Conservation of Ca\textsuperscript{2+}/Calmodulin Regulation across Na and Ca\textsuperscript{2+} Channels

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SUMMARY

Voltage-gated Na and Ca\textsuperscript{2+} channels comprise distinct ion channel superfamilies, yet the carboxy tails of these channels exhibit high homology, hinting at a long-shared and purposeful module. For different Ca\textsuperscript{2+} channels, carboxyl-tail interactions with calmodulin do elaborate robust and similar forms of Ca\textsuperscript{2+} regulation. However, Na channels have only shown subtler Ca\textsuperscript{2+} modulation that differs among reports, challenging attempts at unified understanding. Here, by rapid Ca\textsuperscript{2+} photorelease onto Na channels, we reset this view of Na channel regulation. For cardiac-muscle channels (Na\textsubscript{v}1.5), reported effects from which most mechanistic proposals derive, we observe no Ca\textsuperscript{2+} modulation. Conversely, for skeletal-muscle channels (Na\textsubscript{v}1.4), we uncover fast Ca\textsuperscript{2+} regulation eerily similar to that of Ca\textsuperscript{2+} channels. Channelopathetic myotonia mutations halve Na\textsubscript{v}1.4 Ca\textsuperscript{2+} regulation, and transplanting the Na\textsubscript{v}1.4 carboxy tail onto Ca\textsuperscript{2+} channels recapitulates Ca\textsuperscript{2+} regulation. Thus, we argue for the persistence and physiological relevance of an ancient Ca\textsuperscript{2+} regulatory module across Na and Ca\textsuperscript{2+} channels.

INTRODUCTION

Voltage-gated Na and Ca\textsuperscript{2+} channels constitute two prominent ion channel superfamilies (Jan and Jan, 1989), each subserving distinct functions (Adams and Snutch, 2007; Hille, 2001; Jan and Jan, 1989). Curiously, however, the carboxy tails of these channels (Figure 1A, CI region) demonstrate high sequence homology, hinting at a tangible ancestral blueprint. Babitch first remarked on a conserved vestigial EF hand (Babitch, 1990) (rose shading), and further scrutiny reveals extensive homology throughout. The CI region contains dual vestigial EF hand motifs (Babitch, 1990; Chagot et al., 2009; de Leon et al., 1995; Miloushev et al., 2009) (rose, green) and a calmodulin (CaM)-binding IQ domain (Mori et al., 2000; Zühike and Reuter, 1998) (lavender). If this homology were to support functions of like correspondence, deep mechanistic insights could be gleaned from combined investigation of Na and Ca\textsuperscript{2+} channels and shared principles obtained for approaching related channelopathetic diseases.

Ca\textsuperscript{2+} channels have largely fulfilled this possibility, as the CI regions of channels across this superfamily elaborate rapid and robust Ca\textsuperscript{2+}-dependent regulation (Dunlap, 2007), often manifest as Ca\textsuperscript{2+} current inactivation (Eckert and Chad, 1984) (Ca\textsuperscript{2+}-dependent inactivation, CDI). A single Ca\textsuperscript{2+}-free CaM (apoCaM) preassociates with the IQ and other CI elements in the channel carboxy terminus (Erickson et al., 2001; Mori et al., 2004; Pitt et al., 2001). This arrangement renders CaM as a resident Ca\textsuperscript{2+} sensor poised for modulation. Subsequent Ca\textsuperscript{2+} binding to this CaM triggers CI rearrangements that inhibit channel opening (Ben Johny et al., 2013). Intriguingly, regulation can be induced by Ca\textsuperscript{2+} binding to one lobe of CaM or the other (DeMaria et al., 2001; Peterson et al., 1999; Yang et al., 2006), substantiating a functional bipartition of CaM discovered in Paramecium (Preston et al., 1991). For illustration, Figures 1B and 1C display the CDI of Ca\textsubscript{v}1.3 channels. Ca\textsuperscript{2+} channels themselves convey the Ca\textsuperscript{2+} that induces CDI (Figure 1B), and fluxing Ba\textsuperscript{2+} serves as negative control (Ba\textsuperscript{2+} binds CaM poorly [Chao et al., 1984]). Accordingly, Ca\textsuperscript{2+} currents decline sharply via CDI (Figure 1C, left, red trace), but not Ba\textsuperscript{2+} currents (black trace). The steady-state extent of CDI (\textit{f} \textsubscript{CDI}) thereby exhibits a hallmark U-shaped voltage dependence (Figure 1C, right, red) (Eckert and Chad, 1984). Such regulation influences excitability of heart (Alaseikhan et al., 2002), rhythmicity and neurotransmission in brain (Borst and Sakmann, 1998; Huang et al., 2012), and many other processes (Adams and Snutch, 2007; Crotti et al., 2013).

By contrast, for Na channels (Deschênes et al., 2002; Tan et al., 2002), the existence, functional nature, and postulated mechanisms of Ca\textsuperscript{2+} modulation have eluded consensus. Some find that muscle Na channels are not Ca\textsuperscript{2+} regulated (Herzog et al., 2003); others describe subtle Ca\textsuperscript{2+} modulation of inactivation (Van Petegem et al., 2012). Where Ca\textsuperscript{2+} effects have been observed, the proposed identity of the Ca\textsuperscript{2+} sensor for regulation varies. Unlike Ca\textsuperscript{2+} channels, some propose that Ca\textsuperscript{2+} binding to the first vestigial EF hand in Na channels induces Ca\textsuperscript{2+} regulation (Biswas et al., 2009; Tan et al., 2002; Wingo et al., 2004), but this view has not been universally accepted (Kim et al., 2004b; Miloushev et al., 2009). Instead, others emphasize Ca\textsuperscript{2+} binding to CaM as the trigger (Kim et al., 2004a; Sarhan et al., 2012; Shah et al., 2006), which is consistent with CaM binding to peptide fragments of channels (Feldkamp et al., 2011; Mori et al., 2000; Wang et al., 2012). Also contrasting with Ca\textsuperscript{2+} channels, a key structural determinant of Na channel regulation has
been suggested to reside outside the carboxy tail, in the III-IV loop (Sarhan et al., 2012). Others, however, emphasize a dominant role for the carboxy terminus (Biswas et al., 2009; Shah et al., 2006; Tan et al., 2002; Wingo et al., 2004). Lastly, when observed, Ca2+ regulation of Na channels has only been demonstrated in heterologously expressed recombinant channels, and no modulation has been directly demonstrated in native cells (Deschênes et al., 2002; Feldkamp et al., 2011; Mori et al., 2000; Sarhan et al., 2012; Wang et al., 2012). Compared to Ca2+ channels, this generally inconsistent Na channel landscape (Biswas et al., 2008, 2009; Potet et al., 2009; Sarhan et al., 2012; Tan et al., 2002; Van Petegem et al., 2012; Wingo et al., 2004) suggests divergence, weakening, or even loss of CI regulatory function (Van Petegem et al., 2012).

Here, we introduce two rapid Ca2+ delivery approaches to the Na channel field—Ca2+ photouncaging or Ca2+ influx through

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**Figure 1. Homology but Divergent Function for Ca2+ versus Na Channels**

(A) CI region of Ca2+ (CaV1.3) and Na channels (NaV1.5 and NaV1.4). Dual vestigial EF hands shaded in rose and green. IQ domain, blue.

(B) Ca2+ channel regulation inducible by channel Ca2+ influx. Ba2+ influx as negative control.

(C) Left, CaV1.3 current traces carried by Ca2+ (red) or Ba2+ (black). Vertical bar for Ca2+ trace. Ba2+ trace scaled ~3x downward for kinetic comparison. Right, r300 (fraction of peak current remaining after 300 ms depolarization) versus Vtest potential, plotted as mean ± SEM (eight cells).

(D) Na channels characterized under pipet dialysis with 0 or 10 μM Ca2+.

(E) Schematic of reported Ca2+ effects on inactivation. Left, h∞, fractional current remaining after prepulses (Vhold). Right, purported Ca2+-induced voltage shifts of h∞.

(F) NaV1.5 currents under protocol in (E) (black, 0 Ca2+; red, 10 μM Ca2+ buffered with HEDTA). See Figure S1.

(G and H) Normalized form of h∞ unaffected by Ca2+. Potential Ca2+-induced reduction in NaV1.4 h∞ (rose dashed line). Error bars, SEM throughout. Fit function: h∞ = 1/(1 + exp((Vhold − V1/2)/SF)), where SF = 6.2 (NaV1.4) and 7.5 (NaV1.5).
neighboring Ca$^{2+}$ channels. Results obtained through these methodologies suggest significant revisions to the current view of Ca$^{2+}$ regulation of Na channels. In particular, the bulk of current mechanistic inferences has been drawn from extensive studies of cardiac Na channels (Na$_V$1.5). Yet, under the rapid Ca$^{2+}$ delivery paradigms used here, we fail to detect Ca$^{2+}$ modulation of either heterologously expressed recombinant Na$_V$1.5 channels, or corresponding native Na currents in cardiac myocytes. By contrast, for prevalent skeletal-muscle Na channels (Na$_V$1.4), also reputed to host rather subtle Ca$^{2+}$ effects, we now observe fast and robust Ca$^{2+}$ regulation that strongly resembles the regulation of Ca$^{2+}$ channels. Indeed, transplanting the Na$_V$1.4 carboxy tail onto Ca$^{2+}$ channels recapitulates Ca$^{2+}$ regulation, further establishing this domain as a conserved modular element across channel superfamilies. Biologically speaking, channelopathies for cold- and potassium-aggravated myotonias suppress Na$_V$1.4 Ca$^{2+}$ regulation by 2-fold, and rapid Ca$^{2+}$ delivery methods resolve Ca$^{2+}$ regulation of native Na currents within skeletal myotubes. Thus, the carboxy tail of Na channels presents as a potential molecular therapeutic target for these myotonias and related disease. Altogether, this study highlights the uncommonality of CaM-dependent Ca$^{2+}$ regulation between Na and Ca$^{2+}$ channel superfamilies.

RESULTS

Na Channels Lack Apparent Ca$^{2+}$ Regulation

We initially used current experimental approaches to re-examine Ca$^{2+}$ regulation of the best-studied Na channels—Na$_V$1.5 that prevails in heart and Na$_V$1.4 from skeletal muscle. As a prelude, we carefully considered the chief experimental result from which most conclusions have been drawn—that Ca$^{2+}$ regulation of these channels induces modest shifts in the steady-state properties of a traditional rapid inactivation process (Biswa et al., 2008; Deschênes et al., 2002; Potet et al., 2009; Sarhan et al., 2012; Van Petegem et al., 2012; Wingo et al., 2004). The core paradigm has been to measure the fraction of current (h$_{I_Na}$) remaining at a fixed test voltage ($V_{test}$), following long depolarization to a family of prepulse voltages ($V_{hold}$; Figure 1E, left subpanel). Plotting h$_{I_Na}$ versus $V_{hold}$ then yields the steady-state inactivation relation ($h_{I_Na}$ curve) as schematically diagrammed by the black curve in Figure 1E (right subpanel). Because Na channels do not flux Ca$^{2+}$, testing for Ca$^{2+}$ regulation requires comparison of normalized h$_{I_Na}$ curves measured in cells statically diazylated with a pipet solution containing ∼0 μM free Ca$^{2+}$ concentration ([Ca$^{2+}$]), with those measured in other cells set to ∼10 μM (Figure 1D). Ca$^{2+}$ elevation reportedly shifts h$_{I_Na}$ curves by up to ∼10 mV, rightward in the case of Na$_V$1.5 (Biswa et al., 2009; Potet et al., 2009; Sarhan et al., 2012; Shah et al., 2006; Wingo et al., 2004), and leftward for Na$_V$1.4 (Biswa et al., 2008; Deschênes et al., 2002). These Ca$^{2+}$ effects are cartooned by the red dashed curves in Figure 1E (right subpanel).

Thus appraised, we noted that prior studies used EGTA or BAPTA as Ca$^{2+}$ buffers to nominally set intracellular [Ca$^{2+}$] between 1 and 10 μM (Biswa et al., 2009; Potet et al., 2009; Sarhan et al., 2012; Shah et al., 2006; Tan et al., 2002; Wingo et al., 2004), a range far above their dissociation constants ($K_d$ = 67 nM for EGTA, and $K_d$ = 192 nM for BAPTA [Bers et al., 2010]). This regime could be problematic for controlling Ca$^{2+}$ (Figure S1A available online). We therefore revisited these experiments using the more appropriate Ca$^{2+}$ buffer HEDTA ($K_d$ = 4 μM [Bers et al., 2010]), thus ensuring [Ca$^{2+}$] ∼10 μM. Figures 1F and 1G show exemplar Na currents and population data specifying actual h$_{I_Na}$ curves for Na$_V$1.5 channels, expressed heterologously in HEK293 cells. Surprisingly, no difference is present in the curve measured with [Ca$^{2+}$] ∼0 μM ($V_{1/2}$ = −72.3 ± 3 mV) versus that with [Ca$^{2+}$] ∼10 μM ($V_{1/2}$ = −75.5 ± 1.2 mV). Figure 1H also demonstrates no Ca$^{2+}$ effects for Na$_V$1.4 channels ($V_{1/2}$ = −62 ± 1.8 mV at [Ca$^{2+}$] ∼0 μM; $V_{1/2}$ = −60.8 ± 0.8 mV at [Ca$^{2+}$] ∼10 μM; Figure S1B).

This unexpected lack of Ca$^{2+}$ regulation intensified the seeming deviation of function in Na versus Ca$^{2+}$ channels. Still, closer inspection revealed that Ca$^{2+}$ elevation in Na$_V$1.4 channels appeared to diminish test-pulse current density corresponding to the plateau of h$_{I_Na}$ curves at −120 mV, from −318 ± 98 pA/pF (n = 11) to −189 ± 33 pA/pF (n = 11). Thus, Ca$^{2+}$ might scale down an unnormalized h$_{I_Na}$ curve (Figure 1H, red dashed curve). Such a trend was found for Na$_V$1.5 (−474 ± 98 pA/pF at [Ca$^{2+}$] ∼0 μM; n = 6) versus −424 ± 60 pA/pF at [Ca$^{2+}$] ∼10 μM (n = 11).

Rapid Uncaging of Ca$^{2+}$ Unveils Ca$^{2+}$ Effects on Na Channels

A core limitation of delivering Ca$^{2+}$ via pipet dialysis regards the uncertainty of detecting Ca$^{2+}$-induced changes in current amplitude without corresponding voltage-dependent shifts. Current size may differ in one group of cells versus another for many reasons unrelated to Ca$^{2+}$. To obviate this limitation, we utilized rapid photouncaging of Ca$^{2+}$ to produce step-like increases in intracellular [Ca$^{2+}$], whose magnitude was simultaneously measured via Ca$^{2+}$ fluorescent indicators (Tadross et al., 2013). Figure 2A displays the outcome for Na$_V$1.5 channels. Na currents (i$_{I_Na}$) were evoked every 100 ms by the voltage-pulse train above. Without Ca$^{2+}$ uncaging, peak currents remained steady (gray dots), confirming stability of the preparation. UV uncaging of a large Ca$^{2+}$ step to ∼10 μM (vertical cyan line) failed to perturb subsequent Na currents comprising the black i$_{I_Na}$ trace. On average, plots of steady-state current inhibition (CDI) versus Ca$^{2+}$ step amplitude (bottom subpanel) corroborate the lack of Ca$^{2+}$ regulation of Na$_V$1.5 in our experiments. Detailed kinetic analysis of Na currents within each pulse also showed no change on Ca$^{2+}$ elevation (Figure S2A).

On the other hand, Na$_V$1.4 channels demonstrated a different outcome (Figure 2B). As baseline, peak currents remained steady without Ca$^{2+}$ uncaging (gray dots). Here, however, Ca$^{2+}$ uncaging to ∼2 μM rapidly inhibited peak currents during the pulse train (black i$_{I_Na}$ trace), with an inhibitory time course of ∼100 ms (rose curve). Data averaged from many cells indicated a robust maximal CDI reaching −0.35, with a half-maximal effect achieved at $K_{1/2}$ ∼1.5 μM. The overall CDI ([Ca$^{2+}$]) relation defines a Hill function with steepness coefficient ∼1.8 (black curve, bottom subpanel). This inhibition of Na current upon Ca$^{2+}$ uncaging unveils a CDI whose time course resembles that of Ca$^{2+}$ channels (compare rose curve in Figure 2B with Figure 1C). As expected of a mainly Ca$^{2+}$-dependent process, this CDI was
Insensitive to pulse rate and voltage, whereas onset kinetics were influenced by Ca\textsuperscript{2+} concentration (Figures S2B–S2D, S3, and S4).

To reconcile these effects on Na\textsubscript{v}1.4 with those observed under static Ca\textsuperscript{2+} buffering (Figures 1H and S1B), we evoked Na currents under a modified voltage-pulse protocol that measures \( h_\infty \) curves just before and after Ca\textsuperscript{2+} uncaging. Prior to Ca\textsuperscript{2+} uncaging, peak currents evoked after various holding potentials demonstrated the usual changes affiliated with steady-state inactivation (Figure 2C, black \( h_\text{Na} \) trace). Normalizing these currents by that of the first pulse yielded a baseline \( h_\text{Na} \) curve (Figure 2C, bottom subpanel), here averaged over multiple cells. Figure 2D shows the effect of Ca\textsuperscript{2+} uncaging in same cell. The initial current, obtained just prior to Ca\textsuperscript{2+} uncaging, exhibits the identical amplitude as its analog in Figure 2C, confirming minimal rundown. By contrast, after Ca\textsuperscript{2+} uncaging, the resulting currents (Figure 2D, black \( I_\text{Na} \) trace after UV flash) were uniformly suppressed compared to Figure 2C. Normalizing these responses (after Ca\textsuperscript{2+} uncaging) by that of the first pulse (just before uncaging) yields the Ca\textsuperscript{2+}-regulated \( h_\infty \) curve shown below (Figure 2D, bottom subpanel, red data and fit), as averaged over multiple cells. For reference, the fit to the \( h_\infty \) curve obtained before Ca\textsuperscript{2+} uncaging is reproduced in black in Figure 2D. Importantly, by scaling up the fit to the \( h_\infty \) curve following Ca\textsuperscript{2+} uncaging, we obtain a rose-colored curve that precisely overlays the control relation in black. Hence, Ca\textsuperscript{2+} elevation would have the apparent effect of scaling down the \( h_\infty \) curve without shift along the voltage axis, just as seen in Figure 1H. The actual CDI effect reflects decreased channel open probability, separate from fast inactivation, as shown in the next section.

### Na Channel Regulation by Ca\textsuperscript{2+} Fluxing through Neighboring Ca\textsuperscript{2+} Channels

We next induced Ca\textsuperscript{2+} regulation of Na channels by more physiological means, so as to exclude unsuspected photouncaging effects that might artifactually produce the results in Figure 2 and to permit observations at the level of single Na channel molecules (impractical in the electrical environment of photouncaging equipment).

Accordingly, Na\textsubscript{v}1.4 and Ca\textsubscript{v}2.1 Ca\textsuperscript{2+} channels were coexpressed within the same cells to test whether Ca\textsuperscript{2+} spillover from a Ca\textsuperscript{2+} channel source could inhibit nearby Na channels (Figure 3A). Owing to the higher threshold of voltage activation for Ca\textsubscript{v}2.1 versus Na\textsubscript{v}1.4, Na current alone could be evoked by modest depolarizations to 0 mV (Figures S5A–S5C), which bookend the voltage pulse protocol shown in Figure 3B. Na currents (\( I_\text{Na} \)) evoked in this manner have the same magnitude. By contrast, insertion of an intervening 30 mV pulse activates Ca\textsuperscript{2+} currents, as shown by the red shading in Figure 3C. Importantly, the second Na response is then substantially diminished, as if Ca\textsuperscript{2+} influx through adjacent Ca\textsuperscript{2+} channels triggered Na channel CDI. To exclude voltage-dependent inhibition as the cause of a diminished second response, the intervening voltage pulse was further increased to the Ca\textsuperscript{2+} channel reversal potential (−90 mV), where negligible Ca\textsuperscript{2+} entry would occur. Reassuringly, the second Na response appeared identical to the first (Figure 3D), arguing that the reduction of Na current above (Figure 3C) was due to Ca\textsuperscript{2+} influx and not voltage itself.

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**Figure 2. CDI of Na Channels under Ca\textsuperscript{2+} Photouncaging**

(A) Na\textsubscript{v}1.5 currents unaffected by 10 \( \mu \text{M} \) Ca\textsuperscript{2+}. Gray dots, peak currents before uncaging. Bottom, mean data for CDI versus Ca\textsuperscript{2+}-step amplitude. CDI = \( 1 - \text{average peak } h_\text{Na} \text{ of last three to four responses after } \text{Ca}^{2+} \text{ uncaging/peak } h_\text{Na} \text{ before uncaging.} \) Symbols, mean ± SEM of 1–5 uncaging events compiled from 23 cells. See Figure S2.

(B) Na\textsubscript{v}1.4 peak currents decline during 2 \( \mu \text{M} \) Ca\textsuperscript{2+} step (rose fit). Format as in (A). Bottom, mean CDI plotted versus Ca\textsuperscript{2+}-step amplitude. Each symbol, mean ± SEM of 1–5 uncaging events compiled from 35 cells. See Figures S2, S3, and S4.

(C) Na\textsubscript{v}1.4 currents specifying \( h_\infty \) at 100 nM Ca\textsuperscript{2+}. Bottom, \( h_\infty \) curve (mean ± SEM, five cells).

(D) Approximately 3 \( \mu \text{M} \) Ca\textsuperscript{2+} step uniformly suppresses Na currents. Bottom, corresponding mean \( h_\infty \) curve (red symbols and fit), where symbols plot mean ± SEM (five cells). Upwardly scaled \( h_\infty \) curve (rose) same as before uncaging (black).
Analyzing averaged data for the fraction of current remaining in second versus first Na responses ($r_{Na}$ in Figure 3C) confirms a U-shaped dependence of CDI on intervening pulse potential (Figure 3G, red). Additionally, we examined the effects of substituting Ba$^{2+}$ for Ca$^{2+}$ as charge carrier through Ca$^{2+}$ channels. Because Ba$^{2+}$ binds poorly to CaM (Chao et al., 1984), we expected Na channel CDI to disappear (Figure 3A, right subpanel), as confirmed in Figures 3E–3G (black). As a further test, adding 10 mM BAPTA to the dialyzate eliminated NaV1.4 CDI (Figures 3H–3K and S5D–S5F), demonstrating that Ca$^{2+}$ channel spillover drove the Na channel regulation. Finally, as expected, like experiments with NaV1.5 revealed no CDI (Figures S5G–S5J).

Importantly, this strategy of coexpressing Na and Ca$^{2+}$ channels could be extended from cells to isolated patches of membrane, permitting observations of regulation at the level of individual Na channels, something never before attempted. Figure 4A shows the activity of a patch containing several NaV1.4 channels coexpressed with hundreds of CaV2.1 Ca$^{2+}$ channels. A multichannel stochastic record is shown at the top (multichannel record), along with the voltage-pulse protocol. Only Na channels were activated during test-pulse depolarizations to $-30$ mV at the left (labeled i) and right (labeled ii) ends of the record; Ca$^{2+}$ channels were activated only during the interpulse to a more positive voltage of $30$ mV (shaded in red). The ensemble average of many such records is shown below. Thus oriented, one can clearly appreciate that Na channel activity evoked after intense interpulse Ca$^{2+}$ entry was substantially decreased (pulse ii), compared to the activity before the interpulse (pulse i). Data from a separate patch containing only NaV1.4 channels without CaV2.1 channels demonstrate no such difference between first and second test pulses (Figure 4D).

To distinguish the elementary mechanism of inhibition, we analyzed the unitary current $i$ approximated by the horizontal dashed line on the multichannel record (Figure 4A, labeled $i$ $-1$ pA). This unitary current was not visibly changed in the second test pulse compared to the first, suggesting that single-channel conductance was unaffected by CDI. This outcome is

![Figure 3. Na Channel Regulated by Ca$^{2+}$ Spillover from Ca$^{2+}$ Channels](image-url)
explicitly confirmed in Figure 4B by amplitude histogram analysis, where the smooth curve fit to data (in black) is generated by stochastic simulation of multichannel activity added to Gaussian noise, followed by low-pass filtering present in our system (Prod’hom et al., 1987). Using this method, essentially the same underlying value of $i$ was estimated before and after CDI (vertical red dashed lines). Accordingly, because ensemble average current $I = N P_O i$, and the number of channels $N$ must be the same in first and second test pulses (separated by only hundreds of milliseconds), CDI must occur by decreased open probability $P_O$, just as in Ca$^{2+}$ channel CDI (Imredy and Yue, 1994). To exclude appreciable interaction of the CDI-mediated decrease in open probability with fast inactivation, we confirmed that the time constant of inactivation was not detectably changed by CDI (Figure 4C), echoing whole-cell results in Figure S2B. Thus, CDI and fast inactivation are largely parallel processes.

In all, we emphasize that the whole-cell functional profile in Figure 3G (both Ca$^{2+}$ and Ba$^{2+}$ relations) resembles that for Ca$^{2+}$ channels (Figure 1C) and recapitulates the classic engram of native Ca$^{2+}$ regulation of Ca$^{2+}$ channels historically established by Eckert and colleagues (Eckert and Chad, 1984). Additionally, the single-channel behavior in Figure 3A closely mirrors that observed for native single Ca$^{2+}$ channels (Imredy and Yue, 1994). Therefore, from the functional standpoint, the Ca$^{2+}$ regulation of NaV1.4 channels notably resembles that of Ca$^{2+}$ channels.

**N-Terminal Lobe of CaM as Ca$^{2+}$ Sensor**

With robust functional resolution of Na channel Ca$^{2+}$ regulation in hand, we could appropriately seek after its mechanistic underpinnings, searching first for the Ca$^{2+}$ sensor of NaV1.4 modulation. Prior work has argued that the first vestigial EF hand in the CI region binds Ca$^{2+}$ and triggers modulation (Biswas et al., 2009; Shah et al., 2006; Tan et al., 2002; Wingo et al., 2004). We therefore introduced alanines at two potential Ca$^{2+}$-coordinating residues in the first EF hand of NaV1.4 channels (Figure 1A, “putative Ca binding loops”). If this EF hand were to bind Ca$^{2+}$, introducing these alanines would reduce Ca$^{2+}$ affinity by 10- to 1,000-fold (Linse and Forsén, 1995). However, this mutant channel still exhibited rapid and strong CDI, indistinguishable from wild-type (Figure 5A), as shown by overlaying the wild-type profile (gray) on the CDI –[Ca$^{2+}$] relation. Other mutations historically proposed to disrupt potential Ca$^{2+}$ binding to this EF hand also spared CDI (Figures S6A–S6C). Thus, this EF hand motif is not the Ca$^{2+}$ sensor for CDI, paralleling outcomes in Ca$^{2+}$ channels (Peterson et al., 2000; Zhou et al., 1997). Ca$^{2+}$ binding to a resident CaM serves as the primary Ca$^{2+}$-sensing event in the regulation of Ca$^{2+}$ channels (Peterson et al., 1999; Zühlke et al., 1999). Likewise, some studies of Na channels have argued that CaM may be at least one of the pertinent Ca$^{2+}$ sensors, based on biochemical and structural
throughout the Ca2+ channel superfamily (DeMaria et al., 2001; Linse and Forssén, 1995). We observed in critical N lobe fit well with the Hill steepness coefficient of 1.8 (Linse and Forssén, 1995). A recent crystal structure of Ca2+/CaM complexed with Ca2+ channels (Figures S6D and S6E). Additionally, the strong actions of CaM1234 permitted higher-order tests whether one lobe of CaM or the other suffices to trigger Na channel regulation. Such single-lobe signaling would add to the ranks of a functional bipartition paradigm (Preston et al., 1991), richly observed throughout the Ca2+ channel superfamily (DeMaria et al., 2001; Liang et al., 2003; Peterson et al., 1999; Yang et al., 2006). In this regard, we utilized a mutant CaM12 construct, featuring selective inhibition of Ca2+ binding to the N, but not C, lobe. Coexpressing NaV1.4 channels with CaM12 also fully abolished Ca2+ regulation (Figure 5C), arguing that Ca2+ binding to the N lobe is necessary for CDI. Alternatively, coexpressing NaV1.4 channels with CaM34 (selective inhibition of Ca2+ binding to C lobe) entirely preserved CDI (Figure 5D), with a profile nearly indistinguishable from control (reproduced as gray). Thus, Ca2+ binding to the N lobe of CaM is both necessary and sufficient to trigger Na channel CDI. Indeed, the two Ca2+-binding sites within the critical N lobe fit well with the Hill steepness coefficient of 1.8 observed in CDI –[Ca2+] relations (Linse and Forssén, 1995). We note that our result contrasts with a prior proposal that Ca2+ binding to C lobe triggers regulation of Na channels (Sarhan et al., 2012; Van Petegem et al., 2012). As a final check for the predominance of CaM as Ca2+ sensor, we demonstrated that CaM kinase II inhibition had no effect on Na channel CDI (Figures S6F and S6G).

Structural Determinants of Na Channel Ca2+ Regulation

The CI region of Ca2+-channels (Figure 1A) suffices to confer Ca2+ regulation (de Leon et al., 1995). Yet, prior work in Na channels emphasizes the necessary role of the III-IV loop, a critical determinant for fast inactivation of these channels (Stühmer et al., 1989). A recent crystal structure of Ca2+/CaM complexed with the III-IV loop of NaV1.5 channels further suggests that a conserved tyrosine anchor is necessary for Ca2+/CaM binding and that this binding is required for Ca2+ modulation (Sarhan et al., 2012; Van Petegem et al., 2012). Figure 5G contextualizes the location of this anchor in relation to other Na channel landmarks.

Accordingly, we substituted alanine for the homologous tyrosine in NaV1.4 channels (Y[1311]A) and tested for Ca2+ regulation. In contrast to prior work, we observed that Ca2+ regulation was fully present (Figure 5E), with a functional profile indistinguishable from that of wild-type channels. Indeed, disruption of a similar Ca2+/CaM binding site in the III-IV loop of Ca2+ channels also failed to disrupt CDI (Figures S7B and S7C). Thus, both Na and Ca2+ channels do not require Ca2+/CaM binding to the III-IV loop for Ca2+ regulation.

To explore the role of the Na channel CI region in supporting Ca2+ regulation, we undertook a convenient chimeric-channel approach, exploiting the lack of Ca2+ regulation in NaV1.4 versus NaV1.5. When the carboxy terminus of NaV1.4 was substituted with its NaV1.5 counterpart, Ca2+ regulation was completely eliminated (Figure 5F). Yet more telling were the effects of limited mutations within the IQ element (Figure 1A, blue shaded zone), which potently alter Ca2+ regulation of Ca2+ channels (Bazzazi et al., 2013; Ben Johny et al., 2013; DeMaria et al., 2001; Liang et al., 2003; Yang et al., 2006; Zühlke et al., 1999). When dual alanines were substituted for contiguous isoleucine and glutamine residues in the center of the NaV1.4 IQ element, the Ca2+-dependent inhibition of these channels was not merely eliminated but converted into outright facilitation (CDF, Figure 5G). This effect is eerily similar to that observed upon analogous mutagenesis of certain L-type Ca2+ channels (Zühlke et al., 1999, 2000). Finally, fitting with the preeminence of the CI region, binding of Ca2+-free CaM (apoCaM) to the carboxy tail of NaV1.4 channels has been confirmed (Ben Johny et al., 2012).

In all, like Ca2+ channels, the carboxy tail of Na channels contains the needed structural determinants for CDI, even regarding inversion of regulatory polarity by like mutations therein.

Ca2+ Regulation of Native Na Channels

Encouraged by the recombinant channel findings thus far, we tested for Ca2+ regulation in their native counterparts. Though recombinant NaV1.5 channels were not Ca2+ regulated, cardiac myocytes might furnish added critical auxiliary factors. Thus, we performed Ca2+ uncaging in adult guinea pig ventricular myocytes, where NaV1.5 channels convey the bulk of native Na current. Even here, however, no Ca2+ regulation of Na current was observed (Figure 6A).

By contrast, when testing for Ca2+ regulation of native NaV1.4 channels in skeletal myotubes derived from mouse GLT cells, we observed robust Ca2+ regulation of Na current (Figure 6B), with Ca2+ sensitivity appropriate for physiological Ca2+ transients (Wagner and Maier, 2006). This result may be the first direct demonstration of Ca2+ regulation of endogenous Na currents.

Of further biological concern, channelopathetic mutations occur in the carboxy terminus of Na channels, but the alterations in channel function that underlie pathogenesis have not been fully resolved. Might these mutations affect the Ca2+ regulation in NaV1.4 channels? Figures 6C and 6D investigate this possibility for channelopathetic mutations associated with K- and cold-aggravated myotonias (Kubota et al., 2009; Wu et al., 2005). In both instances, Ca2+ regulation is substantially diminished (but see Biswas et al., 2013), whereas the kinetics of currents remain unchanged by Ca2+ elevation (Figures S7D and S7E). These results offer previously unrecognized dimensions by which Na channel function may influence disease development.
Figure 5. Calmodulin as Ca\(^{2+}\) Sensor for Na\(_{\text{v}}\)1.4 CDI
(A) Mutating putative Ca\(^{2+}\)-coordinating residues in Na\(_{\text{v}}\)1.4 EF hand did not alter CDI. Format as in Figure 2B. Symbols, mean ± SEM of ~3 uncaging events from 12 cells.
(B) CaM\(_{1234}\) abolishes CDI. Symbols, mean ± SEM (~6 uncaging events from 27 cells).

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DISCUSSION

By applying rapid Ca\(^{2+}\) delivery to Na channels (photouncaging of Ca\(^{2+}\) and Ca\(^{2+}\) spillover from neighboring Ca\(^{2+}\) channels), this study significantly refines our understanding of Na channel regulation by Ca\(^{2+}\). First, most prior mechanistic deductions are based on observations on recombinant cardiac Na channels (Na\(_{V1.5}\)), and these deductions have suggested that Ca\(^{2+}\) regulation of Na channels differs at the core from that in Ca\(^{2+}\) channels (Van Petegem et al., 2012). However, by using rapid Ca\(^{2+}\) delivery, our experiments detect no Ca\(^{2+}\) modulation of either recombinant Na\(_{V1.5}\) channels or their native counterparts in cardiac myocytes. This outcome raises questions about prior structure-function deductions (Biswas et al., 2009; Potet et al., 2009; Sarhan et al., 2012; Shah et al., 2006; Tan et al., 2002; Wingo et al., 2004) and may spur revision to the present understanding of Ca\(^{2+}\) regulation in Na channels. Second, more important results concern skeletal-muscle Na\(_{V1.4}\) channels, reputed for only modest Ca\(^{2+}\) regulation via mechanisms that diverge significantly from Ca\(^{2+}\) channels. Here, rapid Ca\(^{2+}\) delivery instead unveils conspicuous Ca\(^{2+}\) regulation of Na\(_{V1.4}\) channels. In like manner, the methods are now at hand to explore potential Ca\(^{2+}\) regulation of the many other Na channel isoforms (Na\(_{V1.1}\)–Na\(_{V1.9}\)). Third, we argue for the persistence of a common Ca\(^{2+}\) regulatory module across Ca\(^{2+}\) and Na channels. In particular, the function and mechanism of Ca\(^{2+}\) regulation of Na\(_{V1.4}\) channels bear remarkable similarity to that of Ca\(^{2+}\) channels. This long-sought commonality suggests that kindred carboxy-tail Ca\(^{2+}\) regulatory modules persist across Ca\(^{2+}\) and Na channels, affording common principles for understanding. Indeed, this persistence can be shown as a latent capability within cardiac Na\(_{V1.5}\) channels by substituting the Na\(_{V1.4}\) carboxy tail onto the Na\(_{V1.5}\) backbone (Figure 7A). This maneuver confers Ca\(^{2+}\) regulation to the resulting chimeric channels, with Ca\(^{2+}\) sensitivity akin to that of Na\(_{V1.4}\) (gray trace). Of greater generality, carboxy-tail transplantation between Ca\(^{2+}\) and Na channels (shown below) fully establishes the carboxy-tail domain as a legitimate module across superfamilies. Finally, channelopathic

(C) Eliminating N-lobe Ca\(^{2+}\) binding (CaM\(_{N}\)) abolishes CDI. Symbols, mean ± SEM of 4 to 5 uncaging events from 12 cells.
(D) Eliminating C-lobe Ca\(^{2+}\) binding (CaM\(_{C}\)) spares CDI. Symbols, mean ± SEM of ~5 uncaging events from 12 cells.
(E) Mutating Na\(_{V1.4}\) III-IV loop spares CDI. Format as in Figure 2B. Symbols, mean ± SEM of 4 to 5 uncaging events from 18 cells.
(F) No CDI in Na\(_{V1.4}\)-1.5CT chimeras. Symbols, mean ± SEM of 4 to 5 uncaging events (13 cells).
(G) Substituting dual alanines for key isoleucine-glutamine residues in Na\(_{V1.4}\) IQ domain yields facilitating Na currents. Bottom, mean data confirm facilitation, shown as negative CDI. Symbols, mean ± SEM of ~13 uncaging events (20 cells).
Figure 7. Persistence of CaM/Cl Module across Na and Ca^{2+} Channel Superfamilies

(A) Transferring Na\textsubscript{V}1.4 carboxy tail to Na\textsubscript{V}1.5 backbone (Na\textsubscript{V}1.5-1.4CT) confers latent Ca^{2+} regulation (wild-type Na\textsubscript{V}1.4, gray fit in bottom subpanel). Format as in Figure 2A. Symbols, mean ± SEM from four to five uncaging events (ten cells).

(B) Phylogenetic tree of the Na and Ca^{2+} channel superfamilies.

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mutations for cold- and K-aggravated myotoniae halve the Ca\textsuperscript{2+} regulation of Na\textsubscript{v}1.4, and Ca\textsuperscript{2+} regulation of native skeletal Na currents is observed. The carboxy tail of Na channels now looms as a molecular target for these myotoniae and related diseases.

**Prior Na Channel Studies of Ca\textsuperscript{2+} Regulation**

Before turning to the implications of newly observed forms of Ca\textsuperscript{2+} regulation, we consider potential explanations for prior observations of Ca\textsuperscript{2+}-dependent shifts in steady-state inactivation curves (i.e., $h_{\infty}$ curves in Figure 1E). Most mechanistic postulates regarding Ca\textsuperscript{2+} regulation of Na channels are based on such shifts (summarized in the Introduction). One can note that the Ca\textsuperscript{2+} chelators EGTA and BAPTA were used to nominally buffer free Ca\textsuperscript{2+} concentrations at levels substantially higher than the dissociation constants for these compounds. If Ca\textsuperscript{2+} concentrations were to far exceed 10 $\mu$M, it would be difficult to undertake whole-cell patch clamp. It is then understandable that most have resorted to intracellular solutions with CaF, which greatly facilitates recording but indiscriminately activates G protein signaling (Sternweis and Gilman, 1982), among numerous other effects (Van Petegem et al., 2012). Moreover, before measurements are taken, time-dependent voltage shifts in $h_{\infty}$ curves are typically allowed to equilibrate following the onset of whole-cell pipet dialysis (Biswas et al., 2009). Ambiguities about actual equilibration may then contribute to conflicts among prior reports. Importantly, the present study does document a lack of Ca\textsuperscript{2+}-dependent shift in $h_{\infty}$ curves using two approaches: static measurements that employ HEDTA to buffer Ca\textsuperscript{2+} at levels close to the corresponding dissociation constant and rapid photoun-caging of Ca\textsuperscript{2+} with concurrent readouts of Ca\textsuperscript{2+}. Thus, the difference in results about voltage shifts in $h_{\infty}$ relations merits ongoing attention in the field.

**Open Frontier for Other Na Channel Isoforms**

That said, it is clear that, by using Ca\textsuperscript{2+} photoun-caging or Ca\textsuperscript{2+} influx via neighboring Ca\textsuperscript{2+} channels, one can now resolve Ca\textsuperscript{2+} regulation of Na\textsubscript{v}1.4 channels that are more rapid, robust, and similar to Ca\textsuperscript{2+} channels than could be previously observed. Likewise, the means of Ca\textsuperscript{2+} delivery used here may facilitate characterization of other superfamily members (Figure 7B, Na\textsubscript{v}1.1–Na\textsubscript{v}1.9, except Na\textsubscript{v}1.5), all possessing high carboxy-tail homology. Many Ca\textsuperscript{2+} channels exhibit variant forms of CaM regulation, where the precise functional behavior can differ; for example, Ca\textsuperscript{2+} facilitates opening of Ca\textsubscript{v}2.1 channels (DeMaria et al., 2001; Lee et al., 1999) but produces CDI in Ca\textsubscript{v}1.3 (Figure 1C). Although we have here investigated the best-studied Na\textsubscript{v}1.4 and Na\textsubscript{v}1.5 channels, it will be interesting to explore other Na channels for various forms of Ca\textsuperscript{2+} regulation. In all, there is the potential for Ca\textsuperscript{2+} regulation across the Na channel superfamily by an array of Ca\textsuperscript{2+} sources like voltage-gated Ca\textsuperscript{2+} channels, ryanodine and IP3 channels, Ca\textsuperscript{2+}-permeable AMPA and NMDA receptors, and store-operated Ca\textsuperscript{2+} channels (Berridge, 2012).

**Synergistic Study of Na and Ca\textsuperscript{2+} Channels**

Given the parallels between Ca\textsuperscript{2+} regulation in Na and Ca\textsuperscript{2+} channels, we attempted a further, patently simple test for the persistence of a common CaM-CI regulatory element among Ca\textsuperscript{2+} and Na channels—the transplantation of the Ca\textsuperscript{2+}-inactivating (CI) module from one superfamily to another. On adjoining the core of a Ca\textsubscript{v}1.3 channel to the carboxy tail of Na\textsubscript{v}1.4 (Figure 7C), not only were sizeable currents expressed, but also the faster decay of Ca\textsuperscript{2+} versus Ba\textsuperscript{2+} currents (top), according to a classic profile ($I_{300}$ plot below), indicates Ca\textsuperscript{2+} regulation (CDI) in this chimera (cf., Figure 1C). Importantly, coexpressing mutant CaM\textsubscript{1234} abolishes this CDI (Figure 7D), just as in Na\textsubscript{v}1.4 (Figure 5B). Hence, the regulatory design of one super-family persists with sufficient congruity to functionally interface with the core of another. This modularity may rival that of voltage-paddle elements transferable from Na\textsubscript{v} to Kv channels (Bosmans et al., 2008).

Thus established, the commonality of the CaM-CI module promises insights from synergistic coinvestigation of Na and Ca\textsuperscript{2+} channels. Structural biological efforts with Na channels have arguably overtaken those with Ca\textsuperscript{2+} channels, given the atomic resolution of nearly intact Na channel CI domains complexed with CaM (Wang et al., 2012). Though results from the present study may spur reinterpretation of inferences drawn from these structures, Na channel structures like these can now be viewed as holding potentially adaptable lessons for Ca\textsuperscript{2+} channels. In particular, recent advances suggest that Ca\textsuperscript{2+} channel CDI arises from a tripartite complex of the channel EF hand segment, the IQ domain, and a single lobe of CaM (Ben Jonny et al., 2013). Emerging Na channel structures may comment on this proposal and whether this Ca\textsuperscript{2+} channel scheme extends in some form to Na channels.

**Antiquity of CaM-CI Module**

More general implications concern the antiquity of the CaM-CI Ca\textsuperscript{2+} regulatory module. Figure 7B depicts the phylogenetic tree of Na and Ca\textsuperscript{2+} channel superfamilies based on carboxy-tail sequences. The CI region is conserved across the top branches of this tree, conferring Ca\textsuperscript{2+} regulation to certain Na and Ca\textsuperscript{2+} channels (Ca\textsubscript{v}1 and Ca\textsubscript{v}2 branches). To further explore historical lineage, we undertake CI sequence alignment and phylogenetic analysis of Ca\textsuperscript{2+} and Na channels from multiple eu-karyotic phyla (Figure 7E), starting with Paramecium. This single-cell organism lacks voltage-gated Na channels but possesses a Ca\textsuperscript{2+} channel in which Ca\textsuperscript{2+} regulation was first discovered (Brehm and Eckert, 1978). From this start, the Ca\textsuperscript{2+} channel clade for more advanced organisms branches toward the top, and the Na channel clade branches toward the bottom. CI

(C) Transplanting Na\textsubscript{v}1.4 carboxy tail onto Ca\textsubscript{v}1.3 backbone (Ca\textsubscript{v}1.3-Na\textsubscript{v}1.4CT) yields chimeric channel that retains Ca\textsuperscript{2+} regulation. Format as in Figure 1C. Symbols, mean ± SEM, seven cells. CDI measured under low Ca\textsuperscript{2+} buffering (see Extended Experimental Procedures).
(D) Coexpressing CaM\textsubscript{1234} with Ca\textsubscript{v}1.3-Na\textsubscript{v}1.4CT abolishes CDI. Format as in Figure 1C. Symbols, mean ± SEM, seven cells. CDI measured as in (C).
(E) Maximum likelihood phylogenetic tree shows conservation among Ca\textsuperscript{2+} and Na channel CI regions, across major eu-karyotic phyla. Format as in Figure 1A. Consensus sequence patterns for motifs on top. Sequence alignment starts at the center with the Paramecium Ca\textsuperscript{2+} channel. Ca\textsuperscript{2+} channels from progressively more advanced organisms branch to the top (pale colors), and those for Na channels branch to the bottom (darker colors).
sequence similarity is conserved throughout. Given this common heritage dating to early eukaryotes (~1 billion years ago), we suggest a persistent link between modern CI elements of Ca2+ and Na channels to a primeval Ca2+ modulatory design.

**CaM-CI Elements as Potential Molecular Therapeutic Targets**

Finally, two results are notable from the disease perspective—channelopathies for mutations of Cys- and K-aggravated myotonia halve the Ca2+ regulation of NaV1.4 channels and the direct demonstration of such modulation in skeletal myotubes. Na channel CDI may thus play a physiological role in activity-dependent feedback control of skeletal-muscle excitability. This CDI might normally raise the threshold for muscle excitation during repetitive activity, protecting against overexcitability caused by rapidly elevating extracellular K levels during contraction (Clausen, 2011). Weakening of CDI by channelopathies may predispose for debilitating myotonia (Cannon, 1997). More broadly, CIs mediated by CaM-CI elements in Ca2+ channels control cardiac action potential duration (Alseikhan et al., 2002; Mahajan et al., 2008), whose dysregulation predisposes for long QT syndrome and life-threatening arrhythmias (Crotti et al., 2013; Limpitikul et al., 2014). Moreover, numerous channelopathies relate to mutations within the CI regions of Na and Ca2+ channels, and these conditions model diseases of more general prevalence (Adams and Snutch, 2007; Kubota et al., 2009; Zim-mer and Surber, 2008). As such, the CaM-CI elements of Na and Ca2+ channels now present as potential molecular therapeutic targets for certain myotonia, cardiac arrhythmias, and other diseases. A collective view of these conditions as perturbations of CaM-CI function may offer fresh insights into pathogenesis and unified screens for small-molecule therapies.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**

The rat NaV1.4 channel (Trimmer et al., 1990) was cloned in pcDNA3 (Invitrogen) via flanking EcoRI sites. The carboxy-tail sequence agrees with clone Y177153.1 (GenBank). Human NaV1.5 corresponds to clone M77235.1 (GenBank). The CaV1.3 construct AF370009 (GenBank), as described (Ben Johny et al., 2013). Construction of chimeras and mutants is detailed in the Supplemental Information.

**Whole-Cell Recording**

Whole-cell recordings were obtained at room temperature (~298 K) with an Axopatch 200A amplifier (Axon Instruments). Electodes were made of borosilicate glass (World Precision Instruments, MTW 150-F4), yielding pipets of 1–2 MΩ resistance, which was compensated by >70%. Pipets were fabricated with a horizontal micropipette puller (model P-97, Sutter Instruments) and fire polished with a microforge (Narishige). Data acquisition utilized an ITC-18 (Instrutech) data acquisition unit controlled by custom MATLAB software (Mathworks). Leak subtraction and four-pole Bessel filter and digitized at 200 kHz with an ITC-18 unit (Instrutech), controlled by custom MATLAB software (Mathworks). Leak subtraction and analysis were previously described (Imredy and Yue, 1992).

**Single-Channel Recording**

All multichannel records were obtained in the on-cell configuration with HEK293 cells (Figure 4). Data were acquired at room temperature using the integrating mode of an Axopatch 200A amplifier (Axon Instruments). Patch pipettes (~14 MΩ) were pulled from ultra-thick-walled borosilicate glass (BF200-116-10; Sutter Instruments) using horizontal puller (P-97, Sutter Instruments), fire polished with a microforge (Narishige), and coated with Sylgard (Dow Corning). Elementary currents were low-pass filtered at 5 kHz with a four-pole Bessel filter and digitized at 200 kHz with an ITC-18 unit (Instrutech), controlled by custom MATLAB software (Mathworks). Leak subtraction and analysis were previously described (Imredy and Yue, 1992).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.04.035.

**AUTHOR CONTRIBUTIONS**

M.B.-J. created mutant channels, performed electrophysiology, conducted flash photolysis experiments, and undertook extensive data analysis. P.S.Y. created mutant Ca2+ channels and performed experiments relating to the potential role of the III-IV loop in mediating Ca2+ channel CDI. M.B.-J. and D.T.Y. conceived the project, refined experimental design and hypotheses, and wrote the paper. J.N. and W.Y. established the GLT cell culture system. R.J.-M. generously provided high-quality adult guinea pig ventricular myocytes.

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Construction of Na\textsubscript{v} and Ca\textsubscript{v} Channel Mutants and Chimeras

To facilitate mutagenesis of Na\textsubscript{v}1.4 carboxy terminus, we first PCR amplified and subcloned an ~900 bp segment containing the CI region (bounded by KpnI and XbaI restriction sites) into zero blunt TOPO II (Invitrogen) vector, yielding a convenient TOPO-Na\textsubscript{v}1.4CT construct. Quikchange (Agilent) was then used to introduce channel EF-hand mutations [D\textsuperscript{1621}A & D\textsuperscript{1623}A in Figure 5A; and 4 x mutations in Figure S6B], alanine substitutions of isoleucine and glutamine residues of IQ domain (Figure 5G), and channelopathetic mutations [Q\textsuperscript{1626}E in Figure 6C; and F\textsuperscript{1698}I in Figure 6D] into the TOPO-Na\textsubscript{v}1.4CT construct. These mutant segments were then transferred to Na\textsubscript{v}1.4 pcDNA3 construct following restriction digest and ligation utilizing KpnI/XbaI sites.

To generate the Na\textsubscript{v}1.4-Na\textsubscript{v}1.5CT chimeric channel (Figure 5F), we first introduced a silent XhoI site immediately downstream of the channel domain IV S6 segment (5212-ctGgag-5217 to 5212-ctCgag-5217) using Quikchange (Agilent) on TOPO-Na\textsubscript{v}1.4CT construct followed by ligation into the full length Na\textsubscript{v}1.4 clone. Subsequently, the entire Na\textsubscript{v}1.5 carboxy-terminus starting at 1772-LENFSV-1778 was PCR amplified and cloned into Na\textsubscript{v}1.4 utilizing restriction sites Xhol and XbaI.

A similar strategy was followed for mutagenesis of the III-IV loop mutation (Y\textsubscript{1311}F, Figure 5E). An ~3300 bp segment bound by unique restriction sites BsiWI and KpnI including the III-IV loop was subcloned into zero blunt TOP II (Invitrogen) vector following PCR amplification. The Y\textsubscript{1311}F mutation was then introduced into this segment using Quikchange (Agilent), and subsequently transferred to the full length channel.

To construct Na\textsubscript{v}1.5-Na\textsubscript{v}1.4CT chimer (Figure 7A), we introduced a silent NruI site into Na\textsubscript{v}1.5 channel by mutagenesis of location 5329-gtggccagcg-5337 to 5329-gtGcgcGacg-5337. Subsequently, the entire Na\textsubscript{v}1.4CT was PCR amplified starting at residue location 1596-VAT-1599 and ligated into full length Na\textsubscript{v}1.5 exploiting the NruI/XbaI sites.

To construct the Ca\textsubscript{v}1.3-Na\textsubscript{v}1.4CT chimeric channel (Figure 7B), we first truncated the Ca\textsubscript{v}1.3 channel immediately upstream of the EF hand region (terminating with residues 1471-ILGPHHLD-1479) and concurrently introduced a silent XbaI site at residue HLD by mutating base pairs, 4426-caccactgagc-4437 into 4426-caccaTCTAgac-4437 using PCR amplification and ligation utilizing BglII/XbaI restriction sites. Subsequently, the Na\textsubscript{v}1.4CT was PCR amplified using primers: (fwd) 5\textsuperscript{\prime}-caccaTCTAgactgttctatgagacctgggag-3\textsuperscript{\prime} and (rev) 5\textsuperscript{\prime}-gatagagtttaaacttagacaagagactctttgacccc-3\textsuperscript{\prime} and ligated into the truncated Ca\textsubscript{v}1.3 construct using XbaI/Pmel restriction sites. This maneuver created the Ca\textsubscript{v}1.3-Na\textsubscript{v}1.4CT chimer with the protein sequence 1471-ILGPHHLDMYEV-1485 at the amino-terminal segment of the EF hand region (Figure 1A).

All segments subjected to PCR amplification and Quikchange mutagenesis were verified by sequencing.

Transfection of HEK293 Cells

For whole-cell patch clamp experiments, HEK293 cells were cultured on 10-cm plates, and channels transiently transfected by calcium phosphate method (Peterson et al., 1999). For experiments involving static Ca\textsuperscript{2+} uncaging experiments (Figures 2, 3, 5, 6C, and 6D), we applied 6-8 µg of cDNA encoding the desired Na channel, 6 µg of eCFP, and 8 µg of rat Ca\textsubscript{M}WT. For experiments involving mutant Ca\textsubscript{M}, we substituted 8 µg of Ca\textsubscript{M}12, Ca\textsubscript{M}34, or Ca\textsubscript{M}1234 instead of Ca\textsubscript{M}WT. For experiments that involved both Ca\textsubscript{V}2.1 and Na channels at the whole-cell level (Figure 3), 6 µg rat Na\textsubscript{V}1.4 or Na\textsubscript{V}1.5, 8 µg of z1 subunit of Ca\textsubscript{V}2.1 Efb 43\textsuperscript{\prime}/44\textsuperscript{\prime} /47\textsuperscript{\prime} isoform (Chaudhuri et al., 2004), 8 µg of rat brain β2a (M80545) auxiliary subunit, 5 µg of z2\textsuperscript{\prime} (NM012919.2), and 8 µg of rat Ca\textsubscript{M}WT or 8 µg of Ca\textsubscript{M}1234 (Figures S6D and S6E) were cotransfected. For like coexpression experiments at the single-channel level (Figure 4), 1-2 µg rat Na\textsubscript{V}1.4, 8-10 µg of z1 subunit of Ca\textsubscript{V}2.1 Efb 43\textsuperscript{\prime}/44\textsuperscript{\prime} /47\textsuperscript{\prime} isoform (Chaudhuri et al., 2004), 8 µg of rat brain β2a (M80545) auxiliary subunit, 5 µg of z2\textsuperscript{\prime} (NM012919.2), and 8 µg of rat Ca\textsubscript{M}WT were cotransfected. For experiments probing Ca\textsuperscript{2+} channel CDI (Figures 1C, 7C, and 7D), we cotransfected 8 µg of Ca\textsubscript{V}1.3 or Ca\textsubscript{V}1.3-Na\textsubscript{V}1.4CT, 8 µg of rat brain β2a auxiliary subunit, 5 µg of z2\textsuperscript{\prime} and 8 µg of rat Ca\textsubscript{M}WT or 8 µg of mutant Ca\textsubscript{M}1234. All of the above cDNA constructs were included within mammalian expression plasmids driven by a cytomegalovirus promoter. To boost expression, cDNA for simian virus 40 T antigen (1-2 µg) was cotransfected. Currents were probed ~1-3 days following transfection.

Detailed Recipes for Pipet and Bath Solutions

For recordings of wild-type Ca\textsubscript{V}1.3 (Figure 1C) and Ca\textsubscript{V}1.3 III-IV\textsubscript{17A} mutant (Figure S7C), we used an internal solution, “0 [Ca\textsuperscript{2+}],” which contained (in mM): CsMeSO\textsubscript{3}, 114; CsCl, 5; MgCl\textsubscript{2}, 1; MgATP, 4; HEPES (pH 7.4), 10; and BAPTA, 10; at 290 mOsm adjusted with glucose. The bath solution contained (in mM): TEA-MeSO\textsubscript{3}, 102; HEPES (pH 7.4), 10; CaCl\textsubscript{2} or BaCl\textsubscript{2}, 40; at 300 mOsm adjusted with TEA-MeSO\textsubscript{3}. The electrophysiological measurements were obtained only after ~10 min of pipet dialysis to permit stabilization of Na channel properties.

For experiments probing Na\textsubscript{v} steady-state inactivation under static Ca\textsuperscript{2+} (Figures 1F–1H), we used either “0 [Ca\textsuperscript{2+}],” solution described above, or a “10 [Ca\textsuperscript{2+}],” solution containing (in mM): CsMeSO\textsubscript{3}, 109; CsCl, 5; MgCl\textsubscript{2}, 1; MgATP, 4; HEPES (pH 7.4), 10; HEDTA, 10; and CaCl\textsubscript{2}, 5, at 290 mOsm adjusted with glucose. The bath solution contained (in mM): TEA-MeSO\textsubscript{3}, 45; HEPES (pH 7.4), 10; NaCl, 100; at 300 mOsm adjusted with TEA-MeSO\textsubscript{3}. The electrophysiological measurements were obtained only after ~10 min of pipet dialysis to permit stabilization of Na channel properties.
For all Ca\textsuperscript{2+}-uncaging experiments, internal solution contained (in mM): CsMeSO\textsubscript{3}, 120; CsCl, 5; HEPES (pH 7.4 with CsOH), 10; Fluo-4FF pentapotassium salt (Invitrogen), 0.01; Alexa 568 succinimidyl ester (Invitrogen), 0.0025; Citrate, 1; DM-Nitrophen EDTA (DMN) and CaCl\textsubscript{2} were adjusted to obtain desired Ca\textsuperscript{2+} flash. Typically, for flashes in range 0.5 – 2 μM, DMN, 1 mM; and CaCl\textsubscript{2}, 0.7 mM. For the 2 – 8 μM range, DMN, 2 mM; and CaCl\textsubscript{2}, 1.4 mM. For larger Ca\textsuperscript{2+} steps, DMN, 4 mM; and CaCl\textsubscript{2}, 3.2 mM. Since DMN can bind Mg\textsuperscript{2+}, all experiments were conducted with 0 mM Mg\textsuperscript{2+} internally. For recombinant Na channel experiments, the bath solution contained (in mM): TEA-MeSO\textsubscript{3}, 45; HEPES (pH 7.4), 10; NaCl, 100; at 300 mOsm, adjusted with TEA-MeSO\textsubscript{3}. For Ca\textsuperscript{2+} uncaging experiments in guinea pig ventricular myocytes, we used a modified bath solution containing (in mM): NaCl, 2; Choline-Cl, 125; CaCl\textsubscript{2}, 5; KCl, 4; HEPES, 10; glucose, 10; adjusted to pH 7.4 with NaOH and 300 mOsm with Choline-Cl. For Ca\textsuperscript{2+}-uncaging experiments in GLT cells, the bath solution contained (in mM): NaCl, 100; HEPES (pH 7.4), 10; Choline-Cl, 35; MgCl\textsubscript{2}, 1; KCl, 4; at 290 mOsm adjusted with Choline-Cl.

For experiments coexpressing both Na\textsubscript{v} and Ca\textsubscript{v} channels (Figure 3), the bath solution contained (in mM): NaCl, 130; CaCl\textsubscript{2}, 15; MgCl\textsubscript{2}, 1; KCl, 4; NaH\textsubscript{2}PO\textsubscript{4}, 0.33; HEPES, 10; with pH 7.4 adjusted with NaOH and at 290 mOsm adjusted with NaCl. For corresponding control experiments that used Ba\textsuperscript{2+} as charge carrier through Ca\textsuperscript{2+} channels, we substituted 15 mM BaCl\textsubscript{2} in place of CaCl\textsubscript{2}. The pipet solution, “0.5 EGTA” contained (in mM): CsMeSO\textsubscript{3}, 124; CsCl, 5; MgCl\textsubscript{2}, 1; MgATP, 4; HEPES (pH 7.4), 10; and EGTA, 0.5; at 290 mOsm adjusted with glucose. For high internal buffering (Figures 3H–3K), we used “0 [Ca\textsuperscript{2+}]” solution described above.

For multi-channel on-cell recordings of Na channels cotransfected with Ca\textsuperscript{2+} channels (Figure 4), the bath solution contained (in mM): K-glutamate, 132; KCl, 5; MgCl\textsubscript{2}, 3; EGTA, 2; Glucose, 6; and HEPES, 10 (pH adjusted to 7.4). The pipet solution contained (in mM): TeA-MeSO\textsubscript{3}, 30; NaCl, 100; CaCl\textsubscript{2}, 10; HEPES, 10 (pH 7.4).

**Ca\textsuperscript{2+} Measurements**

Ca\textsuperscript{2+} measurements were determined from ratio of Fluo4FF/Alexa fluorescence intensities (R), according to the relation [Ca\textsuperscript{2+}] = K\textsubscript{d} \times (R – R\textsubscript{min}) / (R\textsubscript{max} – R). All three parameters K\textsubscript{d}, R\textsubscript{min}, and R\textsubscript{max} were experimentally determined in HEK293 cells dialyzed with reference Ca\textsuperscript{2+} solutions (Tadross et al., 2013) and were assumed to be same for ventricular myocytes and GLT cells. Briefly, R\textsubscript{max} was determined with internal solution containing 40 mM EGTA, and R\textsubscript{max} using 4 mM Ca\textsuperscript{2+}/1 mM EGTA (~3 mM free Ca\textsuperscript{2+}) solution. An R\textsubscript{max} measurement was obtained with internal solution containing [Ca\textsuperscript{2+}] = 20 μM (buffered using NTA). K\textsubscript{d} was experimentally determined by solving the equation above. Calibration measurements were repeated at 1 or 4 mM DMN to account for minor differences in R\textsubscript{max}.

**Construction of Phylogenetic Tree**

For Figure 7B, protein sequences of all human Ca\textsubscript{v}1, Ca\textsubscript{v}2, Ca\textsubscript{v}3 and Na\textsubscript{v}1 channels were obtained from GenBank (Benson et al., 2005). For Figure 7C, protein sequences were acquired from GenBank (Benson et al., 2005), UniProt (UniProt Consortium, 2013), JGI (Grigoriev et al., 2012), or ParameciumDB (Arnaiz and Sperling, 2011) databases. Multiple sequence alignments were made using MUSCLE algorithm (Edgar, 2004) and phylogenetic trees were constructed using MEGA5.2 software (Tamura et al., 2011).

**Isolation of Guinea Pig Ventricular Myocytes**

Ventricular myocytes were isolated from adult guinea pigs in accordance with guidelines established by Johns Hopkins University Animal Care and Use Committee as published in previous publication (Alseikhan et al., 2002). Briefly, hearts were excised and ventricular myocytes were isolated by enzymatic digestion using a Langendorff perfusion apparatus. Whole-cell Na currents were interrogated using patch clamp ~2 hr after isolation.

**GLT Myoblast Culture**

Homozygous mouse dysgenic (mdg/mdg) GLT cell lines were originally generated by stable transfection of mdg myoblasts with plasmid-encoding large-T antigen (Powell et al., 1996). The myoblasts were expanded in growth media based of F-10 Ham’s media containing 20% FBS; HEPES, 25 mM; L-glutamine, 4 mM; Penicillin-Streptomycin, 100 U/ml; and dFGF, 1 ng/ml. After reaching more than 90% confluence (~3 days), the growth media was exchanged for a DMEM based differentiation media that contains 2% horse serum, Penicillin-streptomycin, 100 U/ml; and L-glutamine, 4 mM. Following differentiation (~3 days), the cells were split onto glass coverslips and patch clamp experiments were conducted on the following day.

**SUPPLEMENTAL REFERENCES**


Figure S1. Static Ca²⁺ Buffering to Probe Na Channel Regulation, Related to Figure 1
(A) Theoretical simulation of Ca²⁺ buffering using EGTA, BAPTA, and HEDTA as buffers. The concentration of free [Ca²⁺] is plotted against the ratio of total Ca²⁺ to total buffer ([Ca²⁺]total / [Buffer]total), with [Buffer]total = 10 mM. In general, a buffer is most effective at maintaining free Ca²⁺ concentrations near its Ca²⁺ dissociation constant. Thus, EGTA and BAPTA (black lines) having $K_d = 67$ nM and 192 nM respectively, are effective at buffering Ca²⁺ in the 30–600 nM concentration range, but become rather ineffective at higher Ca²⁺ concentrations. For instance, to attain $[\text{Ca}^{2+}]_{\text{free}}/\text{Ca}^{2+} \approx 10$ mM with these buffers, $[\text{Ca}^{2+}]_{\text{total}}$ must approximately equal $[\text{Buffer}]_{\text{total}}$. In this regime, most buffer molecules are already bound to a Ca²⁺ ion and, therefore, any excess contaminant ions will be unbuffered. Thus, small pipeting errors and stray Ca²⁺ ions from the experimental setting could amount to large fluctuations of $[\text{Ca}^{2+}]_{\text{free}}$ in the pipet. By contrast, HEDTA (red line) with $K_d = 4$ mM is an ideal buffer to clamp $[\text{Ca}^{2+}]_{\text{free}}$ in the 1–20 mM range. This simulation accounts for the precise experimental conditions used in this study (10 mM HEDTA, 4 mM ATP, 5 mM Mg²⁺). So with HEDTA as buffer, $[\text{Ca}^{2+}]_{\text{free}}$ can be effectively maintained near ~10 μM.

(B) Exemplar NaV1.4 currents evoked using steady-state inactivation protocol (Figure 1E) with 0 μM (black) or 10 μM internal [Ca²⁺] (red). The fractional current remaining appears similar in both conditions. Population averages shown in Figure 1H further confirm that the voltage dependence of steady inactivation is unaltered by Ca²⁺.
Figure S2. Extended Data Showing Effects of Ca\(^{2+}\) Uncaging on Na Channels, Related to Figure 2

(A) To probe whether Ca\(^{2+}\) alters the kinetics of Na channel activation and inactivation, we scrutinized the Na current waveform obtained from protocols analogous to those in main text Figures 2A and 2B. Left, Na current obtained prior to Ca\(^{2+}\) uncaging. Right, current evoked 900 ms after the onset of Ca\(^{2+}\) elevation (red trace) overlaid on the waveform before uncaging (black trace underneath, copied from left subpanel). Remarkably, the traces overlay suggesting that Ca\(^{2+}\) has no effect on the Na\(_{v1.5}\) current waveform morphology. Here, the current traces are plotted on an expanded time-base so as to reveal both kinetics of activation and inactivation.

(B) Left, Na\(_{v1.4}\) current before Ca\(^{2+}\) uncaging (Pulse #1 Figure 2B). Middle, Na current amplitude after Ca\(^{2+}\) uncaging (pulse #11 obtain after ~450 ms after Ca\(^{2+}\) uncaging) is reduced by ~30%. Right, normalized current after uncaging shows unperturbed activation and inactivation kinetics in presence of Ca\(^{2+}\) (red trace after uncaging plotted on top of black trace before uncaging).

(C) Exemplar Na\(_{v1.4}\) currents show CDI and recovery from CDI following return of Ca\(^{2+}\) to resting levels. Format as in main text Figure 2B. Left, Na current evoked by pulse train prior to Ca\(^{2+}\) uncaging. Middle, onset of CDI as evident from decrement of Na current following Ca\(^{2+}\) step to ~2 \(\mu\text{M}\). Right, once intracellular Ca\(^{2+}\) returned to basal levels (~150 nM), the Na current amplitude also recovered.

(D) In a different cell, when the cytosolic Ca\(^{2+}\) remained high after uncaging to ~10 \(\mu\text{M}\) Ca\(^{2+}\), the Na current amplitude also remained at the inactivated level, confirming the exquisite Ca\(^{2+}\) dependence of this novel type of modulation.
Figure S3. Effects of Pulse Rate on NaV1.4 Channel CDI, Related to Figure 2

(A) Since Na channels undergo fast inactivation within the first few milliseconds following depolarization, the time course and magnitude of the slower Ca\(^{2+}\)-dependent regulation of Na channels was deduced from the “envelope” of peak Na currents pulsed at regular intervals after Ca\(^{2+}\) uncaging. To ensure that NaV1.4 Ca\(^{2+}\) regulation is independent of this pulse rate, CDI was characterized for Na currents evoked at multiple pulse rates in a single cell. Gray dots and fit correspond to peak currents prior to Ca\(^{2+}\) uncaging. Following Ca\(^{2+}\) uncaging (cyan line), current amplitudes declined rapidly, revealing CDI in response to \( \sim 5 \mu M \) Ca\(^{2+}\) steps. Format as in Figure 2B. Red envelope curves are identical for various pulse rates, confirming that the onset of CDI is independent of pulse rate.

(B) Summary relationship further confirms that CDI magnitude (obtained from records in panel A) is independent of the pulse rate used to evoke NaV1.4 current.
Figure S4. Kinetics of NaV1.4 CDI, Ca2+ Dependence, and Voltage Independence, Related to Figure 2

(A) CDI elicited by Ca2+ steps of different amplitudes. The time constant ($\tau$) for the onset of CDI was estimated by single exponential fits to peak current amplitudes following Ca2+ uncaging (“red envelope”). Left, CDI onset is slower in response to a smaller Ca2+ step. Right, CDI onset is much faster with a larger Ca2+ step. Format as in main text Figure 2B.

(B) The time constant of CDI onset ($\tau$) plotted versus the magnitude of Ca2+ step with holding potential ($V_{\text{hold}}$) near $-90$ mV ($n = 21$ cells). This relation is well approximated (black fit) by system where channels transition from a normal mode of gating to a reduced-probability mode of gating via a rate constant given by $k_{\text{on}} = 3.2 \times 10^{12} \text{M}^{-1}\text{s}^{-1}$, $k_{\text{off}} = 4.7 \text{s}^{-1}$, and Hill coefficient $n = 2$. Indeed, it is reassuring that $(k_{\text{off}} / k_{\text{on}})^{1/2} \sim 1.5 \text{mM}$ is consistent with the measured $K_{\text{d}}$ of steady-state CDI–[Ca2+] relations (Figure 2B). Gray fit, predicted relations for hill coefficient $n = 1$ or $n = 4$ diverge from the experimental data. Overall, these results conform well with the dominance of a single lobe (N-lobe) of CaM in triggering CDI (Figures 5C and 5D). Red symbols, $\tau$–[Ca2+] relation obtained with holding potential set to $-120$ mV also overlay same relation, again consistent with CDI being a largely Ca2+-dependent process.

(C) Steady-state relation for CDI versus Ca2+ concentration, obtained with $V_{\text{hold}} = -120$ mV, overlays standard black relation reproduced from Figure 2B. Thus, the steady-state extent of NaV1.4 CDI also appears independent of the holding potential.

(D) CDI is not reversed by setting holding potential to $-160$ mV. After evoking CDI at $-90$ mV (horizontal gray lines), changing to $-160$ mV does not change the extent of inhibition (horizontal green lines). These results agree with a scaling down of steady-state inactivation (Figure 2D).

Overall, NaV1.4 channel CDI appears primarily a function of Ca2+ binding and unbinding to a resident calmodulin that indwells the channel. CDI is not a strong function of voltage and/or pattern of voltage activation.
Figure S5. Na Channel Ca\textsuperscript{2+} Regulation by Ca\textsuperscript{2+} Spillover from Ca\textsuperscript{2+} Channels, Related to Figure 3

(A) In native systems, voltage-gated Ca\textsuperscript{2+} channels constitute a prominent source for Ca\textsuperscript{2+} influx. Could Ca\textsuperscript{2+} entry through such physiological sources trigger Na channel Ca\textsuperscript{2+} regulation? Accordingly, we coexpress Na\textsubscript{V}1.4 channels with Ca\textsubscript{V}2.1 channels, chosen deliberately for their higher threshold for voltage activation that enables selective measurement of Na currents at lower voltages. The schematic thus illustrates the experimental paradigm used in Figure 3, where Ca\textsuperscript{2+} spillover from Ca\textsuperscript{2+} channels was used to drive Na\textsubscript{V}1.4 CDI.

(B) Top, stimulus protocol used to probe Na\textsubscript{V}1.4 CDI. An initial depolarizing pulse to 0 mV was used to evoke Na current. Immediately following, a family of voltage pulses (\(V_{\text{inter}}\)) was applied to activate Ca\textsuperscript{2+} currents. Since Na channels undergo fast inactivation, the peak current measured during the intervening pulse (\(I_{\text{Ca,peak}}\)) represents the peak Ca\textsuperscript{2+} current at a given voltage (\(I_{\text{Ca,peak}}\)).

(C) Here, peak Ca\textsuperscript{2+} current thus measured during the intervening pulse is plotted against corresponding \(V_{\text{inter}}\) to reveal the current–voltage relationship for Ca\textsubscript{V}2.1. Indeed at 0 mV, Ca\textsuperscript{2+} channels are minimally activated (<5%), thus enabling activation of Na current alone at this potential. Each symbol, mean ± SEM from 4 cells.

(D–F) Restricting Ca\textsuperscript{2+} elevations to the Ca\textsuperscript{2+} channel nanodomain by utilizing fast Ca\textsuperscript{2+} buffering eliminates CDI of Na\textsubscript{V}1.4 driven by Ca\textsuperscript{2+} influx through Ca\textsubscript{V}2.1 (Figures 3H–3K). Here, we furnish control experiments that utilize Ba\textsuperscript{2+} as permeant ion through Ca\textsuperscript{2+} channel. Format as in Figures 3H–3J. Indeed, Na current amplitude is unperturbed by Ba\textsuperscript{2+} influx through Ca\textsuperscript{2+} channel. Similar data were used to calculate black symbols in Figure 3K.

(G–J) A useful aspect of these whole-cell experiments utilizing coexpression of Na and Ca\textsuperscript{2+} channels is that Na channel CDI can be evoked independent of UV uncaging of Ca\textsuperscript{2+} complexed with DM nitrophen, allowing us to control for potential artifacts that may result from release of photolytic products. To complete the control experiments that may be performed in this vein, we coexpress Na\textsubscript{V}1.5 and Ca\textsubscript{V}2.1 channels and utilize a dual-pulse protocol to probe for Ca\textsuperscript{2+} regulation. Indeed even in this mode, we observe no indication of CDI of Na\textsubscript{V}1.5, consistent with results obtained with Ca\textsuperscript{2+} uncaging experiments. Format identical to that in Figures 3A–3D. Importantly, 0.5 mM EGTA is the internal Ca\textsuperscript{2+} buffer, so as to permit robust Ca\textsuperscript{2+} spillover from Ca\textsuperscript{2+} channels.
NaV1.4 CDI Relies on CaM but Is Spared by Channel EF Mutations and CaMKII Inhibition, Related to Figure 5

(A–C) Extended data show that EF-hand region is not the primary Ca\(^{2+}\) sensing element for Na channel regulation. Based on homology modeling, four oxygen-bearing residues of the NaV1.5 EF hand region were historically proposed to support direct binding of Ca\(^{2+}\) to Na channel that presumably triggered channel modulation (Biswas et al., 2009; Wingo et al., 2004). Top, atomic structures (Miloushev et al., 2009; Wang et al., 2012) of NaV1.5 EF hand segment showing these four residues (as labeled) cannot coordinate Ca\(^{2+}\). Bottom, sequence alignment depicting location of these four residues of Na channels argued to bind Ca\(^{2+}\). Nonetheless, we undertook alanine substitutions of corresponding residues on NaV1.4 (E[1621]A, D[1623]A, E[1625]A, E[1632]A – termed 4\(^3\) mutations) to test for Ca\(^{2+}\) regulatory effects.

(B) Ca\(^{2+}\) uncaging experiments, however, show that NaV1.4 channels with the 4\(^3\) mutation can undergo robust CDI, much like the alternative EF hand mutations described in Figure 5A (D[1621]A and D[1623]A). Here, exemplar Na current shows CDI in response to ~3 \(\mu\)M Ca\(^{2+}\) step. Format as in main text Figure 2B.

(C) Population average shows average CDI as a function of [Ca\(^{2+}\)] (black symbols and trace). Gray trace, fit for wild-type channels from Figure 2B. Indeed, these outcomes sharply contrast with the complete knockout of CDI observed using CaM1234 overexpression with wild-type NaV1.4 channels (Figure 5B). The minor reduction in maximal CDI, and the slightly weakened Ca\(^{2+}\) affinity may hint at a potential role for this region as a CDI transduction element. Indeed, in Ca\(^{2+}\) channels, the EF-hand region is thought to bind to the C-lobe of Ca\(^{2+}\)/CaM and the IQ domain to form a tripartite complex that serves as an effector configuration for CDI of these channels (Ben Johny et al., 2013). A variation on this theme may furnish a parsimonious mechanistic basis for Na channel Ca\(^{2+}\) regulation. Each symbol, mean ± SEM from 9-10 uncaging events compiled from 20 cells.

(D–E) Na channel regulation by Ca\(^{2+}\) spillover from nearby Ca\(^{2+}\) channels is mediated by CaM.

(D) Ca\(^{2+}\) regulation of Na channels can be triggered by Ca\(^{2+}\) influx through nearby Ca\(_\text{v}2.1\) channels (Figures 3A–3D). Here, exemplar current records show that coexpression of mutant CaM1234 abolishes this modulation, confirming that CaM is the Ca\(^{2+}\) sensor for regulation of NaV1.4 channels. Format as in Figures 3B–3D. Each symbol represents mean ± SEM.

(E) Population data plots Ca\(^{2+}\) regulation metric \(r_{\text{Na}}\) (Figure 3G) as a function of interpulse voltage \(V_{\text{inter}}\). Format as in main text Figure 3G. Indeed, coexpression of CaM1234 abolishes CDI of NaV1.4 channels, with rose fit reproduced from Figure 3G as reference. These results argue that Ca\(^{2+}\) regulation of NaV1.4 observed with Ca\(^{2+}\) uncaging or Ca\(^{2+}\) spillover from nearby Ca\(_\text{v}2.1\) channels, both depend on the same process as mediated by CaM.

(F and G) Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) has been argued to phosphorylate NaV1.5 channels (Aiba et al., 2010; Ashpole et al., 2012; Wagner and Maier, 2006). Though we found an absence of rapid Ca\(^{2+}\) regulatory effects on NaV1.5 channels, could CaMKII play a role in the CDI of homologous NaV1.4 channels? Here, extended data show that Na channel Ca\(^{2+}\)/CaM regulation does not require activation of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) by application of KN-93, a blocker of CaMKII activity.

(F) Left, exemplar NaV1.4 currents exhibit robust CDI. Right, this robust CDI is preserved following the application of 0.5 \(\mu\)M KN-93 for 3-30 min. Indeed, the kinetics and steady-state extent of CDI appear unaltered by inhibition of CaMKII. Format as in Figure 2A.

(G) Population data (black symbols) confirm that application of KN-93 does not alter the steady-state CDI versus Ca\(^{2+}\) relationship. Gray fit, control CDI versus Ca\(^{2+}\) relationship for NaV1.4 channels (from main text Figure 2B). Overall, these results demonstrate that CDI of Na channels does not require CaMKII activation, further strengthening the case for CaM as a direct modulator of Na channels.
**Figure S7. Ca\(^{2+}\)/CaM Binding to III-IV Loop of Both Ca\(^{2+}\) and Na Channels Nonessential for CDI and Kinetics of Channelopathic Mutant Na\(_{v}1.4\) Channels, Related to Figures 5 and 6**

(A) Top, sequence alignment of Na\(_{v}1.4\) and Na\(_{v}1.5\) III-IV loop. The proximity of this binding site to a primary structural determinant of fast inactivation localized to the tri-residue “IFM” motif (West et al., 1992) (yellow shading) is also diagrammed. Ca\(^{2+}\)/CaM binding site within the Na channel III-IV loop is highlighted in green. The critical Y[1311] residue mutated in main text Figure S6A is shown in red. Bottom, the crystal structure of Na\(_{v}1.5\) III-IV loop bound to Ca\(^{2+}\)/CaM (PDB code: 4DJC [Sarhan et al., 2012]). Engagement of the critical tyrosine residue (Na\(_{v}1.5\), Y[1494]; Na\(_{v}1.4\), Y[1311]) is colored in red. This configuration has been argued to be a trigger for Ca\(^{2+}\) regulation of Na\(_{v}1.5\), an outcome not observed in the present study. By contrast, we find that the carboxy tail of Na\(_{v}\) channels constitutes a prime structural determinant of CDI, not the III-IV loop.

(B) Similar to Na channels, the Ca\(^{2+}\) channel (Ca\(_{v}1.3\)) III-IV loop also contains a Ca\(^{2+}\)/CaM binding site. To evaluate this binding site, we conducted FRET 2-hybrid experiments of Ca\(^{2+}\)/CaM binding to both the full-length III-IV loop segment and a variant with a 15-residue deletion from the carboxy-terminal end of this segment (\(\Delta\)15). Left, cartoon depicts FRET pairs used in this experiment. Right, plotting FRET efficiency (\(E_A\)) as a function of \(D_{\text{free}}\) (relative free concentration of ECFP-CaM) reveals a binding relation for full-length III-IV loop (black symbols and fit). The \(\Delta\)15 mutant of the III-IV loop diminishes Ca\(^{2+}\)/CaM binding significantly (gray symbols and fit). For these experiments, we cotransfected CFP-tagged CaM with YFP-tagged III-IV loop channel segments in HEK293 cells cultured on glass-bottom dishes, and measured fluorescence intensities using an inverted fluorescence microscope as extensively described by our laboratory (Ben Johny et al., 2013; Erickson et al., 2001, 2003). Intracellular Ca\(^{2+}\) was elevated by using an external solution containing 10 mM Ca\(^{2+}\) and bath application of 4 \(\mu\)M ionomycin (Sigma-Aldrich, MO). The 3\(^{\text{rd}}\)-FRET efficiencies (\(E_A\)) were computed as elaborated in our prior publications (Erickson et al., 2001).

(C) The functional relevance of this novel Ca\(^{2+}\)/CaM binding site on Ca\(_{v}1.3\) III-IV loop was probed by substitution of the last 17 residues with alanines, since the deletion of this segment was detrimental to channel expression. Indeed, this manipulation spared CDI of Ca\(_{v}^{2+}\) channels. Left, exemplar currents for this mutant channel. Right, population average of \(r_{300}\) CDI metric defined in Figure 1C. Each symbol corresponds to mean ± SEM. These results reveal yet another commonality between Na and Ca\(_{v}^{2+}\) channels: both channel types contain a Ca\(^{2+}\)/CaM binding site within the III-IV loop, but this binding site does not seem to support CDI in either context. Nonetheless, this site may prove important in other aspects of channel function, such as channel trafficking.

(D) Invariance of kinetics for Na\(_{v}1.4\) Q1626E current upon Ca\(^{2+}\) elevation. Left, Na\(_{v}1.4\) Q1626E current before Ca\(^{2+}\) uncaging (Pulse #1 main text Figure 6C). Middle, Na current amplitude after Ca\(^{2+}\) uncaging (pulse #1 obtained ~450 ms after Ca\(^{2+}\) uncaging) is reduced by ~15%. Right, normalized current after uncaging shows unperturbed activation and inactivation kinetics in presence of Ca\(^{2+}\) (red trace after uncaging plotted on top of black trace before uncaging). (E) Invariance of kinetics for Na\(_{v}1.4\) F1698I current upon Ca\(^{2+}\) elevation. Format as in panel D above.