INTRODUCTION

Voltage-gated sodium channels (Nav) underlie the rising phase and influence the duration of action potentials in excitable cells such as neurons and cardiac and skeletal myocytes. Heritable defects in Na channel expression and gating 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 Nav1.4 has not previously been demonstrated. FRET reveals channel conformation-independent association of CaM with the C terminus of Nav1.4 (CT-Nav1.4) in mammalian cells. Mutation of the Nav1.4 CaM-binding IQ motif (Nav1.4IQ/AA) reduces cell surface expression of Nav1.4 channels and eliminates CaM modulation of gating. Truncations of the CT that include the IQ region abolish Na current. Nav1.4 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 Nav1.4 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. Fragments were directionally cloned into this site and a NotI site in codon of Nav1.4 was replaced with an NheI site, and the EYFP/ECFP fragments were directionally cloned into this site and a NotI site in codon of Nav1.4 was replaced with an NheI site, and the EYFP/ECFP

Electrophysiology

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Cell Culture and Transfection

Construction of Na V 1.4-ECFP/EYFP Deletion Mutants. Inserts containing the Na V 1.4 cDNA were that were truncated at codons 1723 (Na.v 1.4,1723) or 1740 (Na.v 1.4,1740) in frame with either ECFP or EYFP, were generated by PCR using the forward primer Na.v 1.4-BstZF (CTGCGGTCACAGACGACAGCT) and either one of the reverse primers Na.v 1.4,1723NheR (1736 bp) or Na.v 1.4,1740NheR (1787 bp). The reverse primers used to prepare the truncated fragment of Na.v 1.4,1723NheR (1736 bp) or Na.v 1.4,1740NheR (1787 bp). The reverse primers used to prepare the truncated fragment of Na.v 1.4,1723NheR (1736 bp) or

Construction of Na V 1.4,1740 -Glycine-CaM-EYFP. Glycine (G₄)-linkered CaM was inserted into Na V 1.4,IQ-EYFP by cloning a PCR product of full-length CaM flanked by in-frame NheI sites into an NheI site located between the channel and fluorescent protein coding regions. The G₄-CaM insert was prepared by PCR using a calmodulin-containing plasmid as template and the forward primer ₅'-attatctGCTAGGGAGAGGAGGAGGATGCTGACATGAG₃' and the reverse primer ₅'-attatctGCTAGGCCTCTGCTGATTCCGAGGTGGCAGGGGAGTGGCTGACATGAG₃'. The forward primer contains an in-frame NheI site followed by four glycine codons and then the calmodulin start sequence, the reverse primer contains an NheI site and the end of the calmodulin coding region without the stop codon. The G₄ construct was used to prepare the construct with CaM linker with 14 glycines (G₁₄). The linker length was increased from 4 to 14 glycines by mutation of the 5’ NheI site into an AvrII site and the insertion of annealed oligonucleotides encoding 10 glycines and containing AvrII sticky ends.

Cell Culture and Transfection

Approximately 0.75 × 10⁶ 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 mmol/liter), penicillin (100 U/mL), and streptomycin (10 mg/mL). The cells were cotransfected with pBluescript IISK+ (Stratagene) and pCMV-His (Invitrogen) and 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 Na.V 1.4 channels and CaM or CaM 1234 were patch clamped with an Axopatch 200B patch clamp amplifier using pipettes with tip resistances of 5–3 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 BaCl₂, 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 30-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 D535/50× excitation filter and a 1095% mirror (in place of a dichroic mirror). In control experiments this bleaching protocol spared CFP fluorescence. Epifluorescence images were acquired with a PI300 CCD camera (Princeton Instruments) mounted to the side port of Olympus IX70 inverted microscope (60 × 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 (EDET) 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).

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The CaM and Na V 1.4 interaction was further studied using the nondestructive 33 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 artifactual 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 (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 D535/50× excitation filter and a 1095% mirror (in place of a dichroic mirror). In control experiments this bleaching protocol spared CFP fluorescence. Epifluorescence images were acquired with a PI300 CCD camera (Princeton Instruments) mounted to the side port of Olympus IX70 inverted microscope (60 × 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 (EDET) 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).

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Cell Surface Expression and Western Blot Analysis

The cells were cotransfected with equal amounts (0.5 μg/ml) of either wild-type Na.v 1.4-EYFP or Na.v 1.4,1740-EYFP or Na.v 1.4,1740-EYFP plasmids. The concentrations of plasmids were estimated spectrophotometrically and further confirmed by agarose gel electrophoresis (not depicted). The cells were grown to a similar level of confluence and then surface channels were biotinylated and

Calmodulation of Sodium Channels
TABLE I
Effect of CaM and CaM_{1234} Overexpression on NaV_{1.4}, NaV_{1.4-EYFP} and NaV_{1.4-G14-CaM+EYFP}

| Channel/Mutant | V_{1/2} steady state inactivation | Decay time constant $\tau_b$ | V_{1/2} activation | Current density $\mu A/\mu F$ |
|----------------|---------------------------------|-------------------------------|-------------------|-----------------------------|
| NaV_{1.4-EYFP} + $\beta 1$ | $-65.2 \pm 0.4$ (n = 5) | $0.69 \pm 0.04$ | $-32.2 \pm 0.1$ | $-426.4 \pm 93.3$ |
| NaV_{1.4-EYFP} + ECFP-CaM + $\beta 1$ | $-71 \pm 0.2$ (n = 5) | $0.77 \pm 0.05$ | $-32.6 \pm 0.2$ | $-458.0 \pm 54.0$ |
| NaV_{1.4-EYFP} + ECFP-CaM_{1234} + $\beta 1$ | $-64.2 \pm 0.1$ (n = 5) | $0.66 \pm 0.02$ | $-32.9 \pm 0.1$ | $-269.6 \pm 55.5$ |
| NaV_{1.4} + $\beta 1$ | $-65.8 \pm 0.1$ | $0.61 \pm 0.07$ | $-32.1 \pm 0.3$ | $-453.2 \pm 71.5$ |
| NaV_{1.4} + ECFP-CaM + $\beta 1$ | $-69.3 \pm 0.1$ (n = 7) | $0.63 \pm 0.02$ | $-30.6 \pm 0.2$ | $-309.4 \pm 112.0$ |
| NaV_{1.4} + ECFP-CaM_{1234} + $\beta 1$ | $-64.3 \pm 0.5$ (n = 7) | $0.66 \pm 0.07$ | $-31.2 \pm 0.1$ | $-324.1 \pm 80.9$ |
| NaV_{1.4-Q/AA} + $\beta 1$ | $-79.4 \pm 0.2$ (n = 6) | $1.5 \pm 0.2$ | $-40.9 \pm 0.3$ | $-307.8 \pm 89.9$ |
| NaV_{1.4-Q/AA} + ECFP-CaM + $\beta 1$ | $-80.0 \pm 0.4$ (n = 6) | $1.25 \pm 0.15$ | $-40.0 \pm 0.1$ | $-242.5 \pm 60.5$ |
| NaV_{1.4-Q/AA} + ECFP-CaM_{1234} + $\beta 1$ | $-80.7 \pm 0.5$ (n = 5) | $1.12 \pm 0.15$ | $-41.0 \pm 0.3$ | $-254.2 \pm 73.1$ |
| NaV_{1.4-T100} + $\beta 1$ | $-62.7 \pm 0.1$ (n = 6) | $0.69 \pm 0.09$ | $-30.1 \pm 0.2$ | $-437.8 \pm 116.0$ |
| NaV_{1.4-T100} + ECFP-CaM + $\beta 1$ | $-68.0 \pm 0.1$ (n = 8) | $0.62 \pm 0.02$ | $-29.5 \pm 0.1$ | $-294.0 \pm 74.7$ |
| NaV_{1.4-T100} + ECFP-CaM_{1234} + $\beta 1$ | $-64.0 \pm 0.04$ (n = 5) | $0.79 \pm 0.01$ | $-31.1 \pm 0.2$ | $-295.0 \pm 60.9$ |
| NaV_{1.4-T100-G14-CaM + $\beta 1$ | $-65.8 \pm 0.1$ (n = 8) | $0.69 \pm 0.09$ | $-30.8 \pm 0.3$ | $-372.9 \pm 67.4$ |
| NaV_{1.4-T100-G14-CaM + ECFP-CaM + $\beta 1$ | $-71.4 \pm 0.2$ (n = 9) | $0.55 \pm 0.3$ | $-30.7 \pm 0.1$ | $-373.0 \pm 77.5$ |
| NaV_{1.4-T100-G14-CaM + ECFP-CaM_{1234} + $\beta 1$ | $-63.6 \pm 0.2$ (n = 5) | $0.52 \pm 0.1$ | $-32.1 \pm 0.2$ | $-413.5 \pm 118.9$ |
| NaV_{1.4-T100-G14-CaM + ECFP-CaM_{1234} + $\beta 1$ | $-68.4 \pm 0.2$ (n = 9) | $0.60 \pm 0.1$ | $-31.9 \pm 0.3$ | $-201.4 \pm 63.2$ |
| NaV_{1.4-T100-G14-CaM + ECFP-CaM + $\beta 1$ | $-67.7 \pm 0.2$ (n = 9) | $0.51 \pm 0.06$ | $-30.2 \pm 0.3$ | $-391.5 \pm 121.1$ |
| NaV_{1.4-T100-G14-CaM + ECFP-CaM_{1234} + $\beta 1$ | $-66.7 \pm 0.1$ (n = 9) | $0.53 \pm 0.06$ | $-32.0 \pm 0.1$ | $-439.5 \pm 141.9$ |

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 NaV_{1.4 IQ/AA} in the presence of CaM and AIP. Fig. S2 illustrates the effect of the C-terminal truncation NaV_{1.4-G22} on channel trafficking by epifluorescence microscopy and Western blotting.

RESULTS
CT-Na_{1.4} Fusion Constructs Are Functionally Expressed and Interact with CaM
We have previously shown that CaM associates with wild type GST-tagged NaV CT peptides in vitro (Deschênes et al., 2002), this work motivated studies of the interaction between the CT of intact NaV 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 NaV_{1.4} 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.

Confocal microscopy
Approximately 0.75 × 10^6 cells were plated and then transfected on coverslips in a six-well culture dish fixed in 4% formaldehyde 48 h after transfection. Fixed HEK293 cells expressing NaV_{1.4-EYFP} or the IQ mutant NaV_{1.4-Q/AA-EYFP} fusion proteins were imaged using a confocal microscope (LSM510, Carl Zeiss MicroImaging, Inc.) with 63×/1.4 or 100×/1.4 oil differential interference contrast (DIC) plan apochromat objectives (Carl Zeiss MicroImaging, Inc.). EYFP was excited at 514 nm with an LSM510 Argon laser and emitted light was detected with a 530-600-nm band pass filter and beam splitter (HFT 405/514, mirror and NFT 515). Transmitted DIC images were obtained simultaneously.

Biswa et al. 199
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 NaV1.4-EYFP express robust currents (Fig. 1 B) that are indistinguishable from untagged wild-type NaV1.4 (Table I), confirming that labeled channels are functional and targeted to the membrane. Western blots of lysates from cells transfected with NaV1.4-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-CaM1234) alone in HEK293 cells is uniform throughout the cell (Fig. 2 A; ECFP-CaM1234 not depicted). Western blots from cells transfected with ECFP-CaM or ECFP-CaM1234 show uniform expression of CaM (endogenous and ECFP-conjugated) (Fig. 2 B). Western blots of lysates from cells transfected with NaV1.4-EYFP and ECFP-CaM1234 show an additional band of ~225 kD, consistent with membrane targeting of the ECFP-CaM1234 construct (Fig. 2 C). 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 NaV1.4-EYFP.

ECFP-CaM1234 exhibit distinct bands representing labeled CaM and CaM1234, 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
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{1224}. 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. 2C).

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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. 3A; 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. 3A). 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 CaM$_{1234}$ ($V_{1/2} = -32.9 \pm 0.1$ 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-CaM$_{1234}$ with NaV1.4 does not shift the channel availability curve compared with NaV1.4 expression alone in the presence of endogenous CaM (Fig. 3A). 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. Overexpression of both wild-type EYFP-CaM and ECFP-CaM$_{1234}$ with NaV1.4 produced no significant shift in the voltage dependence of the steady-state availability (Fig. 3A; $V_{1/2} = -69.3 \pm 0.6$ mV, $n = 10$) compared with ECFP-CaM overexpression alone, suggesting the possibility that CaM may be
in the absence of AIP (V$_{1/2}$ = 73.9 ± 0.2 mV, n = 7; Fig. 3 B). AIP290-309 has several distinct effects on gating of Na$_V$1.4 IQ/AA mutant channels. AIP290-309 shifts the peak current – voltage relationship to right in Na$_V$1.4 IQ/AA (Fig. 3 C). Channel recovery from inactivated states and entry into slowly recovering inactivated states were evaluated using the pulse protocols in the insets of Fig. 3, D and E, respectively. AIP hastens recovery ($\tau_{\text{rec}}$) from fast inactivation state in IQ/AA mutant channels without affecting the wild-type channel (Fig. 3 D). AIP290-309 significantly reduces $\tau_{\text{rec}}$ from 5.5 ± 0.1 ms (n = 7) to 4.5 ± 0.7 ms in Na$_V$1.4 IQ/AA (n = 10, Fig. 3 D) and decreases $\tau_{\text{entry}}$ from 1592.80 ± 127.4 ms (n = 6) to 815.3 ± 98.2 ms (n = 7, Fig. 3 E). In contrast, AIP290-309 did not significantly alter $\tau_{\text{rec}}$ or $\tau_{\text{entry}}$ of wild-type Na$_V$1.4 (Fig. 3, D and E). Thus the IQ/AA mutation unmasks effects of AIP290-309 that are independent of CaM binding.

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 Na$_V$1.4 CT region. Inclusion of the CaM anti-peptide (AIP290-309) in the pipette solution mitigates the CaM-induced shift in the steady-state inactivation curve (V$_{1/2}$ = 63.7 ± 0.2 mV, n = 7) in cells coexpressing Na$_V$1.4-EYFP and ECFP-CaM (Fig. 3 B). These data are consistent with the peptide effectively competing with CaM binding to the CT of Na$_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 ± 0.2 mV, n = 7) compared with cells expressing only Na$_V$1.4-EYFP (Fig. 3 B). AIP290-309 may be acting by displacement of endogenous CaM from the channel or through inhibition of CaM binding to other sites on Na$_V$1.4 or other effector proteins. However, in cells expressing Na$_V$1.4IQ/AA, AIP290-309 induces an ~+12-mV shift in the steady-state inactivation curve (V$_{1/2}$ = −61.1 ± 0.1 mV, n = 10) compared with cells expressing Na$_V$1.4IQ/AA in the absence of AIP (V$_{1/2}$ = −73.9 ± 0.2 mV, n = 7; Fig. 3 B). AIP290-309 has several distinct effects on gating of Na$_V$1.4IQ/AA that are not observed in the wild-type channel. AIP290-309 shifts the peak current – voltage relationship to right in Na$_V$1.4IQ/AA (Fig. 3 C). Channel recovery from inactivated states and entry into slowly recovering inactivated states were evaluated using the pulse protocols in the insets of Fig. 3, D and E, respectively. AIP hastens recovery ($\tau_{\text{rec}}$) from fast inactivation state in IQ/AA mutant channels without affecting the wild-type channel (Fig. 3 D). AIP290-309 significantly reduces $\tau_{\text{rec}}$ from 5.5 ± 0.1 ms (n = 7) to 4.5 ± 0.7 ms in Na$_V$1.4IQ/AA (n = 10, Fig. 3 D) and decreases $\tau_{\text{entry}}$ from 1592.80 ± 127.4 ms (n = 6) to 815.3 ± 98.2 ms (n = 7, Fig. 3 E). In contrast, AIP290-309 did not significantly alter $\tau_{\text{rec}}$ or $\tau_{\text{entry}}$ of wild-type Na$_V$1.4 (Fig. 3, D and E). Thus the IQ/AA mutation unmasks effects of AIP290-309 that are independent of CaM binding to the channel.
Deletion mutations of the Na\textsubscript{v}1.4 CT that preserve the IQ motif (e.g., Na\textsubscript{v}1.4\textsubscript{1740-EYFP}) expressed in HEK293 cells display fluorescence enrichment at the cell periphery. 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 Na\textsubscript{v}1.4\textsubscript{1740} 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 Na\textsubscript{v}1.4\textsubscript{1740} is similar to intact Na\textsubscript{v}1.4 (Fig. 4 C). The normalized current–voltage (Fig. 4 C) relationships of the Na\textsubscript{v}1.4\textsubscript{1740} mutant and wild-type channels are nearly identical with the peak current amplitudes at −20 mV. τ\textsubscript{rec} from fast inactivation of truncated channel is 1.7 ± 0.1 ms (n = 5), not significantly different from wild-type channel (1.9 ± 0.1 ms, n = 5; Fig. 4 D). Similarly, truncation of the nonstructured part of CT in Na\textsubscript{v}1.4 distal to the IQ region does not significantly alter τ\textsubscript{entry} (1165 ± 17 ms, n = 6), compared with the wild-type channel (1088 ± 20 ms, n = 5; Fig. 4 E). The steady-state inactivation curve of Na\textsubscript{v}1.4\textsubscript{1740} reveals a V\textsubscript{1/2} of −62.7 ± 0.1 mV (n = 6) and coexpression of ECFP-CaM with Na\textsubscript{v}1.4\textsubscript{1740} shifts the V\textsubscript{1/2} ∼5 mV (V\textsubscript{1/2} = −68.0 ± 0.1 mV, n = 8, P < 0.04) in the hyperpolarizing direction (Fig. 4 F). Overexpression of ECFP-CaM\textsubscript{1234} with Na\textsubscript{v}1.4\textsubscript{1740} does not shift the availability curve compared with Na\textsubscript{v}1.4\textsubscript{1740} expression alone (−64.6 ± 0.04 mV, n = 5; Fig. 4 F). Thus the deletion mutant Na\textsubscript{v}1.4\textsubscript{1740} retains the basic biophysical properties of Na\textsubscript{v}1.4 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-Na\textsubscript{v}1.4 (Na\textsubscript{v}1.4\textsubscript{1723}), generated no current (Fig. 4 G). Thus the region containing the IQ motif in Na\textsubscript{v}1.4 is essential to functional expression of the channel.

To define the mechanisms of CaM modulation of Na\textsubscript{v}1.4 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 Na\textsubscript{v}1.4\textsubscript{1740} 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, Na\textsubscript{v}1.4\textsubscript{1740-G4-CaM}) and longer (14 glycines, Na\textsubscript{v}1.4\textsubscript{1740-G14-CaM}). The short linker does not permit CaM to have access to the IQ motif, the longer construct will permit binding. Both constructs exhibit robust currents when expressed in HEK293 cells (Fig. 5 A). The normalized current–voltage (Fig. 5 A) relationships of the Na\textsubscript{v}1.4\textsubscript{1740-G4-CaM} or Na\textsubscript{v}1.4\textsubscript{1740-G14-CaM} and wild-type channel are nearly identical with the peak current amplitudes at −20 mV.
identical with the peak current amplitudes at approximately −20 mV. The $\tau_{\text{rec}}$ of Na$_V$1.4$_{1740}$-G4-CaM (4.2 ± 0.2 ms, $n = 8$) is not significantly different from that of Na$_V$1.4$_{1740}$-G14-CaM (4.6 ± 0.3 ms, $n = 5$; Fig. 5 B), but both are longer than the full-length Na$_V$1.4. Similarly lengthening the glycine linker from 4 to 14 does not significantly alter $\tau_{\text{rec}}$ (Na$_V$1.4$_{1740}$-G4-CaM 700 ± 15 ms, $n = 6$, versus Na$_V$1.4$_{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$_V$1.4$_{1740}$-G4-CaM is −65.1 ± 0.1 mV ($n = 8$) and overexpression of ECFP-CaM with Na$_V$1.4$_{1740}$-G4-CaM shifts the V$_{1/2}$~−5 mV (V$_{1/2}$ −71.5 ± 0.2 mV, $n = 6$) in the hyperpolarizing direction (Fig. 5 D). Overexpression of ECFP-CaM$_{1234}$ with Na$_V$1.4$_{1740}$-G4-CaM does not shift channel availability curve compared with Na$_V$1.4$_{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$_V$1.4$_{1740}$-G4-CaM) retains the CaM-induced modulation of steady-state inactivation of wild-type Na$_V$1.4 channels (Table I). In contrast, lengthening the glycine linker to 14 shifts the steady-state inactivation curve of Na$_V$1.4$_{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$_V$1.4$_{1740}$-G14-CaM (V$_{1/2}$ −67.7 ± 0.2 mV, $n = 9$, Fig. 5 E). Similarly overexpression of ECFP-CaM$_{1234}$ with Na$_V$1.4$_{1740}$-G14-CaM (V$_{1/2}$ −66.7 ± 0.2 mV, $n = 9$) does not shift channel availability curve compared with Na$_V$1.4$_{1740}$-G14-CaM expression alone (Fig. 5 E). CaM linked to the channel by four glycines remained sensitive to overexpression of CaM; in contrast, CaM linked by 14 glycines was no longer sensitive to CaM overexpression, suggesting that a single CaM is sufficient for Na$_V$1.4 modulation. These data further support the role of CaM binding to the CT of Na$_V$1.4 in modulating the voltage dependence of inactivation of the channel.

**Association of CaM with CT-Na$_V$1.4 Assessed by FRET**

The electrophysiological data suggest that CaM is tethered to Na$_V$1.4. We coexpressed Na$_V$1.4-EYFP and ECFP-CaM and used donor dequenching FRET to study the proximity of ECFP-CaM to the CT of Na$_V$1.4. The FRET efficiency (%) using donor dequenching was computed as previously described (Erickson et al., 2001). Cells expressing ECFP alone ($n = 5$) or coexpressing ECFP with expressed in HEK293 cells. EYFP-fused wild-type channels are distributed in both the periphery and center of the cell. The right panel is a DIC image and bottom panel is the merge of the DIC and fluorescence images. (D) In contrast, Na$_V$1.4$_{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.

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**Figure 7.** Influence of CaM on Na$_V$1.4 channel expression and localization. (A) Western blots of lysates from HEK293 cells transfected with wild-type Na$_V$1.4 and mutant Na$_V$1.4$_{IQ/AA}$ channels reveal a reduction in surface expression of Na$_V$1.4 protein by the IQ/AA mutation compared with wild type with no significant change in immunoreactive protein in total cell lysates. The protein is detected with the antibodies described in Fig. 1. (B) The ratio of cell surface expression to total expression of Na$_V$1.4$_{IQ/AA}$ is significantly ($P \leq 0.05$, $n = 4$) reduced compared with wild-type Na$_V$1.4 and Na$_V$1.4$_{1740}$. (C) Confocal images of Na$_V$1.4-EYFP.
CaM to the CT-Na v 1.4. Consistent with the donor de-
ECFP-CaM coexpression (Fig. 6 B; Table I). The pres-
nels were compared in the presence and absence of
reliable voltage control.

studied were highly selected to have peak current densi-
expressed with ECFP. However, we caution that the cells
compared with the channel expressed alone or co-
rent density of any of the Na channel variants studied
ence of ECFP-CaM did not significantly alter the cur-
enced FRET efficiency, current densities of the chan-

the Na V −CT were substituted by alanines (Na v 1.4 IQ/AA ),
while holding the channels at three different potentials:

206  Calmodulation of Sodium Channels

while holding the channel at 300 mV (favoring closed channels), 100 mV (favoring inactivated channels). Coexpressing ECFP-CaM and Na v 1.4-
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 Na v 1.4 under resting condi-
tions and studies describing reduced current density by
expressed Na v channel isoforms that have been mutated
to impair CaM binding (Cormier et al., 2002; Herzog
et al., 2003) suggest that CaM binding influences chan-
nel trafficking and surface expression. In addition to
calmodulation of steady-state inactivation of Na v 1.4,
we assessed whether CaM influenced channel trafficking
and expression. Lysates isolated from cells expressing
wild-type or mutant Na v 1.4 IQ/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
Na v 1.4 IQ/AA channels were labeled with membrane-
permeable biotin and purified with streptavidin beads.
The band intensities of biotinylated proteins, representing
cell surface expression, were normalized to the re-
spective total channel protein expression and plotted in
Fig. 7 B. The Na v 1.4 IQ/AA mutant exhibits significantly
(P ≤ 0.05, n = 4) reduced surface expression compared
with wild-type Na v 1.4 and the truncation mutant distal
to the IQ, Na v 1.4 IQ/AA 1730 (Fig. 7 B). Truncation of Na v 1.4
further upstream at residue 1723 (Na v 1.4 IQ/AA 1723) eliminates
channel function (Fig. 4 G) but does not eliminate cell
surface expression, as detected by Western blot and epi-
fluorescence 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 Na v 1.4 is essential for functional cell sur-
face channel expression. To further examine trafficking of the IQ mutant channel, Na v 1.4-EYFP or Na v 1.4 IQ/AA 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 peri-
nuclear membranes to cell surface (Fig. 7 C). In contrast,
signals from the Na v 1.4 IQ/AA EYFP mutant channels were
predominantly in the perinuclear region, indicating
mutant channels are synthesized but remain trapped in

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 v 1.2 (Erickson et al., 2001). A mean FRET
efficiency 10.5 ± 1.3% (n = 22; Fig. 6 A) was observed
when ECFP-CaM was coexpressed with EYFP-tagged
channels, indicating that the fluorophores in Na v 1.4-
EYFP and ECFP-CaM were separated by <100 Å. In con-
trast, when the IQ residues of the CaM binding motif in
the Na v-CT were substituted by alanines (Na v 1.4 IQ/AA ),
no FRET was observed between the donor ECFP-CaM
and acceptor Na v 1.4 IQ/AA EYFP (Fig. 6 A). Coexpressing
free cytosolic ECFP with Na v 1.4-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 mem-
brane, and nonspecific binding of CaM to the channel.

Moreover, no FRET was evident between the CaM EF
hand mutant ECFP-CaM 1234 and Na v 1.4-EYFP (Fig. 6 A),
suggesting the absence of unconventional binding sites
for apo-CaM in the intact CT of Na v 1.4, as has been
suggested for apo-CaM in the intact CT of Na V 1.4, as has been
suggesting the absence of unconventional binding sites
for apo-CaM in the intact CT of Na v 1.4, as has been
suggesting the absence of unconventional binding sites
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for apo-CaM in the intact CT of Na v 1.4, as has been
suggesting the absence of unconventional binding sites
for apo-CaM in the intact CT of Na v 1.4, as has been
intracellular membranes (Fig. 7 D). Compared with the wild-type Na$_{\text{v1.4}}$ channel, more Na$_{\text{v1.4IQ/AA}}$ channels remain internalized, consistent with the reduced surface expression of the Na$_{\text{v1.4IQ/AA}}$ protein observed in Western blot analysis (Fig. 7, A and B). Although the IQ/AA mutation interferes with cell surface trafficking of Na$_{\text{v1.4}}$, 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$_{\text{v1.4IQ/AA}}$), 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$_{\text{v1.4}}$ 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$_{\text{v1.4}}$ at the IQ motif in a conformation-independent manner and CaM is not displaced by overexpression of the EF hand mutant CaM$_{1254}$ (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$_{\text{v1.4}}$ is indispensable for functional expression (Fig. 4); (d) only one CaM molecule is sufficient to modulate Na$_{\text{v1.4}}$ channel inactivation gating current (Fig. 5, D and E); and (e) mutations in CaM binding IQ motif influence Na$_{\text{v1.4}}$ 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$_{\text{v1.2}}$ (Peterson et al., 1999; Zühlke et al., 1999; Erickson et al., 2001; Van Petegem et al., 2005). An homologous IQ binding domain is present in all Na$_{\text{1}}$ sodium channel isoforms; however, CaM’s interaction with Na$_{\text{v1.4}}$ 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$^{2+}$/CaM/CaMK on Na$_{\text{1}}$ 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$_{\text{v1.4}}$.

CaM Is Tethered to Intact Na$_{\text{v1.4}}$ through an IQ Motif

Our data conclusively demonstrate tethering of CaM to the CT of intact Na$_{\text{v1.4}}$ channels in live cells. Deletion mutations of the Na$_{\text{v1.4}}$ CT that preserve the IQ motif (Na$_{\text{v1.4IQ/AA}}$) generated currents that had voltage dependencies and kinetics of activation and inactivation that were similar to wild-type Na$_{\text{v1.4}}$ (Fig. 4, C and F). In contrast, deletion of the entire nonstructured region and part of the terminal α-helix including the IQ motif (Na$_{\text{v1.4IQ/AA}}$) exhibit no current (Fig. 4 G), indicating that the region containing the IQ motif and CaM binding to this motif in Na$_{\text{v1.4}}$ is indispensable for normal sodium channel functional expression. CaM tethering to the IQ region was further confirmed by the absence of an effect of CaM$_{1254}$ on the CaM-induced shift in gating of Na$_{\text{v1.4}}$ (Fig. 3 A). 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. 3, B–E). AIP290-309 alters the gating of wild-type and IQ/AA mutant Na$_{\text{v1.4}}$ channels. Inactivation of Na$_{\text{v1.4IQ/AA}}$ is destabilized by AIP290-309 independent of the presence of CaM overexpression (Fig. S1), whereas, steady-state inactivation of Na$_{\text{v1.4}}$ 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$_{\text{v1.4}}$ gating are eliminated by overexpression of CaM (Fig. 3 B). Whether AIP290–309 interacts with the channel directly and the site(s) of that interaction remain uncertain. The effects of AIP290-309 on Na$_{\text{v1.4IQ/AA}}$ 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$_{\text{v1.4IQ/AA}}$ gating.

In resting cells, wild-type CaM increased the donor dequenching FRET signal in tagged, presumably inactivated Na$_{\text{v1.4}}$ channels (Fig. 6, A and C). Changing the membrane voltage did not eliminate FRET, suggesting that CaM is tethered to the CT of Na$_{\text{v1.4}}$ 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$_{\text{v1.4}}$ channels. This is in contrast to a previously proposed mechanism of gating for voltage-gated cardiac sodium channels, Na$_{\text{v1.5}}$ (Shah et al., 2006), where CaM has been proposed to bind to the IQ motif at resting Ca$^{2+}$ levels, but later dissociates from the CT upon Ca$^{2+}$ 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$_{\text{v1.4}}$ and mutant Na$_{\text{v1.4IQ/AA}}$ are both regulated by Ca$^{2+}$ in the presence of endogenous CaM (unpublished data). Moreover, CaM-mediated regulation of Na$_{\text{v1.4}}$ is lost in the absence of Ca$^{2+}$, consistent with Ca$^{2+}$-dependent CaM regulation of
Gating by CaM

Further structural characterization of the NaV1.4-CT and at pore mouth that participate in and stabilize inactivation. CT facilitates interactions with other cytoplasmic domains. The relationship of CaM and the IQ motif (Fig. 6 D) is possible. It is pos-sible that the binding of CaM to the more structured CT-NaV1.4 during normal gating. The IQ motifs are highly conserved across NaV iso-forms, suggesting an important functional role for these regions. It is conceivable that the cytoplasmic tail of NaV1.4 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 NaV1.4-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 NaV1.4

We demonstrate that CaM participates in the modulation of cell surface expression of intact NaV1.4 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, CaV1.2, altering both function and expression of this α subunit. Similarly, CaM acts as an obligate subunit of some NaV1 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 NaV1.4. More proximal truncations of the channel that include the IQ motif (NaV1.4I723, Fig. 3 G and Fig. S2) support a role for CaM and the IQ motif in the NaV1.4-CT.

A Functional Model for the Regulation of NaV1.4 Channel Gating by CaM

The conformation-dependent 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 NaV1.4. First, overexpression of CaM1234 does not eliminate the CaM-mediated effect on steady-state inactivation, indicating its only CaM-mediated regulation (Fig. 3 A). Second, overexpression of both CaM and CaM1234 shows similar regulation to that exhibited by CaM alone (Fig. 3 B), indicating that the CaV2 binding–deficient CaM1234 does not displace CaM. Finally, among the variable length glycine–linkered 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 NaV being tethered to the CT-NaV1.4 during normal gating.

The IQ motifs are highly conserved across NaV iso-forms, suggesting an important functional role for these regions. It is conceivable that the cytoplasmic tail of NaV1.4 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 NaV1.4-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.

REFERENCES

Abriel, H., and R.S. Kass. 2005. Regulation of the voltage-gated cardiac sodium channel Nav1.5 by interacting proteins. Trends Cardiovasc. Med. 15:35–40.

Choi, J.S., A. Hudmon, S.G. Waxman, and S.D. Dib-Hajji. 2006. Calmodulin regulates current density and frequency-dependent inhibition of sodium channel Nav1.8 in DRG neurons. J. Neurophysiol. 96:97–108.

Cormier, J.W., I. Rivolta, M. Tateyama, A.S. Yang, and R.S. Kass. 2002. Secondary structure of the human cardiac Na+ channel C terminus: evidence for a role of helical structures in modulation of channel inactivation. J. Biol. Chem. 277:9233–9241.

Deschênes, I., N. Neyroud, D. DiSilvestre, E. Marban, D.T. Yue, and G.F. Tomasselli. 2002. Isoform-specific modulation of voltage-gated Na+ channels by calmodulin. Circ. Res. 90:49–57.

Erickson, M.G., B.A. Alseikhan, B.Z. Peterson, and D.T. Yue. 2001. Preassociation of calmodulin with voltage-gated Ca2+ channels revealed by FRET in single living cells. Neuron. 31:973–985.
Biswas et al. 2003. FRET two-hybrid mapping reveals function and location of L-type Ca\(^{2+}\) channel CaM preassociation. *Neuron*, 39:97–107.

Ghosh, S., D.A. Nunziato, and G.S. Pitt. 2006. KCNQ1 assembly and function is blocked by long-QT syndrome mutations that disrupt interaction with calmodulin. *Circ. Res.*, 98:1048–1054.

Herzog, R.I., C. Liu, S.G. Waxman, and T.R. Cummins. 2003. Calmodulin binds to the C terminus of sodium channels Nav1.4 and Nav1.6 and differentially modulates their functional properties. *J. Neurosci.*, 23:8261–8270.

Joiner, W.J., R. Khanna, L.G. Schlichter, and L.K. Kaczmarek. 2001. Calmodulin regulates assembly and trafficking of SKA/IK1 Ca\(^{2+}\)-activated K\(^+\) channels. *J. Biol. Chem.*, 276:37980–37985.

Kearney, J.A., N.W. Plummer, M.R. Smith, J. Kapur, T.R. Cummins, S.G. Waxman, A.L. Goldin, and M.H. Meisler. 2001. A gain-of-function mutation in the sodium channel gene Scn2a results in seizures and behavioral abnormalities. *Neuroscience*, 102:307–317.

Keating, M.T., and M.C. Sanguinetti. 2001. Molecular and cellular mechanisms of cardiac arrhythmias. *Cell*, 104:569–580.

Kim, J., S. Ghosh, H. Liu, M. Tateyama, R.S. Kass, and G.S. Pitt. 2004. Calmodulin mediates Ca\(^{2+}\) sensitivity of sodium channels. *J. Biol. Chem.*, 279:45004–45012.

Lee, W.S., T.J. Ngo-Anh, A. Bruening-Wright, J. Maylie, and J.P. Adelman. 2003. Small conductance Ca\(^{2+}\)-activated K\(^+\) channels and calmodulin: cell surface expression and gating. *J. Biol. Chem.*, 278:25940–25946.

Lossin, C., D.W. Wang, T.H. Rhodes, C.G. Vanoye, and A.L. George Jr. 2002. Molecular basis of an inherited epilepsy. *Neuron*, 34:877–884.

Mori, M., T. Konno, T. Ozawa, M. Murata, K. Imoto, and K. Nagayama. 2000. Novel interaction of the voltage-dependent sodium channel (VDSC) with calmodulin: does VDSC acquire calmodulin-mediated Ca\(^{2+}\)-sensitivity? *Biochemistry*, 39:1316–1323.

Peterson, B.Z., C.D. DeMaria, J.P. Adelman, and D.T. Yue. 1999. Calmodulin is the Ca\(^{2+}\) sensor for Ca\(^{2+}\)-dependent inactivation of L-type calcium channels. *Neuron*, 22:549–558.

Shah, V.N., T.L. Wingo, K.L. Weiss, C.K. Williams, J.R. Balser, and W.J. Chazin. 2006. Calcium-dependent regulation of the voltage-gated sodium channel hH1: intrinsic and extrinsic sensors use a common molecular switch. *Proc. Natl. Acad. Sci. USA*, 103:3592–3597.

Van Petegem, F., F.C. Chatelain, and D.L. Minor Jr. 2005. Insights into voltage-gated calcium channel regulation from the structure of the CaV1.2 IQ-domain-Ca\(^{2+}\)/calmodulin complex. *Nat. Struct. Mol. Biol.*, 12:1108–1115.

Wang, G.K., C. Russell, and S.Y. Wang. 2003. State-dependent block of wild-type and inactivation-deficient Na channels by flecainide. *J. Gen. Physiol.*, 122:365–374.

Wen, H., and I.B. Levitan. 2002. Calmodulin is an auxiliary subunit of KCNQ2/3 potassium channels. *J. Neurosci.*, 22:7991–8001.

Wu, F.F., E. Gordon, E.P. Hoffman, and S.C. Cannon. 2005. A C-terminal skeletal muscle sodium channel mutation associated with myotonia disrupts fast inactivation. *J. Gen. Physiol.*, 122:365–374.