Cellular/Molecular

A Mechanism for the Direct Regulation of T-Type Calcium Channels by Ca^{2+}/Calmodulin-Dependent Kinase II

Philip J. Welsby,[1]* Hongge Wang,[1]* Joshua T. Wolfe,[1] Roger J. Colbran,[2] Michael L. Johnson,[1] and Paula Q. Barrett[1]

[1]Department of Pharmacology, University of Virginia, Charlottesville, Virginia 22908, and [2]Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee 37232

Low-voltage-activated (LVA) Ca^{2+} channels are widely distributed throughout the CNS and are important determinants of neuronal excitability, initiating dendritic and somatic Ca^{2+} spikes that trigger and shape the pattern of action potential firing. Here, we define a molecular mechanism underlying the dynamic regulation of α_{1H} channels ($Ca_v3.2$), by Ca^{2+}/CaM-dependent protein kinase II (CaMKII). We show that channel regulation is selective for the LVA α_{1H} Ca^{2+} channel subtype, depends on determinants in the α_{1H} II–III intracellular loop, and requires the phosphorylation of a serine residue absent from unregulated α_{1G} ($Ca_v3.1$) channels. These studies identify the α_{1H} channel as a new substrate for CaMKII and provide the first molecular mechanism for the direct regulation of T-type Ca^{2+} channels by a protein kinase. Our data suggest a novel mechanism for modulating the integrative properties of neurons.

Key words: **calcium [Ca]; calmodulin; channel; dendrite; hippocampus; protein kinase; T-type**

Introduction

Low-voltage-activated (LVA), T-type, Ca^{2+} channels are important determinants of neuronal excitability (McCormick, 1992; Huguenard, 1996; Eilers and Konnerth, 1997; Hausser et al., 2000). They support the forward propagation of distal dendritic inputs in neocortical (Markram and Sakmann, 1994) and CA1 hippocampal pyramidal neurons (Magee and Johnston, 1995a,b) and mediate low-threshold spikes that support somatic burst firing in thalamocortical (Huguenard and McCormick, 1992; Destexhe et al., 1996) and CA3 hippocampal pyramidal neurons (Fisher et al., 1990; Migliore and Shepherd, 2002). Both dendritic boosting and neuronal bursting supported by LVA channels amplify subthreshold stimuli (Hausser et al., 2000). LVA Ca^{2+} channels can also facilitate long-term changes in neuronal plasticity as demonstrated in the dorsal horn (Ikeda et al., 2003) and CA1 pyramidal neurons (Su et al., 2002). Thus, LVA Ca^{2+} channel activity can contribute to the complex and diverse firing behavior of neurons.

LVA Ca^{2+} channels belong to the Ca_v3 family, of which there are three family members ($Ca_v3.1$, $Ca_v3.2$, and $Ca_v3.3$: α_{1G}, α_{1H}, and α_{1I}) (Cribbs et al., 1998; Perez-Reyes et al., 1998; Lee et al., 1999). Little is known about the regulation of this Ca^{2+} channel class, in contrast to high voltage-activated Ca^{2+} channels that are well-established targets of G-proteins and kinases (Catterall, 2000). One defined mode of LVA channel regulation depends on the activity of Ca^{2+}/CaM-dependent protein kinase II (CaMKII), an important regulator of ion channel activity (Soderling and Derkach, 2000; Hudmon and Schulman, 2002). Our previous work established that CaMKII modulates α_{1H} but not α_{1G} channel gating, preferentially increasing current at subthreshold potentials (Wolfe et al., 2002). Because the activity of multimeric CaMKII depends on Ca^{2+}/CaM binding and intersubunit autophosphorylation, CaMKII activity can extend beyond the lifetime of the Ca^{2+} signal and enable the kinase to sum and thereby respond to small repeated Ca^{2+} transients (Soderling and Derkach, 2000; Hudmon and Schulman, 2002). Thus, CaMKII-dependent regulation of LVA channel activity could persist during weak synaptic stimulation.

CaMKII is ubiquitously distributed throughout the CNS and PNS and is a major constituent of postsynaptic densities of excitatory synapses (Kennedy et al., 1983; Kelly et al., 1984; Braun and Schulman, 1995; Liu and Jones, 1996; Hudmon and Schulman, 2002). The widespread localization of this kinase (Braun and Schulman, 1995) and LVA channels (Perez-Reyes, 2003) in both dendritic and somatic compartments (Liu and Jones, 1996; Shen et al., 1998; Thiagarajan et al., 2002) and their independent roles in modulating synaptic transmission suggested a direct mechanism for the regulation of α_{1H} channels by CaMKII that would be of general importance to neuronal function.

Here, we show that the regulation of α_{1H} channels by CaMKII depends on structural determinants contained within the intracellular linker connecting channel domains II and III. A serine residue (Ser^{1198}) in the $H_{II–III}$ linker is a preferred CaMKII phosphorylation site and is critical to this modulation. These data identify the α_{1H} Ca^{2+} channel as a direct effector for CaMKII and provide a new CaMKII-dependent mechanism for enhancing neuronal excitability.

Materials and Methods

Preparation of human α_{1G} and α_{1H} chimeric and mutant constructs. All constructs were prepared using a PCR–subcloning strategy in which PCR products were verified by DNA sequencing. A truncated α_{1H} sequence (AF051946 version 1.0) was extended by PCR to add a sequence encoding for

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P.J.W. and H.W. contributed equally to this work.

Correspondence should be addressed to Paula Q. Barrett at the above address. E-mail: pqb4b@virginia.edu.

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50 amino acids using 5′-GACAGCCCTAGGGACAC-3′ [5′-CGAGAATGCACTCGAGCTACACGGGGTCATC-3′ (AF051946 modified)]. The II–III linker regions were amplified from α_{1H} (AF051946 modified) and α_{1G} (AF190860) (Cribbs et al., 2000) were generated by PCR (α_{1H}, 5′-TCCTGTACAACGGCATGG-3′ and 5′-CTCTCTAGATATCAGCGTCTCCACC-3′; α_{1G}, 5′-CCTGTACAATGGTATGGCCTCC-3′ and 5′-CTCTCTAGACACGTGGTCGAACATCTTG-3′) and subcloned into pUC18 at the *Sma*I–*Xba*I site, resulting in pUC18-$H_{II–III}$ and pUC18-$G_{II–III}$. The PCR reaction introduced an upstream *Bsr*GI site in the $H_{II–III}$. The II–III linker of α_{1G} was isolated from pUC18-$G_{II–III}$ by *Bsr*GI–*Pml*I digestion and inserted into pUC18-$H_{II–III}$ at the corresponding site to replace $H_{II–III}$, resulting in pUC18-$G_{II–III}$-1. Finally, the *Bsr*GI–*Eco*RV fragment containing the II–III linker of α_{1G} was isolated from the last construct and subcloned back to pcDNA3-α_{1H}, resulting in construct H-$G_{II–III}$. Similarly, preparation of the construct G-$H_{II–III}$ involved an intermediate construct pUC18-$H_{II–III}$-1 that was generated by subcloning a PCR (5′-CCAATGCATCTTTTCGGCTGCAAGTTC-3′ and 5′-CCATCTAGACACGTGATCAAACATCTTG-3′) product of $H_{II–III}$ into pUC18 at the *Sma*I–*Xba*I site, in which an *Nsi*I site was introduced upstream of $H_{II–III}$. The $H_{II–III}$ linker was isolated from an *Nsi*I–*Pml*I digestion of pUC18-$H_{II–III}$-1 and subcloned into pUC18-α_{1G} replacing the original II–III linker, resulting in pUC18-G-$H_{II–III}$. The chimeric channel was subcloned into pcDNA3.1(−) at the *Eco*RI–*Kpn*I site, resulting in the construct G-$H_{II–III}$. All mutations were introduced into the intermediate constructs pUC18-$H_{II–III}$ or pUC18-$H_{II–III}$-1 using QuikChange XL Site-Directed Mutagenesis Kit (catalog #200517; Stratagene, La Jolla, CA). Last, the construct G-$H_{C\ terminus}$ was prepared by fusing the 5′ section of α_{1H} and the 3′ section of α_{1G} at the *Pml*I site in pcDNA3.

Preparation of glutathione S-transferase fusion proteins. The wild-type DNA construct of the glutathione *S*-transferase (GST)-$H_{II–III}$ fusion protein was generated by subcloning a PCR (5′-CATGGATCCCAGGCGGAGGGCGATG-3′ and 5′-CAAGATATCCGTGGGTCCAGGGACTC-3′) product of α_{1H} ($\alpha_{1H3117–3696}$) into pGEX-2T at the *Bam*HI–*Sma*I site. The mutations S1198A and/or S1153A were introduced by PCR. All PCR products were verified by DNA sequencing.

In vitro *CaMKIIγc phosphorylation.* GST-$H_{II–III}$ fusion proteins were purified from transformed *Escherichia coli* BL21 cell lysates by binding to 200 μl of 50% (w/v) slurry of glutathione-Sepharose 4B (Amersham Biosciences, Arlington Heights, IL). After repeated washes with PBS, the GST-$H_{II–III}$ fusion proteins were eluted from the beads with 20 mM reduced glutathione in a Tris-HCl buffer, pH 7.4. Purity of the isolated proteins was assessed by SDS-PAGE, and concentration was determined using a Coomassie Protein Assay (catalog #1856209; Pierce, Rockford, IL). It was estimated from Coomassie staining of SDS-PAGE samples that 50% of the final protein was full length. Phosphorylation of the GST-$H_{II–III}$ fusion proteins was performed in a final volume of 50 μl containing 1 μM fusion protein, 1 mM DTT, 10 mM HEPES, 0.5 mM $CaCl_2$, 2 μM calmodulin, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 mg/ml BSA, and 100 μM [^{32}P]ATP (10 mCi/ml). Reaction was initiated by the addition of 30 nM purified recombinant porcine CaMKIIγc, and samples were incubated at 37°C for up to 15 min. Reactions were terminated by addition of 20 μl of 4× SDS sample buffer containing 500 mM EGTA, followed by incubation at 95°C for 5 min before separation by SDS-PAGE. Gels were Coomassie stained, and, after autoradiography, bands corresponding to phosphorylated GST-$H_{II–III}$ fusion proteins were cut from the gel, and radioactivity was assessed using liquid scintillation counting.

Electrophysiology. Whole-cell currents were recorded from adherent HEK293 cells using a standard patch electrode voltage-clamp method (Lu et al., 1994). To eliminate K^+ currents and fix free [calcium], we used internal solutions containing the following (in mM): 115 CsCl, 1 tetrabutylammonium chloride, 1 $MgCl_2$, 5 Mg-ATP, 1 Li-GTP, 20 HEPES, pH.7.2 (adjusted with CsOH), and 11 BAPTA; added $CaCl_2$ fixed the free Ca^{2+} at 27 nM (0.9 mM) or 1 μM (8.8 mM) with 2 μM CaM (Lu et al., 1994). HEK293 cells were superfused with a solution containing the following (in mM): 127 TEA-Cl, 10 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, 5 dextrose, and 32 sucrose, pH 7.4 (adjusted with CsOH). Currents were filtered at 2.5 kHz and sampled at 12.5 kHz; leak subtraction was performed on line using P/4 protocol. Activation gating was determined using tail currents in response to 15 test depolarizations in 5 mV increments (−60 to +10 mV; 10.4 msec) from a holding potential of −90 mV during repolarization to −60 mV (45 msec). Interpulsing time was 6 sec to allow for recovery from inactivation. Tail currents were fitted to a single exponential plus a constant using the Chebyshev algorithim in pClamp 6.0 software (Axon Instruments, Foster City, CA). As described previously (Wolfe et al., 2002), activation gating fitted significantly better to a Boltzmann distribution raised to the second power: $[I/I_{max} = 1/(1 + \exp[(V_{0.25} - V_t)/k])^2$, where k is the slope factor (mV/e-fold change), V_t is the test potential, and I_{max} is the maxium current. Half-activation potential was calculated as follows: $V_{0.5} = 0.882(k) + V_{0.25}$. Data obtained with the CaMKII-activating internal solution were fitted to the sum of two Boltzmanns. Averaged data sets obtained with activating or non-activating solutions were analyzed concurrently to obtain a single set of parameters for the non-modulated component that optimally describes both sets of data and another set of parameters for the second modulated component. Parameter estimations were performed by a nonlinear least-squares procedure (Johnson and Frasier, 1985) using boot strapping to determine confidence limits (Efron and Tibshirani, 1993). To evaluate the goodness-of-fit to a single versus a double Boltzmann distribution, the sum of the squared residuals was calculated and mapped into a probability by calculating a Z score for the runs test (Straume and Johnson, 1992).

Results

Activation of CaMKII induces an 11 mV hyperpolarizing shift in the half-activation potential ($V_{1/2}$) of native and recombinant α_{1H} channels (Lu et al., 1994; Schrier et al., 2001; Wolfe et al., 2002). This regulation depends on active kinase (Lu et al., 1994; Barrett et al., 2000) and is not observed with human recombinant α_{1G} channels ($Ca_v3.1$) (Monteil et al., 2000) stably expressed in HEK293 cells (Wolfe et al., 2002). To test for the differential regulation of these two $Ca_v3.0$ family members in a transient transfection paradigm, we cotransfected CaMKII with either the α_{1H} or the α_{1G} channel construct. Channel activity was measured using internal solutions that either promote (1 μM free Ca^{2+}; 2 μM CaM) or impair (27 nM free Ca^{2+}) CaMKII activation (Wolfe et al., 2002). Ca^{2+}/CaM potentiates α_{1H} currents at potentials at which channel activation is incomplete (−60 to 0 mV) (Fig. 1*b*). Neither currents recorded at maximally activating voltages ($V_{test} = +10$ mV; $V_r = -60$ mV) nor currents recorded from α_{1G} channels (Fig. 1*b*,*e*) are increased with CaMKII-activating internal solutions. The gating behavior of α_{1H} channels recorded with CaMKII-activating solutions is poorly fitted by a single squared Boltzmann function but is fitted significantly better ($p < 0.05$) by a double Boltzmann function that defines two channel populations. One population displays properties indistinguishable from channels recorded with impaired CaMKII activity [termed non-modulated (N-M)], whereas the other population [termed modulated (M)] displays a more hyperpolarized half-activation potential ($V_{1/2}$) and an increased voltage sensitivity (k) (Fig. 1*c*, inset). Concurrent analysis of the averaged data sets reveals that CaMKII activation modulates α_{1H} but not α_{1G} channels, inducing a −14.3 ± 1.6 mV ($p < 0.05$) shift in the half-activation potential and an Δ −4.5 ± 1.4 mV/e-fold ($p < 0.05$) increase in the voltage sensitivity of gating, as indicated by the reduction in slope factor (k). A concomitant change in the midpoint and the apparent steepness of gating suggests that CaMKII may change the work required to open the channel, possibly by reducing the electrostatic surface potential sensed by the voltage-sensing module (Hille, 2001). On average, CaMKII modulates 45 ± 9% of the α_{1H} channel population; however, in any given cell, modulation can be near complete or remain submaximal. An additional analysis of the regulation of α_{1H} channels shows that the CaMKII-modulated channel population in individual cells may be as large as 99% or as small as 21% of the total channel population (46 ± 5%). We interpret this variability as a byproduct of transient transfection because the expression of GFP, one cotransfectant, is also

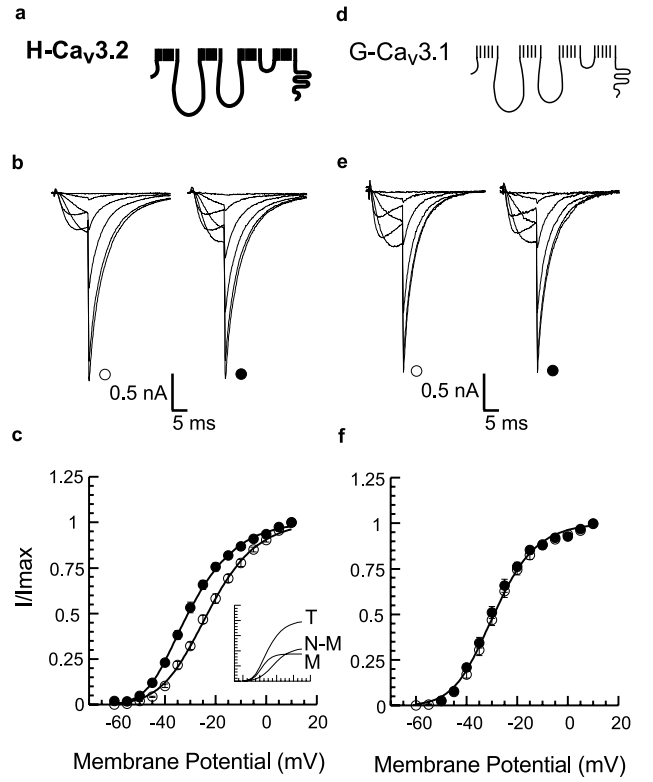

Figure 1. Effect of CaMKII activity on the voltage dependence of activation of T-type Ca^{2+} channels. *a*, *d*, Schematic representation of α_{1H} ($Ca_v3.2$) and α_{1G} ($Ca_v3.1$) channels. *b*, *e*, Whole-cell current traces recorded from α_{1H} (*b*) or α_{1G} (*e*) channels transiently expressed with CaMKII in HEK293 cells. Sample currents at $V_{test} = -55, -45, -35, -25, -10$, and $+10$ mV recorded with intracellular free Ca^{2+} fixed at 27 nM Ca^{2+} (open symbols) to prevent CaMKII activation or 1 μM Ca^{2+} plus 2 μM CaM (filled symbols). Currents were normalized ($I_{max} = 2.5$ nA) to illustrate differences at hyperpolarized V_m between channel subtypes recorded with CaMKII activating solutions. *c*, *f*, Relative conductance (normalized tail current amplitudes, I/I_{max}) plotted (means ± SEM) versus V_t. Data sets were fitted to squared Boltzmann functions; single functions provided good fits to low Ca^{2+} data (open symbols) for α_{1H} (*c*) [$V_{1/2} = -23.2 \pm 0.3$mV; $k = 10.9 \pm 0.4$ mV/*e*-fold change; $n = 17$ (27 nM)] and to both low- and high-Ca^{2+} data for α_{1G} channels (*f*) [$V_{1/2} = -29.2 \pm 0.6$ mV; $k = 9.4 \pm 0.4$ mV/*e*-fold change; $n = 16$ (27 nM) and 11 (1 μM Ca^{2+})]. Fitting to the sum of two functions was significantly better ($p < 0.05$) for the high-Ca^{2+} data for α_{1H}. Inset shows the contributions of the two components fitted to the high-Ca^{2+} α_{1H} data set (T); one component is identical to the low-Ca^{2+} fit and is designated non-modulated (N-M), whereas the second modulated component (M) activates at more hyperpolarized voltages [M: $V_{1/2} = -37.5 \pm 0.3$mV; $k = 6.4 \pm 1.5$ mV/*e*-fold change; $n = 15$ (1 μM Ca^{2+})].

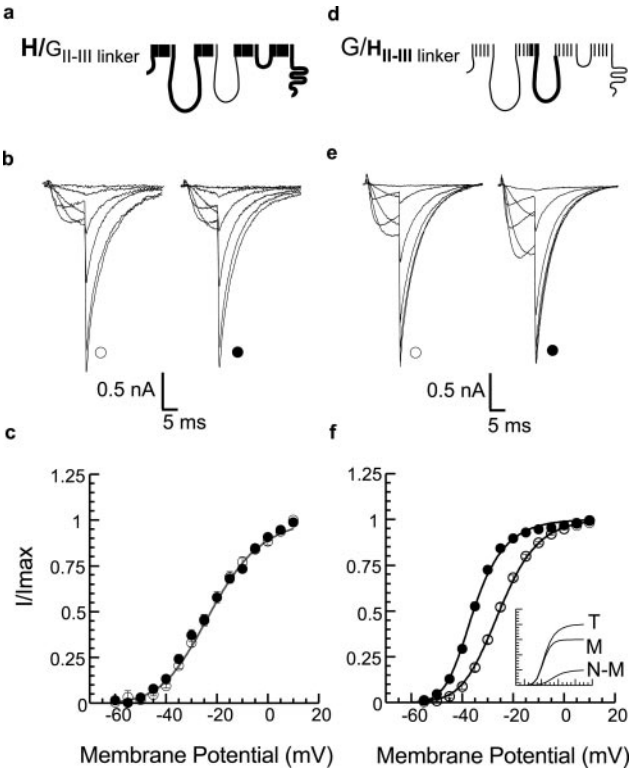

Figure 2. The II–III cytoplasmic linker of α_{1H} channels confers modulation in cells coexpressing CaMKII. *a*, *d*, Schematic representation of channel chimeras in which the II–III linker plus a single transmembrane helix is swapped. *b*, *e*, Sample currents at $V_t = -55, -45, -35, -25, -10$, and $+10$ mV recorded with solutions that impair (open symbols) or promote (filled symbols) CaMKII activation as presented in Figure 1, *b* and *e*. *c*, *f*, Normalized tail current amplitudes (I/I_{max}) plotted (means ± SEM) versus V_t. Single squared Boltzman functions provided good fits for both low- and high-Ca^{2+} data for $H/G_{II-III\ linker}$ (*c*) [$V_{1/2} = -22.4 \pm 0.3$ mV; $k = 11.4 \pm 0.5$ mV; $n = 15$ (27 nM) and 11 (1 μM Ca^{2+})] and low-Ca^{2+} data (open symbols) for $G/H_{II-III\ linker}$. High Ca^{2+} data for $G/H_{II-III\ linker}$ fitted significantly better ($p < 0.05$) to the sum of two functions (filled symbols) (*f*) [$G/H_{II-III\ linker}$, M: $V_{1/2} = -37.8 \pm 0.6$ mV; $k = 5.3 \pm 0.6$ mV; $n = 17$ (1 μM Ca^{2+})]. Inset shows the fitted contribution of modulated (M) and non-modulated (N-M) channels to the voltage dependence of activation during CaMKII simulation (T). T, Sum of both fitted components for high Ca^{2+} data.

variable. Nonetheless, the regulation of α_{1H} but not α_{1G} channels in transient transfection suggests that structural determinants in the channel protein may underlie CaMKII-induced regulation.

To determine the molecular basis for this regulation, we generated chimeric channel constructs from wild-type α_{1H} ($Ca_v3.2$) and α_{1G} ($Ca_v3.1$) channels. Replacement of the $H_{II–III}$ linker with the $G_{II–III}$ linker abolishes CaMKII-dependent regulation (Fig. 2*a–c*) [$H/G_{II–III\ linker}$: $V_{1/2} = -22.4 \pm 0.3$ mV, $k = 11.4 \pm 0.5$ mV; $n = 15$ (27 nM) and 11 (1 μM Ca^{2+})], suggesting that the determinants for the regulation of activation gating by CaMKII are located in the $H_{II–III}$ linker. Despite the absence of regulation, other channel properties remain unchanged. Neither the voltage dependency of inactivation (-65 ± 0.2 mV) nor the τ of deactivation (3.9 ± 0.3 msec at -60 mV) of the $H/G_{II–III}$ linker chimera differs from that of α_{1H} wild-type channels (-64.5 ± 0.2 mV, 4.2 ± 0.5 msec; NS).

On the basis of the previous studies, we predicted that the $H_{II–III}$ linker, when transferred to the α_{1G} backbone, would result in a gain of regulation and tested for CaMKII-dependent regulation of this $G/H_{II–III\ linker}$ chimera. CaMKII activity shifts the half-activation potential of this chimera by -9.4 ± 0.4 mV ($p < 0.05$) and also increases the voltage sensitivity by $\Delta -3.3 \pm 0.6$ mV/*e*-fold change ($p < 0.05$). On average, 75 ± 11% of the G chimeric channel population is modulated, displaying a half-activation potential that is nearly identical to that recorded for CaMKII-modulated α_{1H} wild-type channels [$G/H_{II–III}$ linker: M, $V_{1/2} = -37.8 \pm 0.6$ mV, $k = 5.3 \pm 0.6$ mV, $n = 17$ (1 μM Ca^{2+})]. An additional analysis of the regulation of G-chimeric channels shows that the CaMKII-modulated channel population in individual cells may vary from 89 to 46% of the total channel population (74 ± 4%). This broad distribution differs but overlaps that observed for wild-type α_{1H} channels. The singular importance of the $H_{II–III}$ linker to the CaMKII-dependent change in channel gating was corroborated by the construction of a second G/H chimera that replaces a large segment of the α_{1G} channel from IIIS1 to the C terminus (residues 1294–2353), with the corresponding regions from α_{1H} channels. CaMKII-dependent regulation is not conferred to this $G/H_{C\ terminus}$ chimera (Fig. 3*c*). On the basis of these findings, we conclude that residue(s) critical for CaMKII-dependent regulation are located in the $H_{II–III}$ linker.

The α_{1H} channel is a large 259 kDa membrane protein con-

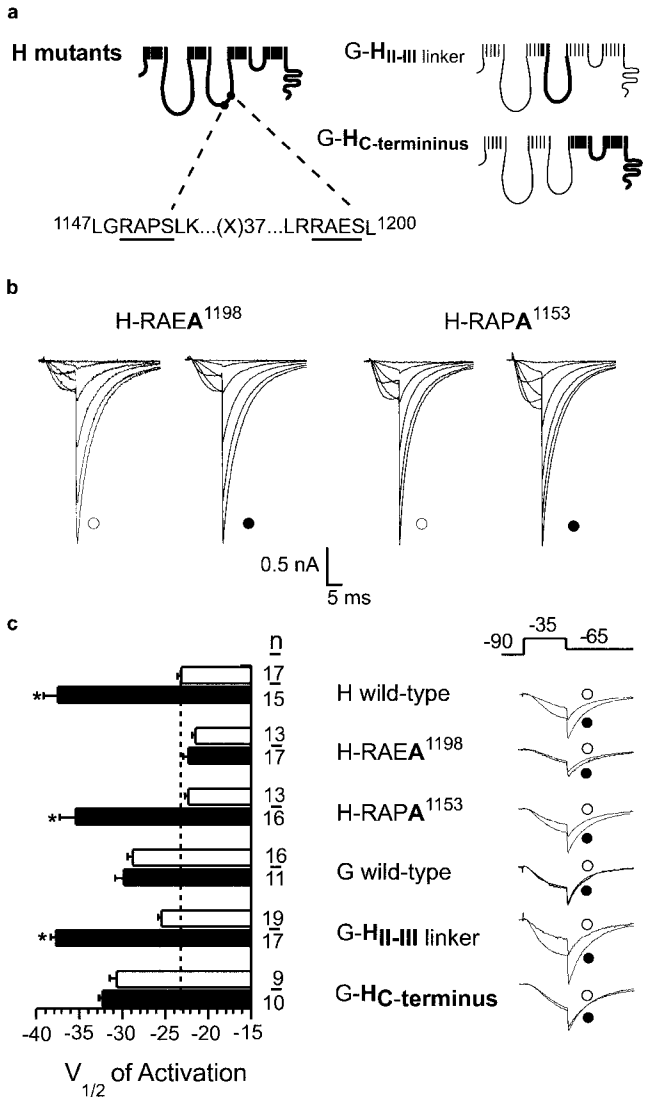

Figure 3. Effect of single point mutations on CaMKII-induced changes in channel gating. *a*, Schematic representation of α_{1H} chimeras in which CaMKII-consensus motifs on the $H_{II–III}$ domain linker are mutated (RAPA[1153] and RAEA[1198]), alone and in combination, and α_{1G} chimeras with transferred α_{1H} domains. *b*, Sample currents at $V_t = -55, -45, -35, -25, -10$, and $+10$ mV recorded with solutions that impair (open symbols) or promote (filled symbols) CaMKII activation as presented in Figure 1, *b* and *e*. *c*, Fitted values for half-activation potential; open bars indicate low-Ca^{2+} fitted parameters (open bars), and filled bars indicate high-Ca^{2+} fitted parameters. Values are mean ± SE; *$p < 0.05$; *n* indicates number of cells. Normalized current waveforms recorded at $V_t = -35$ mV (10 msec, $V_{repolarization}$ of -60 mV) with free Ca^{2+} fixed at 27 nM Ca^{2+} (open symbols) or 1 μM Ca^{2+} plus 2 μM CaM (filled symbols).

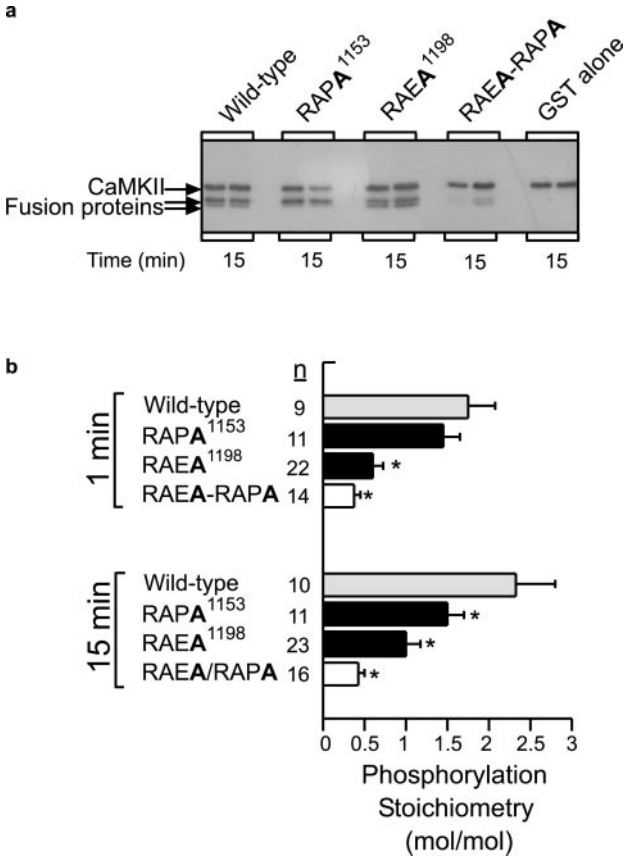

Figure 4. Phosphorylation of GST-domain II–III linker by CaMKII. *A*, GST fusion proteins containing residues 1039–1232 of domain linker II–III (wild-type or mutated at RAPA[1153] and/or RAEA[1198]) were phosphorylated with purified recombinant CaMKIIγ_C in the presence of [γ-^{32}P]ATP. Reactions were subjected to SDS-PAGE analysis. Representative autoradiograph showing phosphorylation state at 15 min ($n = 2$). Autophosphorylated CaMKIIγ_C resolves above the linker proteins. *b*, Analysis of phosphorylation stoichiometry of full-length linker protein (47 kDa) at 1 and 15 min of incubation (mean ± SE). The apparent phosphorylation stoichiometry of the RAES[1198] or RAPS[1153] motifs is indicated by the difference in phosphorylation state between the single-mutated (hatched or stripped bars) and double-mutated (open bars) fusion proteins. Phosphate incorporation into mutant GST-linker fusion proteins were compared with wild-type using ANOVA with *post hoc* Dunnett's testing, in which significance was $p < 0.05$.

taining 22 sites that conform to the minimal consensus sequence for CaMKII phosphorylation, RXXS/T (Soderling, 1996). We considered each site as a putative substrate for CaMKII using criteria established on the basis of the rank order of phosphorylation efficiency (V_{max}/K_m) of a combinatorial peptide library (White et al., 1998). The ^{1192}LRRAE**S**L^{1200} recognition motif, located in the 275 residue $H_{II–III}$ linker, contains hydrophobic residues at -5 and $+1$, a non-basic amino acid residue at -3, and an alanine (Ala) at the -2 position, determinants that are known to enhance substrate recognition (White et al., 1998). Notably, no other motif in the channel protein has as many advantageous residues for substrate recognition, and this motif is not found in the α_{1G} sequence. To determine whether the LRRAE**S**[1198]L recognition motif on the $H_{II–III}$ linker is a site of CaMKII phosphorylation, we expressed GST fusion proteins containing ~70% of the $H_{II–III}$ linker that includes Ser[1198] as well as Ser[1153], a strong but less optimal substrate site (^{1147}LGRAP**S**LK^{1156}). Recombinant CaMKII (at 30 nM) phosphorylates the full-length wild-type linker protein (47 kDa) as well as a proteolytic fragment (44 kDa) lacking the RAES[1198] motif that resolves as a lower band of a doublet on SDS-PAGE (Fig. 4*a*,*b*). The full-length wild-type linker protein is phosphorylated to a stoichiometry of 1.7 mol of PO_4 incorporated per moles of protein at 1 min, indicating that more than a single residue is phosphorylated, and reaches saturation at 15 min, to attain a final stoichiometry of 2.3. Despite the presence of five CaMKII phosphorylation motifs on the $H_{II–III}$ linker protein, the majority of incorporated phosphate at 1 and 15 min is distributed between Ser[1198] and Ser[1153]. The combined mutation of these residues to Ala reduces phosphorylation by ~80%. At 1 min, Ser[1198] is preferentially phosphorylated to full stoichiometry as determined by mutational analysis. In contrast, mutation of Ser[1153], which is embedded in a less optimal substrate recognition motif, does not result in significant loss of PO_4

incorporation at 1 min. Longer incubation allows for the slower phosphorylation of the RAPS[1153] motif and the recognition of other poorer substrates. Because rapid stoichiometric phosphorylation of a motif is a signature feature of a preferred kinase substrate, our findings suggested that the phosphorylation of Ser[1198] on the $H_{II–III}$ linker could be a critical event that mediates CaMKII-dependent changes in channel gating.

Finally, to test the functionality of the RAES[1198] motif, we mutated Ser[1198] in the full-length channel protein and assayed for CaMKII-dependent regulation of channel activity. Mutation of Ser[1198] to Ala abrogates CaMKII-dependent modulation of α_{1H} channels (Fig. 3*c*). Neither the half-activation potential nor the voltage sensitivity differs from non-modulated wild-type α_{1H} channels [$V_{1/2} = -21.8 \pm 0.4$ mV, $k = 11.5 \pm 0.5$ mV; $n = 17$ (1 μM Ca^{2+}); NS]. In contrast, mutant α_{1H} channels harboring a Ser[1153] to Ala mutation retain CaMKII-dependent modulation (M: $V_{1/2} = -35.4 \pm 1.9$ mV; $k = 5.3 \pm 2.0$ mV/*e*-fold change; $34 \pm 11\%$; $p < 0.05$).

Modification of protein function by phosphorylation can be the result of the accommodation of the bulk of the phosphate moiety (Busch et al., 2002) or the introduction of negative charge (Buchbinder et al., 1997). We mutated Ser[1198] to glutamine (Gln) to test first for the possible importance of the loss of serine hydroxyl interactions during phosphate incorporation. Gln[1198] failed to change either the half-activation potential or the voltage sensitivity of this mutant α_{1H} channel [$V_{1/2} = -23.5 \pm 1.2$ mV; $k = 9.4 \pm 0.3$ mV; $n = 10$ (27 nM)]. Moreover, as observed previously with mutation of the RAES[1198] motif, the loss of Ser[1198] prevents regulation by CaMKII [$V_{1/2} = -25.2 \pm 0.7$ mV; $k = 9.4 \pm 0.2$ mV; $n = 11$ (1 μM)]. In a similar manner, mutation of Ser[1198] to glutamic acid (Glu) creates a channel with gating properties that mimic those of the Gln[1198] mutant. Both the $V_{1/2}$ of activation and the voltage sensitivity of this mutant channel also remain unaltered in the absence and presence of CaMKII activation [$V_{1/2} = -24.8 \pm 0.8$ mV, $k = 9.8 \pm 0.4$ mV, $n = 12$ (27 nM); $V_{1/2} = -23.3 \pm 0.5$ mV, $k = 9.7 \pm 0.4$ mV, $n = 11.$, (1 μM)], suggesting that the monoanionic carboxylate group of Glu cannot replace the dianionic phosphate of phosphoserine in reproducing either the exact charge balance or the phosphopeptide positioning that is required to change channel gating (Buchbinder et al., 1997). Collectively, our mutagenesis studies provide support for our *in vitro* phosphorylation data and identify the key role played by Ser[1198] in the observed changes in α_{1H} channel gating induced by CaMKII.

Discussion

The present data provide a molecular explanation for how CaMKII differentially regulates LVA Ca^{2+} channels. We described a remarkably simple way by which CaMKII changes α_{1H} activation gating. Here, we identify a single serine residue (Ser[1198]) within the II–III cytoplasmic linker that is unique to α_{1H} channels, phosphorylated by CaMKII, and critical for channel regulation. This mechanism for Ca^{2+} channel regulation differs from that reported for α_{1C} ($Ca_v1.2$) channels. Feedforward regulation (facilitation) of α_{1C} channels can be supported by a CaMKII-independent mechanism that requires CaM binding to an IQ motif (Zuhlke et al., 2000), a domain absent from the T-type Ca^{2+} channel family, and/or by a CaMKII-dependent mechanism that may require cytoskeletal intermediates (Yuan and Bers, 1994; Dzhura et al., 2000, 2002; Wu et al., 2001). However, the critical substrate(s) targeted by CaMKII remains undetermined. Our data support previous observations showing that the opening frequency of single α_{1H} channels in excised patches can be increased by membrane-associated CaMKII (Barrett et al., 2000). These studies showed that the participation of a cytosolic kinase cascade is not required for CaMKII-dependent regulation and implied the possibility of a direct modulation of α_{1H} Ca^{2+} channels by CaMKII. Our data provide the first evidence for direct regulation of the pore-forming subunit of a voltage-dependent Ca^{2+} channel by CaMKII.

The II–III cytoplasmic linker of the pore-forming channel subunit of other voltage-gated Ca^{2+} channels forms important structural associations with signaling effectors. In the Ca_v1 family, the II–III linker of α_{1S} couples the voltage sensor to the ryanodine-sensitive Ca^{2+} release channel (Tanabe et al., 1988, 1990), whereas in the Ca_v2 family, the II–III linker facilitates efficient delivery of Ca^{2+} to sites of neurotransmitter release by binding SNARE (soluble *N*-ethylmaleimide-sensitive factor attached protein receptor) proteins that concomitantly modify inactivation gating (Sheng et al., 1994; Catterall, 2000; Jarvis and Zamponi, 2001). Our data highlight a role for the II–III linker of T-type Ca^{2+} channels and show that, in the Ca_v3 family, the II–III linker is an important site for the control of activation gating. Recent studies from our laboratory indicate that the $H_{II–III}$ linker also transduces current inhibition by directly binding G-protein β_2-containing $\beta\gamma$-dimers (Wolfe et al., 2003). Thus, in the Ca_v3 family, the $H_{II–III}$ linker may serve as a center for integrating effects of multiple stimuli.

Although mRNA transcripts encoding each of the T-type Ca^{2+} channel subtypes are widely expressed throughout the CNS and PNS, the pattern of expression of each channel gene is unique, albeit overlapping (Talley et al., 1999). α_{1H} mRNA is expressed at high levels in restricted regions of the hippocampus (pyramidal cell layers and granule layer of the dentate gyrus), cerebral cortex (a subset of layer V neocortical cells), and thalamus (reticular cells), as well as in sensory ganglia, dorsal horn neurons (external lamina), and the olfactory bulb. In contrast to other members of the Ca_v3 Ca^{2+} channel family whose currents are blocked by Ni^{2+} with an IC_{50} that overlaps that for block of HVA Ca^{2+} channels, α_{1H} channels are inhibited by a Ni^{2+} concentration that is 20- to 50-fold lower (IC_{50} of 10 μM) (Perez-Reyes, 2003).

The Ni^{2+} sensitivity of low-threshold, slowly deactivating Ca^{2+} channel currents has revealed the specific importance of the α_{1H} channel isoform in the following: hippocampal–neocortical dendritic low-threshold Ca^{2+} spike initiation (Markram and Sakmann, 1994; Magee and Johnston, 1995b), regenerative potential conduction, and somatic burst firing (Gillessen and Alzheimer, 1997; Larkum et al., 2001), as well as in both synaptic (lamina I projection neurons of the dorsal horn) and nonsynaptic (CA1 pyramidal neurons) forms of activity-dependent potentiation (Su et al., 2002; Ikeda et al., 2003). Given the broad distribution of α_{1H} channels and CaMKII in nervous tissue, the operation of the direct phosphorylation-dependent regulatory mechanism described here may be widespread, supporting the enhanced participation of α_{1H} channels in these diverse neuronal settings.

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