lt; 0.001 v. Control</td>
</tr>
<tr>
<td></td>
<td>CaM1234</td>
<td>4/2</td>
<td>-10 ± 2</td>
<td>p &lt; 0.001 v. CaM WT</td>
<td>p &lt; 0.001 v. CaM WT</td>
</tr>
<tr>
<td></td>
<td>+ W-13</td>
<td>6/1</td>
<td>-10 ± 2</td>
<td>p =0.04 v. Control</td>
<td>p &lt; 0.001 v. Control</td>
</tr>
</tbody>
</table>

Table 5. Whole-cell currents were recorded from HEKT cells transiently transfected with Cav1.2 WT or variants co-expressed with Cavβ2a, Cavα2δ1, and CaM1234. Activation properties (E0.5, act) were estimated from the I-V relationships and fitted to a BoltzIV equation as described in Experimental Procedures. 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.
Table 6. Electrophysiological properties of Cav1.2 Gly449 variants with CaM phosphorylation surrogates

<table>
<thead>
<tr>
<th>Cav1.2</th>
<th>CaM</th>
<th>n / N</th>
<th>Peak Current density (pA/pF)</th>
<th>E_{0.5, act} (mV)</th>
<th>R100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Native</td>
<td>12/2</td>
<td>-35 ± 8</td>
<td>-18 ± 3</td>
<td>0.55 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>CaM WT</td>
<td>22/4</td>
<td>-31 ± 8</td>
<td>-15 ± 2</td>
<td>0.57 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>CaM T79A</td>
<td>7/1</td>
<td>-11 ± 2</td>
<td>-13 ± 2</td>
<td>0.70 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>CaM T79D</td>
<td>6/1</td>
<td>-28 ± 8</td>
<td>-18 ± 2</td>
<td>0.55 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>CaM S81A</td>
<td>13/2</td>
<td>-14 ± 3</td>
<td>-13 ± 2</td>
<td>0.70 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>CaM S81D</td>
<td>19/2</td>
<td>-30 ± 9</td>
<td>-14 ± 3</td>
<td>0.58 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Native</td>
<td>17/2</td>
<td>-14 ± 4</td>
<td>-15 ± 2</td>
<td>0.62 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>CaM WT</td>
<td>17/2</td>
<td>-27 ± 8</td>
<td>-17 ± 2</td>
<td>0.56 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>CaM T79A</td>
<td>16/1</td>
<td>-15 ± 4</td>
<td>-16 ± 3</td>
<td>0.59 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>CaM T79D</td>
<td>14/1</td>
<td>-29 ± 6</td>
<td>-18 ± 2</td>
<td>0.55 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>CaM S81A</td>
<td>21/2</td>
<td>-16 ± 4</td>
<td>-15 ± 2</td>
<td>0.60 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>CaM S81D</td>
<td>10/1</td>
<td>-33 ± 7</td>
<td>-18 ± 2</td>
<td>0.52 ± 0.02</td>
</tr>
</tbody>
</table>

For CaM T79A, p < 0.001 v. native CaM; p < 0.001 v. CaM WT.
For CaM T79D, p < 0.001 v. native CaM; p < 0.001 v. CaM WT.
For CaM S81A, p < 0.001 v. native CaM; p < 0.001 v. CaM WT.
For CaM S81D, p < 0.001 v. native CaM; p < 0.001 v. CaM WT.

For CaM T79A, p = 0.002 v. native CaM.
For CaM T79D, p = 0.007 v. native CaM.
For CaM S81A, p = 0.03 v. native CaM.
For CaM S81D, p = 0.01 v. CaM WT.
Table 6. Cav1.2 Gly449 variants were co-expressed with Cavβ2a, Cavα2δ1, and CaM WT or CaM T79A, CaM T79D, CaM S81A, or CaM S81D. Activation properties (E_{0.5,act}) were estimated from the I-V relationships as described in Experimental Procedures. 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, Cav1.2 WT, G449D, and G449P were modulated by CaM phosphovariants. whereas the properties of G449K remained unaffected. G449A was not upregulated by CaM WT but was downregulated by phospho-resistant CaM variants.
Figure 1

A.

B.

AID = α-interacting domain

432-**GVLSGEFSKEREKAKARGDFQKLREK**-457

458-**QQLEEDLKGYLDWI**-471
Figure 2
Figure 3
FIGURE 4
Figure 5
Figure 6

A. Ca\textsubscript{v}1.2 WT + CaM T79A

B. Ca\textsubscript{v}1.2 WT + CaM T79D

C. Ca\textsubscript{v}1.2 WT + CaM S81A

D. Ca\textsubscript{v}1.2 WT + CaM S81D

E. Peak Current Density (pA/pF)

F. E\textsubscript{0.5, act} (mV)
Figure 7

A. Ca_{v}1.2 G449R + CaM T79A

B. Ca_{v}1.2 G449R + CaM T79D

C. Ca_{v}1.2 G449R + CaM S81A

D. Ca_{v}1.2 G449R + CaM S81D

E. Control, W-13

F. Control, W-13

Peak Current Density (pA/pF)

E_{0.5, act} (mV)
Figure 8

A. TOTAL PROTEINS

CaM WT T79A T79D

250

75

50

25

20

15

50

37

CaV1.2 WT GR WT GR WT GR

B. IP-PROTEINS

CaM WT T79A T79D

CaV1.2

CaVβ2

CaM

GAPDH

CaV1.2 WT GR WT GR WT GR

Journal Pre-proof
Figure 9

A G449P + CaM WT CaM T79A CaM T79D CaM S81A CaM S81D

B

-80 -60 -40 -20 20 40 60 mV

-15

-10

-5

no CaM CaM WT CaM T79A CaM T79D CaM S81A CaM S81D

5 0 -5 -10 -15 E0.5, act (mV)

Peak Current Density (pA/pF)

D

5

0

-5

-10

E0.5, act (mV)

No CaM WT T79A T79D S81A S81D

C

Figure 9
AUTHOR CONTRIBUTIONS
L.P. designed and supervised the study. J.Z., E.S. and M.M. carried out the experiments and analyzed data. J.Z. and L.P. wrote the paper with inputs from all other authors. All authors read and approved the final version of this manuscript.