UltraRapid Communication

Isoform-Specific Modulation of Voltage-Gated Na\(^+\) Channels by Calmodulin

Isabelle Deschênes, Nathalie Neyroud, Deborah DiSilvestre, Eduardo Marbán, David T. Yue, Gordon F. Tomaselli

Abstract—Calmodulin (CaM) is a calcium-sensing protein that binds to Na\(^+\) channels, with unknown functional consequences. Wild-type CaM produced a hyperpolarizing shift in the steady-state availability of expressed skeletal muscle (\(\mu l\)) but not cardiac (hH1) Na\(^+\) channels. Mutant CaM\(_{1234}\) did not alter the voltage dependence or the kinetics of gating of either \(\mu l\) or hH1. Mutation of the highly conserved IQ motif in the carboxyl terminus of both isoforms (IQ/AA) slowed the kinetics of current decay and abolished the effect of wild-type CaM on \(\mu l\), but did not alter hH1 currents. The IQ/AA mutation eliminated CaM binding to the carboxyl terminus of both \(\mu l\) and hH1 channels. Inhibition of Ca\(^{2+}\)/CaM kinase (CaM-K) slowed the current decay, the rate of entry into inactivation, and shifted the voltage dependence of hH1 in the depolarizing direction independent of CaM overexpression with no effect on \(\mu l\) Na\(^+\) channels. CaM signaling modulates Na\(^+\) currents in an isoform-specific manner, via direct interaction with skeletal muscle Na\(^+\) channels and through CaM-K in the case of the cardiac isoform. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2002;90:e49-e57.)

Key Words: sodium channels ■ calmodulin ■ cardiac ■ skeletal muscle ■ Ca\(^{2+}\)/calmodulin-dependent kinase

Voltage-gated Na\(^+\) channels are essential for the generation of action potentials and cell excitability.\(^1\) Na\(^+\) channels are responsible for the upstroke of the action potential in excitable cells and are the target of local anesthetic drugs. Na\(^+\) channels are composed of a pore-forming \(\alpha\)-subunit and smaller \(\beta\)-subunits that have been implicated in assembly, trafficficking, and posttranslational modulation of the \(\alpha\)-subunit.\(^2\)

A number of interacting signaling pathways modulate the Na\(^+\) channel. The function of neuronal and cardiac Na\(^+\) channels is altered by cAMP-dependent protein kinase (PKA) phosphorylation of residues in the linker between domains I and II. PKA-dependent phosphorylation of the neuronal channel reduces current density,\(^3,4\) but directionally opposite effects are seen with cardiac Na\(^+\) channels.\(^5\) The \(\alpha\)-subunit of the skeletal muscle channel is not affected by PKA despite the presence of consensus phosphorylation sites.\(^6,7\) In contrast to PKA, protein kinase C (PKC) alters the function of all mammalian sodium channel isoforms.\(^8–13\) PKC reduces channel conductance and alters gating in an isoform-specific manner; an effect largely attributed to phosphorylation of a highly conserved serine residue in the linker between domains III to IV of the channel. There is also evidence that neuronal sodium channel gating is modulated by a G-protein–coupled pathway acting through the carboxyl terminus.\(^14\)

Calmodulin (CaM) is a ubiquitous, small (16.7 kDa) calcium-binding protein that acts as a Ca\(^{2+}\)-sensor translating changes in cytoplasmic Ca\(^{2+}\) into cellular responses by interacting with a diverse group of signaling molecules.\(^15\) A group of kinases, Ca\(^{2+}\)/CaM-dependent kinases (CaM-K) are activated by CaM and phosphorylate a number of functionally diverse effectors from ion channels and receptors to transcriptional activators. Ion channels are prominent targets of CaM; for example, CaM is bound to L-type voltage–dependent Ca\(^{2+}\) channels and mediates Ca\(^{2+}\)-dependent inactivation.\(^16–19\) The possibility that CaM modulates the Na\(^+\) current was suggested by yeast 2-hybrid experiments demonstrating the interaction of voltage-dependent sodium channels and CaM through an IQ-like motif in the carboxyl terminus of the \(\alpha\)-subunit.\(^20\)

The functional consequences of CaM binding to Na\(^+\) channels are unknown. We demonstrate that coexpression of CaM with Na\(^+\) channels alters the function of the skeletal muscle (\(\mu l\)) channels but not the cardiac (hH1) isoform. Further, mutation of the IQ motif in the carboxyl terminus of the \(\mu l\) skeletal muscle Na\(^+\) channel alters inactivation gating and eliminates the effect of CaM on the expressed current. Inhibition of CaM-K by KN-93, but not the CaM-KII–specific inhibitor autocamtide-2 peptide (AIP), slowed cardiac Na\(^+\) current decay, shifted the availability curve to more...
depolared potentials, and slowed entry into inactivated states. Mutating the IQ motif eliminated CaM binding to carboxyl terminal peptides of both isoforms.

Materials and Methods

Transfections of HEK293 and C2C12 Cell Lines

Human embryonic kidney cells (HEK293; American Type Culture Collection, Manassas, Va) were used to express the cloned sodium channels µ1 and hH1 in presence of wild-type or mutant CaM1234. C2C12 cells (American Type Culture Collection) are a subclone derived from a mouse myoblast cell line expressing characteristic muscle proteins. Cells were grown in DMEM high glucose supplemented with FBS 10%, L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (10 mg/mL) in a humidified 5% CO2 atmosphere. Transfections of the cells were performed using lipofectamine according to the manufacturer’s instructions. Cells were patch-clamped or stained 1 to 2 days after transfection.

Patch-Clamp Methods

Macroscopic Na+ currents from transfected cells were recorded using the whole-cell configuration of patch-clamp technique. Cells with currents no larger than 6 nA were clamped with an Axopatch 200 patch-clamp amplifier using low-resistance electrodes and typical series resistance compensation of >80% that minimized voltage-clamp errors. Membrane currents were filtered at 5 kHz and digitized with 12-bit resolution. In all experiments, recording was begun 10 minutes after whole-cell access was attained with the exception of the CaM-K inhibitor experiments where recording was begun 15 minutes after patch rupture to ensure stabilization of the time-dependent shift in gating.

Solutions and Reagents

The patch pipette contained (in mmol/L) 35 NaCl, 105 CsCl, and 10 Cs-HEPES (pH 7.3) with no Ca2+ buffering. All of the experiments were initially performed using 105 mmol/L CsF in the pipette; however, due to possible confounding effects on phosphorylation reactions and Ca2+ buffering, the experiments were repeated with the CsCl-containing intracellular solution. No differences in the reactions and Ca2+ dependent shift in gating.

Statistical Analysis

Data are expressed as mean±SEM. A 2-tailed Student’s t test was used to assess statistical significance when appropriate. Differences were considered to be significant at a value of P<0.05.

For the experiments with 504 mmol/L free Ca2+ clamp errors. Membrane currents were filtered at 5 kHz and digitized with 12-bit resolution. In all experiments, recording was begun 10 minutes after whole-cell access was attained with the exception of the CaM-K inhibitor experiments where recording was begun 15 minutes after patch rupture to ensure stabilization of the time-dependent shift in gating.

The patch pipette contained (in mmol/L) 35 NaCl, 105 CsCl, and 10 Cs-HEPES (pH 7.3) with no Ca2+ buffering. All of the experiments were initially performed using 105 mmol/L CsF in the pipette; however, due to possible confounding effects on phosphorylation reactions and Ca2+ buffering, the experiments were repeated with the CsCl-containing intracellular solution. No differences in the reactions and Ca2+ dependent shift in gating.

Figure 1. Biophysical properties of µ1 expressed in HEK293 cells in presence of CaM or mutant CaM1234. A, Representative sodium currents through µ1 skeletal muscle channels in the absence (solid line) and presence of overexpression of CaM (dashed line) or a Ca2+ -binding deficient mutant CaM1234 (dotted line). Currents were elicited by 30-ms steps to −20 mV from a holding potential of −120 mV. B, The normalized current-voltage (IV) relationships elicited by voltage-clamp steps from −80 to +60 mV in 10-mV increments (mean±SEM) for µ1 (squares), µ1 + CaM (circles), and µ1 + mutant CaM1234 (triangles). C, Voltage dependence of steady-state inactivation measured by a 2-pulse protocol with 500-ms conditioning pulses from −140 to −30 mV followed by a test pulse to −30 mV. The data were fit to a Boltzmann distribution. D, Voltage dependence of steady-state inactivation, as described in C, but measured in the presence of 0.1 mmol/L EGTA in the bath solution and 10 mmol/L BAPTA in the intracellular solution.
TABLE 1. Functional Effects of CaM and Mutant CaM1234 on μ1 Expressed in HEK293 Cells and on Skeletal Muscle Sodium Currents Recorded From C2C12 Cells

<table>
<thead>
<tr>
<th>Biophysical Parameters</th>
<th>Steady-State Inactivation (V1/2 in mV)</th>
<th>Decay Time Constant (τd in ms at −20 mV)</th>
<th>Recovery From Inactivation (τrec in ms)</th>
<th>Entry Into Inactivation (Time Constant in ms)</th>
<th>Activation (V1/2 in mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>μ1</td>
<td>−68.0±0.9</td>
<td>n1hm=0.72±0.09</td>
<td>2.03±0.14</td>
<td>931±59</td>
<td>−32.2±2.5</td>
</tr>
<tr>
<td>μ1+CaM</td>
<td>−73.8±0.6*</td>
<td>n1hm=0.66±0.05</td>
<td>1.89±0.20</td>
<td>884±77</td>
<td>−31.1±2.7</td>
</tr>
<tr>
<td>μ1+CaM1234</td>
<td>−68.3±0.7</td>
<td>n1hm=0.71±0.04</td>
<td>1.91±0.13</td>
<td>879±65</td>
<td>−31.7±2.2</td>
</tr>
<tr>
<td>μ1+β</td>
<td>−69.4±0.6</td>
<td>n1hm=0.66±0.09</td>
<td>1.95±0.15</td>
<td>953±24</td>
<td>−33.1±3.0</td>
</tr>
<tr>
<td>μ1+β+CaM</td>
<td>−74.7±0.3†</td>
<td>n1hm=0.62±0.02</td>
<td>2.02±0.26</td>
<td>871±67</td>
<td>−32.9±2.6</td>
</tr>
<tr>
<td>μ1+β+CaM1234</td>
<td>−68.7±0.2</td>
<td>n1hm=0.59±0.05</td>
<td>1.89±0.23</td>
<td>973±23</td>
<td>−33.3±3.1</td>
</tr>
<tr>
<td>μ1 (0 Ca²⁺)</td>
<td>−73.2±0.2</td>
<td>n1hm=0.66±0.06</td>
<td>2.22±0.34</td>
<td>901±35</td>
<td>−33.7±2.6</td>
</tr>
<tr>
<td>μ1+CaM (0 Ca²⁺)</td>
<td>−75.0±0.4</td>
<td>n1hm=0.61±0.05</td>
<td>2.05±0.25</td>
<td>890±29</td>
<td>−34.3±3.2</td>
</tr>
<tr>
<td>μ1+CaM1234 (0 Ca²⁺)</td>
<td>−75.9±0.6</td>
<td>n1hm=0.64±0.08</td>
<td>1.99±0.46</td>
<td>884±41</td>
<td>−34.1±3.6</td>
</tr>
<tr>
<td>C2C12</td>
<td>−87.5±0.2</td>
<td>n1hm=1.32±0.11</td>
<td>5.93±0.82</td>
<td>811±94</td>
<td>−30.7±3.0</td>
</tr>
<tr>
<td>C2C12+CaM</td>
<td>−94.2±0.7†</td>
<td>n1hm=1.30±0.09</td>
<td>6.78±1.05</td>
<td>760±67</td>
<td>−32.4±2.7</td>
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<tr>
<td>C2C12+CaM1234</td>
<td>−85.8±0.7</td>
<td>n1hm=1.24±0.13</td>
<td>6.32±0.82</td>
<td>779±74</td>
<td>−32.0±3.2</td>
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<tr>
<td>μ1-IQ/AA</td>
<td>−78.6±1.0*</td>
<td>n1hm=0.68±0.03*</td>
<td>2.65±0.54*</td>
<td>901±44</td>
<td>−40.4±3.1</td>
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<tr>
<td>μ1-IQ/AA+CaM</td>
<td>−78.1±1.2*</td>
<td>n1hm=0.72±0.04*</td>
<td>3.19±0.69*</td>
<td>851±36</td>
<td>−42.1±3.7</td>
</tr>
<tr>
<td>μ1+KN-92</td>
<td>−67.2±1.2</td>
<td>n1hm=0.74±0.08</td>
<td>1.92±0.21</td>
<td>915±58</td>
<td>−34.1±2.8</td>
</tr>
<tr>
<td>μ1+KN-93</td>
<td>−66.0±0.9</td>
<td>n1hm=0.79±0.09</td>
<td>2.06±0.11</td>
<td>907±49</td>
<td>−32.8±3.1</td>
</tr>
<tr>
<td>μ1+CaM+KN-92</td>
<td>−73.4±1.0†</td>
<td>n1hm=0.69±0.07</td>
<td>2.00±0.15</td>
<td>889±61</td>
<td>−33.1±2.2</td>
</tr>
<tr>
<td>μ1+CaM+KN-93</td>
<td>−73.8±0.7†</td>
<td>n1hm=0.70±0.08</td>
<td>1.95±0.09</td>
<td>874±38</td>
<td>−32.5±2.1</td>
</tr>
<tr>
<td>μ1+AIP</td>
<td>−66.9±0.9</td>
<td>n1hm=0.71±0.08</td>
<td>2.01±0.44</td>
<td>907±61</td>
<td>−32.8±2.4</td>
</tr>
<tr>
<td>μ1+CaM+AIP</td>
<td>−73.8±1.3†</td>
<td>n1hm=0.69±0.12</td>
<td>1.92±0.69</td>
<td>887±101</td>
<td>−32.1±3.2</td>
</tr>
</tbody>
</table>

n indicates number of cells.

*Significantly different from μ1 (P<0.05); †significantly different from μ1+β (P<0.05); §significantly different from C2C12 (P<0.05); ‡significantly different from μ1+KN-92 (P<0.05); ‡‡significantly different from μ1+KN-93 (P<0.05); and §§significantly different from μ1+AIP (P<0.05).

**Results**

**Effects of CaM Coexpression With μ1 Skeletal Muscle Na⁺ Channels**

To assess the effect of CaM binding on skeletal muscle sodium channels, we cotransfected HEK293 cells with μ1 and wild-type CaM or a mutant form of CaM with strongly impaired Ca²⁺ binding (CaM1234). In the absence of intracellular Ca²⁺ buffering, CaM overexpression did not significantly affect the whole-cell current (Figure 1A), current-voltage relationship (Figure 1B), voltage dependence of activation, time constants of inactivation (τi), recovery from inactivation, or the rate of entry into inactivation (Table 1). However, the steady-state availability relation was significantly shifted in the hyperpolarizing direction (Figure 1C). In contrast to wild-type CaM, coexpression of mutant CaM1234 with the μ1 Na⁺ channel produced no significant change in steady-state availability compared with the μ1 α-subunit expressed alone. The presence of the β₁-subunit did not significantly alter the steady-state availability curve of the μ1 channel compared with the α-subunit alone, nor did it alter the hyperpolarizing shift produced by CaM. Table 1 summarizes the effects of wild-type and mutant CaM on the functional properties of the μ1 skeletal muscle Na⁺ channel expressed in the presence and absence of the β₁-subunit with CaM.

The action of CaM on effector molecules often requires Ca²⁺; therefore, we studied the effect of different free intracellular Ca²⁺ concentrations on the CaM modulation of μ1 sodium channels. Our standard recording conditions included no intracellular Ca²⁺ buffering and 2 mmol/L external Ca²⁺. There was no significant difference in the effect of CaM on μ1 Na⁺ current when [Ca²⁺], was buffered to 504 mmol/L compared with control recording conditions (data not shown). However, when extracellular Ca²⁺ was buffered with 0.1 mmol/L EGTA and 10 mmol/L BAPTA was added to the intracellular solution to ensure the removal of free Ca²⁺, the CaM-induced shift of the steady-state inactivation curve was no longer present (Figure 1D). Thus, the effect of CaM on gating of the skeletal muscle Na⁺ current is Ca²⁺-dependent (Table 1).

**Effects of CaM Coexpression on the hH1 Cardiac Na⁺ Current**

The carboxyl terminal IQ-like binding motif is conserved across all isoforms of the mammalian Na⁺ channel. Therefore, we examined the effect of CaM on the function of the human heart sodium channel, hH1. When coexpressed with hH1, neither wild-type CaM nor mutant CaM1234 significantly altered any whole-cell property of the current (Table 2). Expression of wild-type CaM and mutant CaM1234 proteins
was confirmed by immunofluorescence staining (data not shown). CaM coexpression had no effect on the hH1 Na⁺ current either in the absence or presence of the β₁-subunit at any intracellular Ca²⁺ concentration. A cell line stably expressing hH1 was used for the experiments. An \( \approx -10 \) mV shift in the steady-state inactivation curve is observed in these cells compared with cells transiently transfected with hH1; however, CaM overexpression had no effect on the voltage dependence of inactivation. The rates of entry into and recovery from inactivation of the stably expressed channels was slower than transiently transfected hH1, but CaM overexpression had no significant effect on the kinetics of inactivation (Table 2).

### Effects of CaM Binding on Skeletal Muscle Na⁺ Currents Recorded From C2C12 Cells

In order to study the effect of CaM on native skeletal muscle Na⁺ channels, we transfected wild-type CaM and mutant CaM\(_{1234}\) into C2C12 cells, a mouse myoblast cell line. C2C12 cells showed typical skeletal muscle sodium currents (Figure 2 and Table 1), as previously reported. The time constants of decay \( (\tau_d) \) and recovery from inactivation are modestly but significantly slower in C2C12 cells compared with expressed \( \mu \)1 currents recorded under identical conditions (Table 1). In the absence of exogenous CaM, the steady-state availability of C2C12 Na⁺ currents is shifted \( \approx -20 \) mV compared with expressed \( \mu \)1 currents. Expression of wild-type CaM in C2C12 cells significantly shifts the steady-state availability of the Na⁺ currents in the hyperpolarizing direction (Figure 2C). However, coexpression of mutant CaM\(_{1234}\) did not shift the voltage dependence of the steady-state availability relationship (Figure 2C). Neither wild-type nor mutant CaM\(_{1234}\) altered the other whole-cell properties of C2C12 Na⁺ current (Figure 2, Table 1).

### Mutation of CaM Binding Site

The isoleucine-glutamate (IQ) CaM binding domain is essential for CaM-mediated Ca²⁺-dependent inactivation of the L-type Ca²⁺ current. Yeast 2-hybrid assays have established CaM binding to an IQ-like motif on the carboxyl terminal region of Na⁺ channels. In order to determine the role of the Na⁺ channel IQ motif in CaM-induced shifts in

### Table 2. Functional Effects of CaM and Mutant CaM\(_{1234}\) on hH1 Expressed in HEK293

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Steady-State Inactivation (( V_{in} ) in mV)</th>
<th>Decay Time Constant (( \tau_{in} ) in ms at (-30) mV)</th>
<th>Recovery From Inactivation (( \tau_{rec} ) in ms)</th>
<th>Entry Into Inactivation (Time Constant in ms)</th>
<th>Activation (( V_{in} ) in mV)</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>hH1</td>
<td>-82.6 ± 0.8</td>
<td>( \tau_{in} = 0.89 \pm 0.05 )</td>
<td>4.25 ± 0.51</td>
<td>1265 ± 138</td>
<td>-53.4 ± 3.1</td>
<td>4</td>
</tr>
<tr>
<td>hH1 + CaM</td>
<td>-80.6 ± 0.4</td>
<td>( \tau_{in} = 0.81 \pm 0.10 )</td>
<td>4.77 ± 0.30</td>
<td>1225 ± 131</td>
<td>-52.1 ± 2.5</td>
<td>5</td>
</tr>
<tr>
<td>hH1 + CaM(_{1234})</td>
<td>-80.3 ± 0.5</td>
<td>( \tau_{in} = 0.77 \pm 0.06 )</td>
<td>4.29 ± 0.36</td>
<td>1201 ± 121</td>
<td>-52.9 ± 2.7</td>
<td>5</td>
</tr>
<tr>
<td>hH1 + ( \beta )</td>
<td>-90.1 ± 0.6</td>
<td>( \tau_{in} = 0.84 \pm 0.06 )</td>
<td>8.89 ± 0.76</td>
<td>1308 ± 137</td>
<td>-57.5 ± 3.5</td>
<td>4</td>
</tr>
<tr>
<td>hH1 + ( \beta ) + CaM</td>
<td>-89.6 ± 0.5</td>
<td>( \tau_{in} = 0.83 \pm 0.07 )</td>
<td>8.22 ± 0.64</td>
<td>1303 ± 111</td>
<td>-57.1 ± 3.6</td>
<td>4</td>
</tr>
<tr>
<td>hH1-IQ/AA</td>
<td>-83.2 ± 0.9</td>
<td>( \tau_{in} = 0.85 \pm 0.13 )</td>
<td>4.89 ± 0.29</td>
<td>1198 ± 135</td>
<td>-53.7 ± 3.4</td>
<td>6</td>
</tr>
<tr>
<td>hH1-IQ/AA + CaM</td>
<td>-81.6 ± 1.1</td>
<td>( \tau_{in} = 0.90 \pm 0.11 )</td>
<td>4.99 ± 0.31</td>
<td>1167 ± 147</td>
<td>-54.6 ± 3.2</td>
<td>6</td>
</tr>
<tr>
<td>hH1 + KN-92</td>
<td>-87.1 ± 0.9</td>
<td>( \tau_{in} = 0.91 \pm 0.11 )</td>
<td>14.77 ± 0.95</td>
<td>1123 ± 145</td>
<td>-54.3 ± 4.0</td>
<td>6</td>
</tr>
<tr>
<td>hH1 + KN-93</td>
<td>-72.4 ± 0.7*</td>
<td>( \tau_{in} = 1.90 \pm 0.11^* )</td>
<td>7.49 ± 0.92*</td>
<td>3064 ± 245*</td>
<td>-51.5 ± 4.5</td>
<td>5</td>
</tr>
<tr>
<td>hH1 + CaM + KN-92</td>
<td>-86.4 ± 1.1</td>
<td>( \tau_{in} = 0.98 \pm 0.07 )</td>
<td>13.05 ± 0.68</td>
<td>1390 ± 205</td>
<td>-54.6 ± 3.2</td>
<td>5</td>
</tr>
<tr>
<td>hH1 + CaM + KN-93</td>
<td>-70.7 ± 1.2†</td>
<td>( \tau_{in} = 1.95 \pm 0.09^† )</td>
<td>6.79 ± 0.89†</td>
<td>3114 ± 231†</td>
<td>-52.1 ± 3.2</td>
<td>5</td>
</tr>
<tr>
<td>hH1 + AIP</td>
<td>-83.2 ± 0.9</td>
<td>( \tau_{in} = 0.84 \pm 0.08 )</td>
<td>4.46 ± 0.78</td>
<td>1214 ± 105</td>
<td>-52.2 ± 3.4</td>
<td>6</td>
</tr>
<tr>
<td>hH1 + CaM + AIP</td>
<td>-82.9 ± 1.3</td>
<td>( \tau_{in} = 0.86 \pm 0.09 )</td>
<td>4.34 ± 0.71</td>
<td>1270 ± 111</td>
<td>-52.9 ± 3.0</td>
<td>5</td>
</tr>
</tbody>
</table>

\( n \) indicates number of cells.

*Significantly different from hH1 + KN-92 (\( P<0.05 \)); †significantly different from hH1 + CaM + KN-92 (\( P<0.05 \)).
availability, we mutated IQ to alanines in both μ1 (I1727A-Q1728A, μ1-IQ/AA) and hH1 (I1908A-Q1909A, hH1-IQ/AA) Na^+ channels and then coexpressed these mutant channels with wild-type CaM and mutant CaM 1234. The IQ/AA mutations in the μ1 background significantly altered the whole-cell current compared with the wild-type current. The current decay became more obviously biexponential with the addition of a slow component of decay that resulted in a pedestal of current of ≈5% of the peak at 30 ms after the voltage step (Figure 3A). The peak IV relationship (Figure 3B) was not altered but the steady-state availability curve of μ1-IQ/AA was shifted ≈10 mV compared with μ1-WT (Figure 3C, Table 1). Interestingly, by mutating the IQ motif, we abolished the CaM-induced shift of the steady-state availability relationship seen with coexpression of wild-type CaM and wild-type μ1 (Figure 3C, Table 1).

In contrast to μ1-IQ/AA, hH1-IQ/AA did not exhibit changes in whole-cell current decay compared with wild-type hH1. The wild-type hH1 current decay was best fitted with a biexponential function, and neither the time constants nor amplitudes of the components were altered by the IQ/AA mutations. Not surprisingly, coexpression of wild-type CaM with hH1-IQ/AA had no effect on any of the whole-cell properties of the current (Table 2).

CaM Binding to the IQ Motif

The colocalization of CaM and the Na^+ channel is further supported by protein binding experiments. GST-fusion proteins of carboxyl-terminal regions containing the wild-type IQ motifs and the IQ/AA mutants of the hH1 and μ1 Na^+ channels were made. The carboxyl terminal μ1 and hH1 fusion proteins with an intact IQ binding motif interact with CaM. In contrast, neither of the IQ/AA mutant proteins are able to pull-down CaM (Figure 4). Therefore, binding of CaM to the IQ motif appears to be necessary for the modulation of μ1 channel gating but is not sufficient in that binding to the hH1 carboxyl terminus does not alter gating of the cardiac isoform.

Effect of CaM-K Inhibition on Na^+ Currents

The cellular effects of CaM often involve downstream signaling through CaM-K. The activity of Na^+ channels is known to be modulated through different phosphorylation pathways. Because CaM binds to an IQ-like motif located in the C-terminus of Na^+ channel, phosphorylation of the channel by CaM-K is possible. The isoform-specific differences in the response to CaM may result from differences in the channels as substrates for phosphorylation by CaM-K. To assess this possibility, we used a nonselective CaM-K inhibitor, KN-93, and its inactive analog, KN-92. We applied KN-93 and KN-92 intracellularly at a concentration of 10 μmol/L to μ1 Na^+ channels expressed alone and in the presence of CaM.

KN-93 and the inactive analog KN-92 had no effect on the voltage dependence or kinetics of μ1 gating either in the presence or absence of CaM overexpression. Specifically, neither compound altered the rate of current decay (Figure 5B), rates of recovery from or entry into inactivated states (Table 1). Further, neither compound altered the CaM-induced hyperpolarizing shift of the steady-state availability relationship of μ1 Na^+ channels (Figure 5A). The CaM-KII–specific inhibitor AIP (100 nmol/L) was applied to expressed μ1 currents with results similar to the KN compounds. AIP did not alter the voltage dependence or kinetics of gating of μ1 channels and did not

Figure 2. Biophysical properties of sodium currents recorded from C2C12 cells in presence of CaM or mutant CaM 1234. A, Representative currents through C2C12 Na^+ channels in the absence (solid line) or presence of overexpression of CaM (dashed line) or mutant CaM 1234 (dotted line) were recorded as shown in Figure 1. B, Normalized IV relationships, similar to Figure 1B, show no change in C2C12 cells overexpressing CaM (circles) or mutant CaM 1234 (triangles) compared with C2C12 Na^+ currents alone (squares). C, Steady-state inactivation relationship is obtained as described in Figure 1 and exhibits a shift similar to that of expressed μ1 currents in the presence of wild-type CaM.

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KN-92 also had effects on hH1, but not μ1 currents: it shifted the steady-state inactivation curve in the hyperpolarizing direction and produced an apparent slowing of recovery at −120 mV. However, the currents are not fully reprimed at this voltage in the presence of KN-92. KN-92, and to a lesser extent KN-93, retards recovery from inactivation compared with hH1 alone; therefore, KN-induced slowed recovery from inactivation cannot be considered a specific effect of CaM-K inhibition. The slowing of recovery by these compounds was not observed in expressed μ1 channels.

In contrast, AIP, a selective CaM-KII inhibitor, had no effect on channel activity and did not block the depolarizing shift in the steady-state inactivation curve by the subsequent addition of KN-93 to the bath (data not shown). Thus, a CaM-K other than CaM-KII, possibly CaM-KIV, which is richly expressed in the heart, may modulate the shift in the voltage dependence of inactivation of cardiac Na⁺ channels.

Discussion

CaM is the most ubiquitous Ca²⁺-sensing protein in mammalian cells. The Ca²⁺/CaM complex directly modulates the function of a number of proteins including a family of serine/threonine kinases (CaM-K), ion channels, and receptors. CaM that is constitutively tethered to L-type Ca²⁺ channels mediates Ca²⁺-dependent inactivation and facilitation of the current.16–18 On Ca²⁺ binding, CaM associates with a conserved IQ motif23 in the carboxyl terminus of the channel. The presence of a highly conserved IQ-like motif in the carboxyl terminus of the Na⁺ channel suggested the possibility of CaM-mediated modulation of Na⁺ currents. Supportive evidence for such a mechanism included the demonstration of Ca²⁺-dependent and Ca²⁺-independent binding of CaM to a carboxyl terminal fragment of the Na⁺ channel containing the IQ motif.20 The functional consequences, if any, of CaM binding to the Na⁺ channel were previously unknown.

We demonstrate that CaM does indeed modify the function of the μ1 skeletal muscle isoform of the Na⁺ channel, shifting the steady-state inactivation curve in the hyperpolarizing direction (Figure 1, Table 1). The CaM effect on gating requires intact Ca²⁺ binding because a mutant, CaMΔ175, with impaired Ca²⁺ binding does not alter the gating of the μ1 Na⁺ channel. In addition, buffering of Ca²⁺ eliminates the wild-type CaM-mediated shift in steady-state availability (Figure 1D, Table 1). As expected, buffering Ca²⁺ produced a hyperpolarizing shift in channel gating, and we cannot exclude the possibility that this shift masks the CaM effect. CaM modulation of the skeletal muscle Na⁺ channel does not require the presence of the β₁-subunit, nor does β₁ alter the effect of CaM on the expressed current (Table 1). The modulation of the expressed μ1 current was recapitulated in C2C12 cells, a bona fide skeletal muscle cell line. These data suggest that native skeletal muscle channels, regardless of subunit composition, are targets for CaM.

Analogous to the Ca²⁺ channel, an IQ-like motif is a crucial component of the functional effects of CaM on skeletal muscle Na⁺ channels. Mutation of the IQ motif eliminates CaM binding to (Figure 4) and modulation (Figure 3) of μ1

Figure 3. Biophysical properties of μ1-IQ/AA mutant channels expressed in HEK293 cells in presence of CaM. A, Representative sodium currents through wild-type (solid line) and μ1-IQ/AA channels (−CaM dashed line, +CaM dotted line) recorded during steps to −20 mV; compared with Figure 1A, the currents exhibited a delayed decay and a pedestal of residual current independent of the presence of CaM 30 ms after the pulse. B, There is no significant shift in the normalized IV relationship elicited from a holding potential of −120 mV for μ1-IQ/AA in the presence (circles) or absence of CaM (squares). C, μ1-IQ/AA shifts the steady-state inactivation curve in the hyperpolarizing direction independent of the presence of CaM (−CaM filled squares, +CaM filled circles) compared with wild-type μ1 (−CaM open squares, +CaM open circles).

diminish the hyperpolarizing shift of the steady-state inactivation relationship induced by CaM overexpression (Table 1).

In contrast, KN-93 had robust effects on hH1 current, independent of CaM coexpression. KN-93 slowed the current decay with the most prominent effect on the fast time constant (Figure 6A, Table 2), shifted the steady-state inactivation relationship in depolarizing direction (Figure 6B), and slowed entry into inactivated states (Figure 6D, Table 2).
skeletal muscle currents. Our data indicate that the IQ motif has important functional effects on skeletal muscle Na⁺ currents independent of CaM binding. Indeed, the μ1-IQ/AA mutation itself slows the decay of the whole-cell current and shifts the steady-state inactivation curve to hyperpolarized potentials relative to wild-type μ1. In fact, the shift in the availability curve produced by mutating IQ to AA exceeds the shift produced by coexpression of CaM with wild-type μ1. It is unlikely that the effect of the IQ to AA mutation is simply the result of a loss of CaM binding because the direction of the shift is opposite to what would be predicted by the elimination of CaM binding (Figure 3C). Instead, this suggests that the IQ region of the skeletal muscle Na⁺ channel is an important component of the channel’s inactivation machinery that is modulated by CaM-mediated signaling.

IQ-mediated binding of CaM to Na⁺ channels is a necessary but not sufficient condition for modulation of channel function. hH1 and μ1 Na⁺ channels have IQ motifs with similar CaM binding capacity (Figure 4), yet CaM coexpression does not alter the function of hH1 channels. The difference in the functional effects of CaM on the Na⁺ channel isoforms may be the result of the conformation of CaM bound to the channel. It has been shown that CaM can bind to the IQ motif of the rat brain II Na⁺ channel in its Ca²⁺-bound and Ca²⁺-free (apo-CaM) states. 20 Indeed, Ca²⁺ buffering eliminates CaM modulation of μ1 channel gating (Figure 1D). However, the functional roles of the IQ motifs in skeletal muscle and cardiac Na⁺ channels appear to differ independent of CaM binding, as revealed by the IQ/AA mutant channels. Unlike the μ1 mutant channel, hH1-IQ/AA does not alter gating (Tables 1 and 2).

Alternatively, binding of CaM to the Na⁺ channel may serve to facilitate phosphorylation of the channel complex by CaM-K. We do not have direct evidence for phosphorylation of either channel isoform; however, inhibition of CaM-K (possibly CaM-KIV) modifies cardiac Na⁺ channel inactivation, shifting the steady-state inactivation curve in the depolarizing direction, slowing entry into inactivated states, and hastening recovery when compared with the inactive analog KN-92. KN-92 itself has effects on the cardiac Na⁺ channel (shifting the inactivation curve in the hyperpolarizing direction and slowing recovery) that cannot be ascribed to CaM-K inhibition. The differential effects of CaM-K inhibition on skeletal muscle and cardiac channels suggest the possibility of distinct functionally significant phosphorylation sites on each of the channel isoforms. However, the existence and precise identity of such sites remain to be demonstrated.

CaM signaling appears to fine-tune the function of voltage-dependent Na⁺ channels in isof orm-specific ways. The physiological significance of such modulation is uncertain, but recruitment of CaM signaling tends to stabilize inactivation of skeletal muscle and cardiac channels, albeit by distinct molecular mechanisms.

Interestingly, the C-terminal region of the cardiac Na⁺ channel is a hot spot for mutations that cause inherited cardiac arrhythmias, such as long-QT and Brugada syndromes. 24–30 We have recently demonstrated that the C-terminus of the Na⁺ channel plays a role in fast inactivation. 31 A common theme is that these disease-causing mutations alter gating and often disrupt inactivation of the Na⁺ channel. The mutations may directly alter Na⁺ channel function; however, it is possible that the effect of the mutation is through interruption of CaM/CaM-kinase signaling leading to destabilization of inactivation of the Na⁺ channel.
In the heart under pathophysiological conditions associated with increased intracellular Ca$^{2+}$, CaM-KII appears to enhance the arrhythmogenic transient inward current. Our data suggest that CaM may also regulate activation and repolarization of heart cells through effects on Na$^{+}$ channels altering the susceptibility to arrhythmias. Similarly, modulation of Na$^{+}$ channel availability may regulate the frequency of action potential firing in skeletal muscle, a possible mechanistic link to myotonia.

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References


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