Calmodulin Regulation of Na\textsubscript{v}1.4 Current: Role of Binding to the Carboxyl Terminus

Subrata Biswas, Isabelle Deschênes, Deborah DiSilvestre, Yanli Tian, Victoria L. Halperin, and Gordon F. Tomaselli

Department of Medicine, Division of Cardiology, Johns Hopkins University School of Medicine, Baltimore, MD 21205

Calmodulin (CaM) regulates steady-state inactivation of sodium currents (Na\textsubscript{v}1.4) in skeletal muscle. Defects in Na current inactivation are associated with pathological muscle conditions such as myotonia and paralysis. The mechanisms of CaM modulation of expression and function of the Na channel are incompletely understood. A physical association between CaM and the intact C terminus of Na\textsubscript{v}1.4 has not previously been demonstrated. FRET reveals channel conformation-independent association of CaM with the C terminus of Na\textsubscript{v}1.4 (CT-Na\textsubscript{v}1.4) in mammalian cells. Mutation of the Na\textsubscript{v}1.4 CaM-binding IQ motif (Na\textsubscript{v}1.4IQ/AA) reduces cell surface expression of Na\textsubscript{v}1.4 channels and eliminates CaM modulation of gating. Truncations of the CT that include the IQ region abolish Na current. Na\textsubscript{v}1.4 channels with one CaM fused to the CT by variable length glycine linkers exhibit CaM modulation of gating only with linker lengths that allowed CaM to reach IQ region. Thus one CaM is sufficient to modulate Na current, and CaM acts as an ancillary subunit of Na\textsubscript{v}1.4 channels that binds to the CT in a conformation-independent fashion, modulating the voltage dependence of inactivation and facilitating trafficking to the surface membrane.
site-directed mutagenesis of the Na V 1.4-ECFP/EYFP construct. The IQ/AA mutants (I1727A/Q1728A) were made by codon of Nav1.4 was replaced with an NheI site, and the EYFP/ECFP fragments were directionally cloned into this site and a Nofl site in the GWHI. The IQ/AA mutants (I1727A/Q1728A) were made by site-directed mutagenesis of the Na V 1.4-ECFP/EYFP construct.

Construction of NaV1.4-ECFP/EYFP Deletion Mutants. Inserts containing the NaV1.4 cDNA that were truncated at codons 1723 (NaV1.4 1723 ) or 1740 (NaV1.4 1740 ) in frame with either ECFP or EYFP, were generated by PCR using the forward primer NaV1.4-BstZF (CTGGGTCAACACCACTC) and either one of the reverse primers NaV1.4-1723NheR (1736bp) or NaV1.4-1740NheR (1787 bp). The reverse primers used to prepare the 1723 and 1740 truncations were TCGAATTCAGCTAGCAACACTCTCTGGTTCC and TCGAATTCAGCTAGCAACACATCCTCCTGGTCCC, respectively. The PCR products that generated the truncations were ligated into pGWIH-NaV1.4 digested with BstZ17 and NheI. All the clones were sequence confirmed.

Construction of NaV1.4 1723 -Glycine-CaM-EYFP. Glycine (G4)-linked CaM was inserted into NaV1.4 1723 EYFP by cloning a PCR product of full-length CaM flanked by in-frame Nhe1 sites into an Nhel site between the channel and fluorescent protein coding regions. The G4-CaM insert was used as template and the forward primer 5’-attactGCTAGGACGAGGCGGATGCTGACACTG3’ and the reverse primer 5’-attactGCTAGCCCTGCGTGATCATTTTGACGTGCAGG, respectively. The PCR products that generated the truncations were ligated into pGWIH-NaV1.4 digested with BstZ17 and NheI. All the clones were sequence confirmed.

Cell Culture and Transfection
Approximately 0.75 x 10^9 human embryonic kidney cells (HEK293; American Type Culture Collection) were cultured in six-well tissue dishes in DMEM supplemented with FBS 10%, l-glutamine (2 mM/liter), penicillin (100 U/ml), and streptomycin (10 mg/ml). The cells were cotransfected with BAC and plasmids encoding wild-type sodium channels NaV1.4-EYFP, NaV1.4-ECFP or mutant NaV1.4-1723 ECFP, NaV1.4-1723 EYFP, wild-type ECFP/EYFP/CaM or mutant ECFP/EYFP/CaM 1234 alone or in combination. Cells were transfected using Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions and were studied 48–72 h post-transfection. The total amount of DNA for all transfections was kept constant.

Electrophysiology
HEK293 cells expressing wild-type or mutant tagged NaV1.4 channels and CaM or CaM 1234 were patch clamped with an Axopatch 200B patch-clamp amplifier using pipettes with tip resistances of 1–3 MΩ and typical series resistance compensation of >90% to minimize voltage clamp errors. Transfected cells were identified for patch clamping by ECFP, EYFP, or GFP fluorescence. Current recording was initiated 10 min after establishing the whole-cell configuration to avoid time-dependent shifts in gating. The bath solution contained (in mM) 150 NaCl, 2 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, and 10 Na-HEPES (pH 7.4). The patch pipette contained (in mM) 35 NaCl, 3.7 CaCl2, 5 BaPta, and 10 Cs-HEPES (pH 7.3). The free [Ca2+] in the pipette was ~0.5 μM; the bath and pipette solutions were adjusted to same osmolarity using glucose.

For simultaneous FRET measurements cells were held at −140, −60, and −20 mV during the collection of emitted light. A standard two-pulse protocol (500 ms pulse from −140 to −50 mV and a 50-ms test pulse to −20 mV) was used to generate the steady-state inactivation curves. Recovery from inactivation was assessed with a two-pulse protocol with a 30-ms first pulse to −20 mV followed by a variable interpulse interval and a second 30-ms pulse to assay recovery from predominantly fast inactivated states. Entry into inactivated states was assessed by a variable first test pulse to −20 mV (1–10,000 ms), followed by a 10-ms interpulse interval and a second test pulse of 50 ms to −20 mV to assay entry into slowly recovering (intermediate and slow) inactivated states.

FRET Measurements
Measurements of single-cell FRET based on aggregate (non-spatial) fluorescence recordings with donor dequenching were performed as previously described (Erickson et al., 2001) with minor modifications. Donor dequenching experiments were performed using a CFP filter cube (D440/20M (excitation), 455DCLP (dichroic), D480/30M (emission; Chroma Technology Corp.) before and 5 min after intense illumination using a custom YFP photobleaching cube (Chroma Technology Corp.), consisting of a D535/50v excitation filter and a 100% mirror (in place of a dichroic mirror). In control experiments this bleaching protocol spared CFP fluorescence. Epifluorescence images were acquired with a P11300 CCD camera (Princeton Instruments) mounted to the side port of Olympus IX70 inverted microscope (60 x 1.4 objective; oil) driven by Metamorph software (Version7.0, Molecular Devices). The exposure time was set during the prebleach image acquisition and was not changed throughout the acquisition. Intensities were measured from a user-defined region of interest within the area of YFP fluorescence. Each image was background adjusted by subtracting the average pixel intensity from a region in the same field located outside the cell. Donor dequenching FRET efficiency (E_{donor}) was calculated using a previously described method (Erickson et al., 2001) in SigmaPlot (Systat Software Inc.). Movies of cell fluorescence intensities were recorded by stream acquisition (600 frames, 50-ms exposure time). The CaM and NaV1.4 interaction was further studied using the nondestructive 33 triple cube) FRET method, which has been previously used in CFP/YFP FRET to show interaction of CaM with the L-type Ca2+ channel. 33 FRET is highly concordant with donor dequenching FRET, thus is used as a complementary method to verify results of donor dequenching FRET. 33 FRET avoids the need for photolysis of the acceptor, thus reducing the possibility of artificial changes in the quantum yield of the donor. The 33 method measures the FRET ratio, a fractional increase in YFP emission due to FRET, by eliminating direct excitation of YFP and contaminating CFP emission using different filter cubes. 33 FRET images were acquired with the following filter cubes (excitation, dichroic, emission): CFP filter cube (D440/20M, 455DCLP, D480/30M, Chroma Technology Corp.); the YFP filter cube (HQ500/20×, 515LPLP, HQ505/30, Chroma Technology Corp.); and the FRET filter cube (D440/20M, Chroma Technology Corp.), 33FRET measurements were performed with equal amounts (0.5 μg/ml) of either wild-type NaV1.4-ECFP or NaV1.4-1723 EYFP, and wild-type ECFP/EYFP and CaM 1234 alone or in combination. Cells were transfected using Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions and were studied 48–72 h post-transfection. The total amount of DNA for all transfections was kept constant.

Cell Surface Expression and Western Blot Analysis
The cells were cotransfected with equal amounts of either wild-type NaV1.4-ECFP or NaV1.4-1723 EYFP, and wild-type ECFP/EYFP and CaM 1234 alone or in combination. Cells were transfected using Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions and were studied 48–72 h post-transfection. The total amount of DNA for all transfections was kept constant.
**TABLE I**  
*Effect of CaM and CaM<sub>1234</sub> Overexpression on Na<sub>V1.4</sub>, Na<sub>V1.4</sub>-EYFP and Na<sub>V1.4-EYFP</sub>*

<table>
<thead>
<tr>
<th>Channel/Mutant</th>
<th>V&lt;sub&gt;i&lt;/sub&gt; steady state inactivation</th>
<th>Decay time constant τ&lt;sub&gt;h&lt;/sub&gt;</th>
<th>V&lt;sub&gt;i&lt;/sub&gt; activation</th>
<th>Current density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;-EYFP + β1</td>
<td>−65.2 ± 0.4 (n = 5)</td>
<td>0.69 ± 0.04</td>
<td>−32.2 ± 0.1</td>
<td>−426.4 ± 93.3</td>
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<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;-EYFP + ECFP-CaM + β1</td>
<td>−71 ± 0.2 (n = 5)</td>
<td>0.77 ± 0.05</td>
<td>−32.6 ± 0.2</td>
<td>−458.0 ± 54.0</td>
</tr>
<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;-EYFP + ECFP-CaM&lt;sub&gt;1234&lt;/sub&gt; + β1</td>
<td>−64.2 ± 0.1 (n = 5)</td>
<td>0.66 ± 0.02</td>
<td>−32.9 ± 0.1</td>
<td>−269.6 ± 55.5</td>
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<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt; + β1</td>
<td>−65.8 ± 0.1 (n = 6)</td>
<td>0.61 ± 0.07</td>
<td>−32.1 ± 0.3</td>
<td>−453.2 ± 71.5</td>
</tr>
<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt; + ECFP-CaM + β1</td>
<td>−69.3 ± 0.1 (n = 7)</td>
<td>0.63 ± 0.02</td>
<td>−30.6 ± 0.2</td>
<td>−309.4 ± 112.0</td>
</tr>
<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt; + ECFP-CaM&lt;sub&gt;1234&lt;/sub&gt; + β1</td>
<td>−64.3 ± 0.5 (n = 7)</td>
<td>0.66 ± 0.07</td>
<td>−31.2 ± 0.1</td>
<td>−324.1 ± 80.9</td>
</tr>
<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;&lt;sub&gt;Ct&lt;/sub&gt;/AA + β1</td>
<td>−79.4 ± 0.2 (n = 6)</td>
<td>1.3 ± 0.2</td>
<td>−40.9 ± 0.3</td>
<td>−307.8 ± 89.9</td>
</tr>
<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;&lt;sub&gt;Ct&lt;/sub&gt;/AA + ECFP-CaM + β1</td>
<td>−80.0 ± 0.4 (n = 6)</td>
<td>1.25 ± 0.15</td>
<td>−40.0 ± 0.1</td>
<td>−242.5 ± 60.5</td>
</tr>
<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;&lt;sub&gt;Ct&lt;/sub&gt;/AA + ECFP-CaM&lt;sub&gt;1234&lt;/sub&gt; + β1</td>
<td>−80.7 ± 0.5 (n = 5)</td>
<td>1.12 ± 0.15</td>
<td>−41.0 ± 0.3</td>
<td>−254.2 ± 73.1</td>
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<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;&lt;sub&gt;Ct&lt;/sub&gt;/β1</td>
<td>−62.7 ± 0.1 (n = 6)</td>
<td>0.69 ± 0.09</td>
<td>−30.1 ± 0.2</td>
<td>−437.8 ± 116.0</td>
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<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;&lt;sub&gt;Ct&lt;/sub&gt;/β1 + ECFP-CaM + β1</td>
<td>−68.0 ± 0.1 (n = 6)</td>
<td>0.62 ± 0.02</td>
<td>−29.5 ± 0.1</td>
<td>−294.0 ± 74.7</td>
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<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;&lt;sub&gt;Ct&lt;/sub&gt;/β1 + ECFP-CaM&lt;sub&gt;1234&lt;/sub&gt; + β1</td>
<td>−64.0 ± 0.04 (n = 5)</td>
<td>0.79 ± 0.01</td>
<td>−31.1 ± 0.2</td>
<td>−295.0 ± 60.9</td>
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<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;&lt;sub&gt;Ct&lt;/sub&gt;/G4+CaM + β1</td>
<td>−65.8 ± 0.1 (n = 8)</td>
<td>0.69 ± 0.09</td>
<td>−30.8 ± 0.3</td>
<td>−372.9 ± 67.4</td>
</tr>
<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;&lt;sub&gt;Ct&lt;/sub&gt;/G4+CaM + ECFP-CaM + β1</td>
<td>−71.4 ± 0.2 (n = 9)</td>
<td>0.55 ± 0.3</td>
<td>−30.7 ± 0.1</td>
<td>−373.0 ± 77.5</td>
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<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;&lt;sub&gt;Ct&lt;/sub&gt;/G4+CaM + ECFP-CaM&lt;sub&gt;1234&lt;/sub&gt; + β1</td>
<td>−63.6 ± 0.2 (n = 5)</td>
<td>0.52 ± 0.1</td>
<td>−32.1 ± 0.2</td>
<td>−413.5 ± 118.9</td>
</tr>
<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;&lt;sub&gt;Ct&lt;/sub&gt;/G4+CaM + β1</td>
<td>−68.4 ± 0.2 (n = 9)</td>
<td>0.60 ± 0.1</td>
<td>−31.9 ± 0.3</td>
<td>−201.4 ± 63.2</td>
</tr>
<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;&lt;sub&gt;Ct&lt;/sub&gt;/G4+CaM + ECFP-CaM + β1</td>
<td>−67.7 ± 0.2 (n = 9)</td>
<td>0.51 ± 0.06</td>
<td>−30.2 ± 0.3</td>
<td>−391.5 ± 121.1</td>
</tr>
<tr>
<td>Na&lt;sub&gt;V1.4&lt;/sub&gt;&lt;sub&gt;Ct&lt;/sub&gt;/G4+CaM + ECFP-CaM&lt;sub&gt;1234&lt;/sub&gt; + β1</td>
<td>−66.7 ± 0.1 (n = 9)</td>
<td>0.53 ± 0.06</td>
<td>−32.0 ± 0.1</td>
<td>−439.5 ± 141.9</td>
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**Data Analysis**

Electrophysiological, protein expression, and FRET data were expressed as the means ± SEM and inactivation data were fit to a Boltzmann function. Data for entry into inactivation and recovery from inactivation were fit to exponential functions. Significance was assessed using a t test (Microcal Origin, Microcal Software Inc.).

**Online Supplemental Material**

The online supplemental material (available at http://www.jgp.org/cgi/content/full/jgp.200709863/DC1) contains two figures. Fig. S1 summarizes the biophysical properties of Na<sub>V1.4</sub><sub>Ct</sub>/AA in the presence of CaM and AIP. Fig. S2 illustrates the effect of the C-terminal truncation Na<sub>V1.4</sub><sub>Ct</sub> on channel trafficking by epifluorescence microscopy and Western blotting.

**RESULTS**

**CT-Na<sub>V1.4</sub> Fusion Constructs Are Functionally Expressed and Interact with CaM**

We have previously shown that CaM associates with wild type GST-tagged Na<sub>V</sub> CT peptides in vitro (Deschênes et al., 2002), this work motivated studies of the interaction between the CT of intact Na<sub>V</sub> channels and CaM in live cells using FRET. We designed channel fusions with fluorescent proteins linked to the CT, which is close in the linear amino acid sequence to the IQ motif, optimizing the possibility of FRET detection on CaM binding. One of the major concerns about fusion of a fluorophore to CT is the possibility of functional alterations of the sodium current by tagging the channel. The CT EYFP/ECFP-tagged Na<sub>V1.4</sub> channels were fully characterized and did not exhibit significant changes in current–voltage relationship or the voltage dependence of activation and steady-state inactivation compared
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ECFP-CaM 1234 exhibit distinct bands representing labeled CaM and CaM 1234, which can be distinguished from either ECFP or endogenous native CaM expression (Fig. 2 B). The levels of endogenous CaM are comparable to that of fluorophore-conjugated CaM in Western blots (Fig. 2 B). Thus, analysis based on single-cell voltage clamp recording and fluorescence microscopy

Figure 1. Na\textsubscript{v}1.4-EYFP expression. (A) Na\textsubscript{v}1.4-EYFP expression in HEK293 cells. Epifluorescence image and intensity profile of an HEK293 cell expressing Na\textsubscript{v}1.4-EYFP and Na\textsubscript{v}\beta1 subunits. The peaks in the fluorescence profiles indicate membrane targeting. (B) A family of Na\textsubscript{v}1.4-EYFP currents elicited by the voltage protocol shown in the inset. (C) Western blot of total cell lysates from Na\textsubscript{v}1.4-EYFP-transfected (1 \( \mu \)g/ml) HEK293 cells and nontransfected controls. Lysates isolated 48 h after transfection, separated by Western blot, and probed with an anti-Na\textsubscript{v} antibody exhibit a band near 197 kD representing Na\textsubscript{v}1.4-EYFP expression.

Figure 2. ECFP-CaM overexpression in HEK293 cells. (A) Epifluorescence image and intensity profile of an HEK293 cell expressing ECFP-CaM alone shows uniform cytosolic expression of CaM. (B) Immunoblot of HEK293 lysates probed with an anti-CaM antibody demonstrates endogenous CaM, ECFP-CaM, and ECFP-CaM\textsubscript{1234} (left). Immunoreactive ECFP, ECFP-CaM, and ECFP-CaM\textsubscript{1234} are shown with an anti-GFP antibody. (C) Epifluorescence image and intensity profile of Na\textsubscript{v}1.4-EYFP coexpressed with ECFP-CaM in HEK293 cells; the peaks indicate membrane targeting. In the top panel, the cell is excited with 535-nm light and the bottom panel is the same cell excited with 440-nm light showing enrichment of ECFP-CaM at the cell membrane when coexpressed with Na\textsubscript{v}1.4-EYFP.

with untagged wild-type channels expressed in HEK293 cells (Table I).

The channel fusion constructs expressed in HEK293 cells display a distinct fluorescent enrichment at the cell perimeter consistent with surface membrane expression (Fig. 1 A). Cells transfected with Na\textsubscript{v}1.4-EYFP express robust currents (Fig. 1 B) that are indistinguishable from untagged wild-type Na\textsubscript{v}1.4 (Table I), confirming that labeled channels are functional and targeted to the membrane. Western blots of lysates from cells transfected with Na\textsubscript{v}1.4-ECFP or -EYFP reveal a band of \(~\)225 kD, consistent with the expression of labeled channels (Fig. 1 C).

In contrast to channel expression, ECFP-CaM expression or the EF hand mutant of CaM (ECFP-CaM\textsubscript{1234}) alone in HEK293 cells is uniform throughout the cell (Fig. 2 A; ECFP-CaM\textsubscript{1234} not depicted). Western blots from cells transfected with ECFP-CaM or
responding to surface membrane enrichment of ECFP-CaM (Fig. 2C).

We characterized Na\textsubscript{v} 1.4-EYFP currents in the presence and absence of overexpressed ECFP-CaM and its EF hand mutant ECFP-CaM\textsubscript{1251}. The currents generated by expression of the EYFP-fused channel construct is necessary to evaluate the effect of overexpressed CaM. If CaM is associated with Na\textsubscript{v} 1.4, one would predict enrichment of CaM at the cell membrane based on the distribution of Na\textsubscript{v} 1.4 in the surface membrane (Fig. 1A). Cells expressing both labeled channels and ECFP-CaM show a small peak in the image profile corresponding to surface membrane enrichment of ECFP-CaM (Fig. 2 C).

We characterized Na\textsubscript{v} 1.4-EYFP currents in the presence and absence of overexpressed ECFP-CaM and its EF hand mutant ECFP-CaM\textsubscript{1251}. The currents generated by expression of the EYFP-fused channel construct...
have activation and inactivation kinetics and peak amplitudes that were not different from the wild-type NaV1.4 channel (Table I). The voltage dependence of steady-state inactivation was not altered by fusion of EYFP at the CT of NaV1.4 (Fig. 3 A; Table I). The V_{1/2} of steady-state inactivation of NaV1.4-EYFP is $-65.2 \pm 0.4$ mV ($n = 5$), coexpression of ECFP-CaM significantly (P < 0.04) shifts the V_{1/2} $\sim 5$ mV ($V_{1/2} = -71 \pm 0.2$, $n = 5$) in the hyperpolarizing direction compared with NaV1.4-EYFP expressed alone (Fig. 3 A). The voltage dependence of activation of the NaV1.4-EYFP expressed in HEK293 cells ($V_{1/2} = -32.2 \pm 0.1$ mV, $n = 5$) was not altered by overexpression of CaM ($V_{1/2} = -32.6 \pm 0.2$ mV; $n = 5$) or CaM1234 ($V_{1/2} = -32.9 \pm 0.1$ mV, $n = 5$) (Table I). Thus fusion of ECFP/EYFP to CT of NaV1.4 and CaM does not alter the CaM-induced steady-state inactivation shift.

CaM Is Tethered to CT-NaV1.4

We next asked if CaM or apo-CaM is tethered to the CT of intact NaV1.4 channels and if there are alternate apo-CaM binding site(s) with functional effects on the Na current as in the L-type Ca\(^{2+}\) channel (Erickson et al., 2001, 2003). Overexpression of ECFP-CaM1234 with NaV1.4 does not shift the channel availability curve compared with NaV1.4 expression alone in the presence of endogenous CaM (Fig. 3 A). These data indicate that apo-CaM does not affect steady-state inactivated channel availability; however, it does not exclude the possibility of apo-CaM binding to the channel. Overexpressing both wild-type EYFP-CaM and ECFP-CaM1234 with NaV1.4 produced no significant shift in the voltage dependence of the steady-state availability (Fig. 3 A; $V_{1/2} = -69.3 \pm 0.6$ mV, $n = 10$) compared with ECFP-CaM overexpression alone, suggesting the possibility that CaM may be
durably tethered to CT-Na\(_{\text{V}}\)1.4 and that apo-CaM does not displace CaM binding.

Inclusion of the CaM anti-peptide (AIP290-309) in the pipette solution mitigates the CaM-induced shift in the steady-state inactivation curve (\(V_{1/2} = -63.7 \pm 0.2\) mV, \(n = 7\)) in cells coexpressing Na\(_{\text{V}}\)1.4- EYFP and ECFP-CaM (Fig. 3 B). These data are consistent with the peptide effectively competing with CaM binding to the CT of Na\(_{\text{V}}\)1.4 and supports the hypothesis that CaM binding to the CT is essential for modulation of the voltage dependence of inactivation gating. In the absence of CaM overexpression, AIP290-309 induced a +7-mV shift in the steady-state inactivation curve (\(V_{1/2} = -58.5 \pm 0.2\) mV, \(n = 7\)) compared with cells expressing only Na\(_{\text{V}}\)1.4-EYFP (Fig. 3 B). AIP290-309 may be acting by displacement of endogenous CaM from the channel or through inhibition of CaM binding to other sites on Na\(_{\text{V}}\)1.4 or other effector proteins. However, in cells expressing Na\(_{\text{V}}\)1.4\(_{\text{IQ/AA}}\), AIP290-309 induces an \(\sim +12\)-mV shift in the steady-state inactivation curve (\(V_{1/2} = -61.1 \pm 0.1\) mV, \(n = 10\)) compared with cells expressing Na\(_{\text{V}}\)1.4\(_{\text{IQ/AA}}\) in the absence of AIP (\(V_{1/2} = -73.9 \pm 0.2\) mV, \(n = 7\); Fig. 3 B). AIP290-309 has several distinct effects on gating of Na\(_{\text{V}}\)1.4\(_{\text{IQ/AA}}\) (Fig. 3 C). Channel recovery from inactivated states is similar in Na\(_{\text{V}}\)1.4\(_{\text{IQ/AA}}\) and wild-type Na\(_{\text{V}}\)1.4 channels. (D) Overexpression of wild-type CaM (○) but not CaM\(_{\text{1234}}\) (△) shifts the steady-state inactivation curve in the hyperpolarizing direction compared with Na\(_{\text{V}}\)1.4\(_{\text{IQ/AA}}\) (○) or wild-type Na\(_{\text{V}}\)1.4 (□) expressed alone. (E) The steady-state inactivation curve of Na\(_{\text{V}}\)1.4\(_{\text{IQ/AA}}\) is unaffected by overexpression of CaM (△) or CaM\(_{\text{1234}}\) (□).
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unmasks effects of AIP290-309 that are independent of CaM binding to the channel.

To further elucidate role of the IQ region in CaM binding and channel function, we studied deletion mutants with and without the IQ motif of the NaV1.4 CT region. Deletion mutations of the NaV1.4 CT that preserve the IQ motif (e.g., NaV1.41720–G4–CaM) in HEK293 cells display fluorescence enrichment at the cell perim-eter. Line scans of fluorescence intensity are consistent with surface membrane expression of this truncation mutant (Fig. 4 A). Further qualitative analysis of Western blots of purified biotinylated NaV1.41720 protein demonstrates expression of the truncated channels at the surface membrane (Fig. 4 B). The family of currents elicited by voltage-clamp pulses of the deletion mutant NaV1.41740 is similar to intact NaV1.4 (Fig. 4 C). The normalized current–voltage (Fig. 4 C) relationships of the NaV1.41740 mutant and wild-type channels are nearly identical with the peak current amplitudes at \( V \) = 0 mV.

\[ \tau \text{ inact} = 1.7 \pm 0.1 \text{ ms (} n = 5 \text{), not significantly different from wild-type channel (1.9} \pm 0.1 \text{ ms, } n = 5; \text{ Fig. 4 D)}. \]

Similarly, truncation of the nonstructured part of CT in NaV1.4 distal to the IQ region does not significantly alter \( V \) = 0 mV entry (1165 \pm 17 ms, \( n = 6 \)), compared with the wild-type channel (1088 \pm 20 ms, \( n = 5 \); Fig. 4 E). The steady-state inactivation curve of NaV1.41740 reveals a \( V \text{,}1/2 \) of 62.7 \pm 0.1 mV (\( n = 6 \)) and coexpression of ECFP-CaM with NaV1.41740 shifts the \( V \text{,}1/2 \) \( \sim 5 \) mV (\( V \text{,}1/2 \) = -68.0 \pm 0.1 mV, \( n = 8 \), \( P < 0.04 \)) in the hyperpolarizing direction (Fig. 4 F).

Overexpression of ECFP-CaM1234 with NaV1.41740 does not shift the availability curve compared with NaV1.41740 expression alone (\( -64.6 \pm 0.04 \text{ mV}, n = 5; \text{ Fig. 4 F) \). Thus the deletion mutant NaV1.41740 retains the basic biophysical properties of NaV1.4 channels and CaM-induced modulation of steady-state inactivation (Table I). These data suggest that the nonstructured distal portion of the CT (after amino acid 1740) is not critical for modulation of channel gating by CaM. In contrast, deletion after amino acid 1723, which includes the entire nonstructured region and the IQ motif of CT-NaV1.4 (NaV1.41723), generated no current (Fig. 4 G). Thus the region containing the IQ motif in NaV1.4 is essential to functional expression of the channel.

To define the mechanisms of CaM modulation of NaV1.4 current we sought to determine the number of CaM molecules required for the channel modulation. We created a channel with CaM fused to the CT of NaV1.41740 through variable length glycine linkers to promote the association of CaM with the channel from the earliest stages of biosynthesis and to increase the local concentration of CaM in the vicinity of IQ motif. Two different lengths of glycine linkers were used to tether CaM to the channel, short (4 glycines, NaV1.41740–G4–CaM) and longer (14 glycines, NaV1.41740–G14–CaM). The short linker does not permit CaM to have access to

![Figure 6. In vivo association of CaM with CT-NaV1.4 by FRET.](image-url)

(A) Donor dequenching FRET in live HEK293 cells expressing the indicated channel constructs. The top three bars in the graph are the FRET efficiencies of control experiments in cells not expressing channels, but expressing ECFP alone, ECFP and EYFP, or the linked chameleon (ECFP-EYFP). NaV1.4-EYFP coexpressed with ECFP or ECFP-CaM1234 or NaV1.4IQ/AA coexpressed with ECFP-CaM yield no detectible FRET. NaV1.4-EYFP coexpressed with ECFP-CaM exhibits significant FRET (>5% efficiency). (B) Peak current densities of expressed NaV1.4 and NaV1.4IQ/AA channels used for FRET measurements. The densities of NaV1.4-EYFP coexpressed with ECFP-CaM or ECFP-CaM1234 or NaV1.4IQ/AA coexpressed with ECFP-CaM are not significantly different from the current density of NaV1.4-EYFP coexpressed with ECFP alone. (C) Interaction of CaM and NaV1.4 assayed by the 33 FRET method. This nondestructive technique confirms the proximity of the fluorophores and the production of FRET in cells expressing NaV1.4-EYFP and ECFP-CaM by a FRET ratio of almost 2. (D) Cells coexpressing NaV1.4-EYFP and ECFP-CaM are held at different membrane voltages to populate different channel conformations. FRET is comparable in cells held at \(-140 \) and \(-60 \) mV, and is present but reduced at a holding voltage of \(-20 \) mV.
mately -20 mV. The \( \tau_{\text{rec}} \) of Na\textsubscript{v}1.4\textsubscript{1740-G4-CaM} (4.2 ± 0.2 ms, \( n = 8 \)) is not significantly different from that of Na\textsubscript{v}1.4\textsubscript{1740-G14-CaM} (4.6 ± 0.3 ms, \( n = 5 \); Fig. 5 B), but both are longer than the full-length Na\textsubscript{v}1.4. Similarly lengthening the glycine linker from 4 to 14 does not significantly alter \( \tau_{\text{rec}} \) (Na\textsubscript{v}1.4\textsubscript{1740-G4-CaM} 700 ± 15 ms, \( n = 6 \), versus Na\textsubscript{v}1.4\textsubscript{1740-G14-CaM} 760 ± 10.4 ms, \( n = 6 \), Fig. 5 C). We then examined the voltage-dependent shift in steady-state inactivation mediated by CaM binding to the CT. The \( V_{1/2} \) of the steady-state inactivation curve of Na\textsubscript{v}1.4\textsubscript{1740-G4-CaM} is -65.1 ± 0.1 mV (\( n = 8 \)) and overexpression of ECFP-CaM with Na\textsubscript{v}1.4\textsubscript{1740-G4-CaM} shifts the \( V_{1/2} \sim 5 \) mV (\( V_{1/2} = 71.5 ± 0.2 \) mV, \( n = 6 \)) in the hyperpolarizing direction (Fig. 5 D). Overexpression of ECFP-CaM\textsubscript{1234} with Na\textsubscript{v}1.4\textsubscript{1740-G4-CaM} does not shift channel availability curve compared with Na\textsubscript{v}1.4\textsubscript{1740-G4-CaM} expression alone (-63.6 ± 0.2 mV, \( n = 5 \); Fig. 5 D). Thus the shorter length glycine linker fused CaM to channel (Na\textsubscript{v}1.4\textsubscript{1740-G4-CaM}) retained the CaM-induced modulation of steady-state inactivation of wild-type Na\textsubscript{v}1.4 channels (Table I). In contrast, lengthening the glycine linker to 14 shifts the steady-state inactivation curve of Na\textsubscript{v}1.4\textsubscript{1740-G14-CaM} in the hyperpolarizing direction (\( V_{1/2} = 68.8 ± 0.1 \) mV, \( n = 5 \)) and overexpression of ECFP-CaM does not alter the voltage dependence of inactivation of Na\textsubscript{v}1.4\textsubscript{1740-G14-CaM} (\( V_{1/2} = 67.7 ± 0.2 \) mV, \( n = 9 \); Fig. 5 E). Similarly overexpression of ECFP-CaM\textsubscript{1234} with Na\textsubscript{v}1.4\textsubscript{1740-G14-CaM} (\( V_{1/2} = 66.7 ± 0.2 \) mV, \( n = 9 \)) does not shift channel availability curve compared with Na\textsubscript{v}1.4\textsubscript{1740-G14-CaM} expression alone (Fig. 5 E). CaM linked to the channel by four glycines remained sensitive to overexpression of CaM; in contrast, CaM linked by 14 glycines was no longer sensitive to CaM overexpression, suggesting that a single CaM is sufficient for Na\textsubscript{v}1.4 modulation. These data further support the role of CaM binding to the CT of Na\textsubscript{v}1.4 in modulating the voltage dependence of inactivation of the channel.

Association of CaM with CT-Na\textsubscript{v}1.4 Assessed by FRET

The electrophysiological data suggest that CaM is tethered to Na\textsubscript{v}1.4. We coexpressed Na\textsubscript{v}1.4-EYFP and ECFP-CaM and used donor dequenching FRET to study the proximity of ECFP-CaM to the CT of Na\textsubscript{v}1.4. The FRET efficiency (%) using donor dequenching was computed as previously described (Erickson et al., 2001). Cells expressing ECFP alone (\( n = 5 \)) or coexpressing ECFP with EYFP (\( n = 15 \)) exhibited no FRET (Fig. 6 A); however, HEK293 cells expressing the linked chameleon ECFP-EYFP yield a FRET efficiency of 28.3 ± 2.8\% (\( n = 12 \); Fig. 6 A), which is consistent with previously reported FRET results in Ca\textsubscript{v}1.2 (Erickson et al., 2001). A mean FRET efficiency 10.3 ± 1.3\% (\( n = 22 \); Fig. 6 A) was observed when ECFP-CaM was coexpressed with EYFP-tagged channels, indicating that the fluorophores in Na\textsubscript{v}1.4-EYFP and ECFP-CaM were separated by <100 Å. In contrast, when the IQ residues of the CaM binding motif in

Figure 7. Influence of CaM on Na\textsubscript{v}1.4 channel expression and localization. (A) Western blots of lysates from HEK293 cells transfected with wild-type Na\textsubscript{v}1.4 and mutant Na\textsubscript{v}1.4\textsubscript{IQ/AA} channels reveal a reduction in surface expression of Na\textsubscript{v}1.4 protein by the IQ/AA mutation compared with wild type with no significant change in immunoreactive protein in total cell lysates. The protein is detected with the antibodies described in Fig. 1. (B) The ratio of cell surface expression to total expression of Na\textsubscript{v}1.4\textsubscript{IQ/AA} is significantly (\( P < 0.05 \), \( n = 4 \)) reduced compared with wild-type Na\textsubscript{v}1.4 and Na\textsubscript{v}1.4\textsubscript{IQ/AA}. (C) Confocal images of Na\textsubscript{v}1.4-EYFP expressed in HEK293 cells. EYFP-fused wild-type channels are distributed in both the periphery and center of the cell. The right panel is a DIC image and bottom panel is the merge of the DIC and fluorescence images. (D) In contrast, Na\textsubscript{v}1.4\textsubscript{IQ/AA} mutant channels fused to EYFP are predominantly distributed in the perinuclear region of the cell, indicating mutant channels are synthesized but remain trapped in internal membranes. The images are as described in C.
the NaV-CT were substituted by alanines (NaV1.4IQ/AA), no FRET was observed between the donor ECFP-CaM and acceptor NaV1.4IQ/AA-EYFP (Fig. 6 A). Coexpressing free cytosolic ECFP with NaV1.4-EYFP does not yield FRET (Fig. 6 A). The absence of FRET between the mutant IQ channel and CaM, and the wild-type channel and free cytosolic ECFP, excludes possibility of FRET due to generalized enrichment of CaM at the cell membrane, and nonspecific binding of CaM to the channel. Moreover, no FRET was evident between the CaM EF hand mutant ECFP-CaM1234 and NaV1.4-EYFP (Fig. 6 A), suggesting the absence of unconventional binding sites for apo-CaM in the intact CT of NaV1.4, as has been observed in L-type Ca2+ channels (Erickson et al., 2001, 2003). Mutant CaM1234 does not bind Ca2+ at physiological intracellular concentrations (Xia et al., 1998), suggesting that the interaction of CaM with NaV1.4 under resting conditions is Ca2+ dependent.

To determine if channel surface expression influenced FRET efficiency, current densities of the channels were compared in the presence and absence of ECFP-CaM coexpression (Fig. 6 B; Table I). The presence of ECFP-CaM did not significantly alter the current density of any of the Na channel variants studied compared with the channel expressed alone or coexpressed with ECFP. However we caution that the cells studied were highly selected to have peak current densities in a range that allows for adequate detection and reliable voltage control.

The association of CaM with the CT of NaV1.4 was tested with a complementary, nondestructive FRET measurement technique, 3F FRET (Erickson et al., 2001). Expression of CT-NaV1.4-EYFP with ECFP-CaM resulted in a FRET ratio (FR) of >1, with a mean FRET efficiency \( n = 12 \) (Fig. 6 C), confirming the association of CaM to the CT-NaV1.4. Consistent with the donor dequenching FRET results, the 3F FRs were <1 in cells expressing the NaV1.4IQ/AA channel with CaM (Fig. 6 C). Thus FRET is the result of a specific CaM-channel interaction at the IQ motif.

To understand the mechanism of modulation of inactivation of NaV1.4 we examined the voltage dependence of the interaction of CaM and its proximity to the CT fluorescent Na channel tag. We measured donor dequenching FRET between NaV1.4-EYFP and ECFP-CaM while holding the channels at three different potentials: −140 mV (favoring closed channels), −60 mV (\( \sim V_{1/2} \) of the inactivation curve), and −20 mV (favoring inactivated channels). Coexpressing ECFP-CaM and NaV1.4-EYFP enhanced the ECFP signal in patch-clamped cells held at −140 mV, leading to a mean 22.7 ± 2.0% (\( n = 15 \)) FRET efficiency, which is significantly (\( P < 0.0006 \)) higher than the FRET efficiency of unpatched cells (Fig. 6 D). Similarly, at a holding voltage of −60 mV, a mean FRET efficiency of 25.4 ± 1.8% (\( n = 9 \)) was observed (Fig. 6 D). However, holding at −20 mV yielded a FRET efficiency (9.9 ± 2.3%, \( n = 15 \)) similar to FRET of unpatched cells (Fig. 6 D). CaM does not appear to dissociate from the channel in the closed or inactivated conformations.

**CaM Influences Channel Trafficking**

CaM binding to the CT of NaV1.4 under resting conditions and studies describing reduced current density by expressed NaV channel isoforms that have been mutated to impair CaM binding (Cormier et al., 2002; Herzog et al., 2003) suggest that CaM binding influences channel trafficking and surface expression. In addition to calmodulation of steady-state inactivation of NaV1.4, we assessed whether CaM influenced channel trafficking and expression. Lysates isolated from cells expressing wild-type or mutant NaV1.4IQ/AA channel proteins were separated on SDS-PAGE gels (Fig. 7 A) and band intensities were measured to estimate the level of total channel expression. To assess effect of CaM binding on surface expression, cells expressing wild-type or mutant NaV1.4IQ/AA channels were labeled with membrane-impermeable biotin and purified with streptavidin beads. The band intensities of biotinylated proteins, representing cell surface expression, were normalized to the respective total channel protein expression and plotted in Fig. 7 B. The NaV1.4IQ/AA mutant exhibits significantly (\( P < 0.05, \ n = 4 \)) reduced surface expression compared with wild-type NaV1.4 and the truncation mutant distal to the IQ, NaV1.4IQ/AA2140 (Fig. 7 B). Truncation of NaV1.4 further upstream at residue 1723 (NaV1.4IQ/AA1723) eliminates channel function (Fig. 4 G) but does not eliminate cell surface expression, as detected by Western blot and epifluorescence microscopy (Fig. S2, available at http://www.jgp.org/cgi/content/full/jgp.200709863/DC1). The data suggest that the CaM binding region of the intact CT of NaV1.4 is essential for functional cell surface channel expression. To further examine trafficking of the IQ mutant channel, NaV1.4-EYFP or NaV1.4IQ/AA1723-EYFP were expressed in HEK293 cells, and the cellular channel protein distribution was examined with confocal microscopy. Fluorescence from EYFP-fused wild-type channels was distributed in both the periphery and center of the cell, consistent with trafficking from perinuclear membranes to cell surface (Fig. 7 C). In contrast, signals from the NaV1.4IQ/AA-EYFP mutant channels were predominantly in the perinuclear region, indicating mutant channels are synthesized but remain trapped in intracellular membranes (Fig. 7 D). Compared with the wild-type NaV1.4 channel, more NaV1.4IQ/AA channels remain internalized, consistent with the reduced surface expression of the NaV1.4IQ/AA protein observed in Western blot analysis (Fig. 7, A and B). Although the IQ/AA mutation interferes with cell surface trafficking of NaV1.4, truncation of the CT proximal to IQ generates nonfunctional channels that are expressed on the cell surface. It is possible that the IQ/AA mutation creates a signal that mediates retention in subcellular membranes such
as the ER. This mutation-mediated retention signal is eliminated by truncation of the IQ motif (i.e., Na\textsubscript{v1.4\textsubscript{1723}}).

resulting in normal surface membrane trafficking of nonfunctional channels. Either the IQ motif itself or CaM binding to IQ promotes a structure of this region of the CT, likely involving residues between 1723 and 1740, that is trafficked to the cell surface.

**DISCUSSION**

The cytoplasmic domain of the voltage-gated sodium channel participates in the formation of a multiprotein complex (Abriel and Kass, 2005). Protein-protein interactions involving the CT influence the expression, membrane trafficking, subcellular localization, and gating properties of the pore-forming subunit. Mutations of the IQ motif in the CT of Na\textsubscript{v1.4} channels have been shown to alter slow inactivation kinetics, generate sustained current, and reduce current density (Cormier et al., 2002; Deschênes et al., 2002; Herzog et al., 2003; Kim et al., 2004). In this study we have demonstrated, that (a) CaM is bound to the CT of intact, expressed Na\textsubscript{v1.4} at the IQ motif in a conformation-independent manner and CaM is not displaced by overexpression of the EF hand mutant CaM\textsubscript{1234} (Figs. 3, 5, and 6); (b) CaM binding alters the voltage dependence of steady-state inactivation (Fig. 3A); (c) the IQ region in CT-Na\textsubscript{v1.4} is indispensable for functional expression (Fig. 4); (d) only one CaM molecule is sufficient to modulate Na\textsubscript{v1.4} channel inactivation gating current (Fig. 5, D and E); and (e) mutations in CaM binding IQ motif influence Na\textsubscript{v1.4} surface channel protein expression (Fig. 7).

CaM acts as a channel subunit binding to a C-terminal IQ motif, and a calcium sensor for calcium-dependent inactivation of Ca\textsubscript{v1.2} (Peterson et al., 1999; Zuhlke et al., 1999; Erickson et al., 2001; Van Petegem et al., 2005). An homologous IQ binding domain is present in all Na\textsubscript{v} sodium channel isoforms; however, CaM’s interaction with Na\textsubscript{v} channels and its functional effects appear to be isoform specific (Mori et al., 2000; Deschênes et al., 2002; Kim et al., 2004; Young and Caldwell, 2005; Choi et al., 2006). To begin to understand the isoform-specific functional effects of Ca\textsuperscript{2+}/CaM/CaMK on Na\textsubscript{v} isoforms and the role of the IQ motif and alternate sites of CaM and apo-CaM interaction we have examined the effect of CaM binding to the CT of skeletal muscle channel, Na\textsubscript{v1.4}.

CaM Is Tethered to Intact Na\textsubscript{v} 1.4 through an IQ Motif

Our data conclusively demonstrate tethering of CaM to the CT of intact Na\textsubscript{v1.4} channels in live cells. Deletion mutations of the Na\textsubscript{v1.4} CT that preserve the IQ motif (Na\textsubscript{v1.4\textsubscript{1720}}) generated currents that had voltage dependences and kinetics of activation and inactivation that were similar to wild-type Na\textsubscript{v1.4} (Fig. 4, C and F).

In contrast, deletion of the entire nonstructured region and part of the terminal \(\alpha\)-helix including the IQ motif (Na\textsubscript{v1.4\textsubscript{1725}}) exhibit no current (Fig. 4 G), indicating that the region containing the IQ motif and CaM binding to this motif in Na\textsubscript{v1.4} is indispensible for normal sodium channel functional expression. CaM tethering to the IQ region was further confirmed by the absence of an effect of CaM\textsubscript{1235} on the CaM-induced shift in gating of Na\textsubscript{v1.4} (Fig. 3A). In contrast, the effects of AIP290-309 (CaM anti-peptide) are more complex and independent of CaM binding to the channel at the IQ motif (Fig. 3B–E). AIP290-309 alters the gating of wild-type and IQ/AA mutant Na\textsubscript{v1.4} channels. Inactivation of Na\textsubscript{v1.4\textsubscript{IQ/AA}} is destabilized by AIP290-309 independent of the presence of CaM overexpression (Fig. S1), whereas, steady-state inactivation of Na\textsubscript{v1.4} is shifted to depolarized potentials by AIP290-309 with no effect on the IV curve or recovery from inactivation. The effects of AIP290-309 on wild-type Na\textsubscript{v1.4} gating are eliminated by overexpression of CaM (Fig. 3B). Whether AIP290–309 interacts with the channel directly and the site(s) of that interaction remain uncertain. The effects of AIP290-309 on Na\textsubscript{v1.4\textsubscript{IQ/AA}} do not appear to be the result of a general inhibition of endogenous CaM function as overexpression of CaM does not mitigate the AIP290-309-induced changes in Na\textsubscript{v1.4\textsubscript{IQ/AA}} gating.

In resting cells, wild-type CaM increased the donor dequenching FRET signal in tagged, presumably inactivated Na\textsubscript{v1.4} channels (Fig. 6, A and C). Changing the membrane voltage did not eliminate FRET, suggesting that CaM is tethered to the CT of Na\textsubscript{v1.4} in all channel conformations; however, the proximity of the fluorophores may be different in channels subjected to depolarized voltages for extended periods of time (slow inactivated states). In any case, these data confirm the importance of the IQ motif in the CT for CaM binding and modulation of the voltage dependence of gating of Na\textsubscript{v1.4} channels. This is in contrast to a previously proposed mechanism of gating for voltage-gated cardiac sodium channels, Na\textsubscript{v1.5} (Shah et al., 2006), where CaM has been proposed to bind to the IQ motif at resting Ca\textsuperscript{2+} levels, but later dissociates from the CT upon Ca\textsuperscript{2+} binding, thus enabling the CaM-free IQ motif to interact with an EF hand motif in the CT of the channel. However we observed wild-type Na\textsubscript{v1.4} and mutant Na\textsubscript{v1.4\textsubscript{IQ/AA}} are both regulated by Ca\textsuperscript{2+} in the presence of endogenous CaM (unpublished data). Moreover, CaM-mediated regulation of Na\textsubscript{v1.4} is lost in the absence of Ca\textsuperscript{2+}, consistent with Ca\textsuperscript{2+}-dependent CaM regulation of Na\textsubscript{v1.4} gating (Deschênes et al., 2002). Finally, Na\textsubscript{v1.4\textsubscript{IQ/AA}} mutant channels are not regulated by CaM but retain sensitivity to Ca\textsuperscript{2+}, thus at least one form of Ca\textsuperscript{2+}-mediated regulation of channel gating does not require an interaction between the IQ motif and the EF hand. The loss of FRET between CaM and Na\textsubscript{v1.4\textsubscript{IQ/AA}}, and the absence of an interaction between wild-type Na\textsubscript{v1.4} and CaM\textsubscript{1235} indicates that CaM-Ca\textsuperscript{2+} binds to the CT via the IQ motif. CaM-Ca\textsuperscript{2+} regulation of channel gating is
mediated principally through Ca\textsuperscript{2+} binding to CaM complexes with IQ in the Na\textsubscript{V}1.4-CT.

A Functional Model for the Regulation of Na\textsubscript{V}1.4 Channel Gating by CaM

The conformation-independent tethering of CaM to the IQ motif is central to the model presented. Additional electrophysiological evidence supports the idea that CaM does not dissociate from the IQ motif in Na\textsubscript{V}1.4. First, overexpression of CaM\textsubscript{1234} does not eliminate the CaM-mediated effect on steady-state inactivation, indicating that only CaM-mediated regulation (Fig. 3 A). Second, overexpression of both CaM and CaM\textsubscript{1234} shows similar regulation to that exhibited by CaM alone (Fig. 5 B), indicating that the Ca\textsuperscript{2+} binding–deficient CaM\textsubscript{1234} does not displace CaM. Finally, among the variable length glycine–linked CaM fused to CT only the linker length that allows CaM to reach IQ region exhibits CaM modulation of gating, indicating one CaM molecule is sufficient for regulation of inactivation (Fig. 5, D and E). Overall the data are consistent with CaM being tethered to the CT-Na\textsubscript{V}1.4 during normal gating.

The IQ motifs are highly conserved across Na\textsubscript{V} isoforms, suggesting an important functional role for these regions. It is conceivable that the cytoplasmic tail of Na\textsubscript{V}1.4 exists in a disordered form under basal depolarized conditions, when subjected to more polarized voltages, the cytoplasmic tail could be more structured due to an increase in helicity, leading to an altered binding relationship of CaM and the IQ motif (Fig. 6 D). It is possible that the binding of CaM to the more structured CT facilitates interactions with other cytoplasmic domains at pore mouth that participate in and stabilize inactivation. Further structural characterization of the Na\textsubscript{V}1.4-CT and its interactions with CaM are required to critically test this hypothesis and will aid in understanding the role of CaM binding in various cardiac and muscular disorders.

Role of CaM Binding in Surface Expression of Na\textsubscript{V}1.4

We demonstrate that CaM participates in the modulation of cell surface expression of intact Na\textsubscript{V}1.4 channels by interaction with the IQ motif similar to other channels such as KCNQ1 (Ghosh et al., 2006), SK4 channels (Joiner et al., 2001; Lee et al., 2003), and KCNQ2/Q3 potassium channels (Wen and Levitan., 2002). It has been suggested that CaM behaves as an obligate subunit of the calcium channel, Ca\textsubscript{V}1.2, altering both function and expression of this α subunit. Similarly, CaM acts as an obligate subunit of some Na\textsubscript{V}1 channels, necessary for proper folding (Kim et al., 2004). Our data reveal that mutations in the CaM binding motif reduce total and cell surface expression of Na\textsubscript{V}1.4. More proximal truncations of the channel that include the IQ motif (Na\textsubscript{V}1.4\textsubscript{1723}, Fig. 3 G and Fig. S2) support a role for other regions of the CT of the Na channel in both function and expression. It is possible that the IQ/AA mutation leads to defects in proper channel folding; thus the role of CaM could be in stabilization of proper channel folding and facilitating trafficking of the channel to the cell surface. CaM regulation of channel trafficking to the cell surface and its role in the early stages of channel formation could be a general phenomenon further adding to the diversity of the CaM signaling on ion channel function.

Our data demonstrate an intimate relationship between intact Na\textsubscript{V}1.4 channels and CaM that influences channel gating and functional expression, and that a single CaM molecule is sufficient to regulate Na\textsubscript{V}1.4 inactivation gating. In live cells, CaM is associated with CT-Na\textsubscript{V}1.4 under resting conditions and CaM remains tethered to the CT-Na\textsubscript{V}1.4 over a wide range of voltages, indicating no significant displacement of CaM from any channel conformation. The Na\textsubscript{V}1.4\textsubscript{IQ/AA} mutant eliminates the proximity of CaM to CT-Na\textsubscript{V}1.4 and reduces Na\textsubscript{V}1.4 surface expression. Thus CaM is tethered to CT-Na\textsubscript{V}1.4 at the IQ motif and is essential for normal inactivation gating of Na\textsubscript{V}1.4, functions to stabilize channel conformation, and promotes membrane trafficking of the channel protein.

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