Calmodulin kinase is functionally targeted to the action potential plateau for regulation of L-type Ca^{2+} current in rabbit cardiomyocytes

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L-type Ca^{2+} current ($I_{Ca\text{-}L}$) triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) and both SR and $I_{Ca\text{-}L}$ are potential sources of intracellular Ca^{2+} (Ca_i^{2+}) for feedback regulation of $I_{Ca\text{-}L}$. Ca_i^{2+} bound to calmodulin (Ca^{2+}–CaM) can inhibit $I_{Ca\text{-}L}$, while Ca^{2+}–CaM can also activate Ca^{2+}–CaM-dependent protein kinase II (CaMK) to increase I_{Ca}. However, it is not known whether $I_{Ca\text{-}L}$ or the SR is the primary source of Ca^{2+} for $I_{Ca\text{-}L}$ regulation. The L-type Ca^{2+} channel C terminus is implicated as a critical transduction element for $I_{Ca\text{-}L}$ responses to Ca^{2+}–CaM and CaMK, and the C terminus undergoes voltage-dependent steric changes, suggesting that Ca_i^{2+} control of $I_{Ca\text{-}L}$ may also be regulated by cell membrane potential. We developed conditions to separately test the relationship of Ca^{2+}–CaM and CaMK to $I_{Ca\text{-}L}$ and SR Ca_i^{2+} release during voltage clamp conditions modelled upon time and voltage domains relevant to the cardiac action potential. Here we show that CaMK increases $I_{Ca\text{-}L}$ after brief positive conditioning pulses, whereas Ca^{2+}–CaM reduces $I_{Ca\text{-}L}$ over a broad range of positive and negative conditioning potentials. SR Ca^{2+} release was required for both Ca^{2+}–CaM and CaMK $I_{Ca\text{-}L}$ responses after strongly positive conditioning pulses (+10 and +40 mV), while Ca_i^{2+} from $I_{Ca\text{-}L}$ was sufficient for Ca^{2+}–CaM during weaker depolarizations. These findings show that $I_{Ca\text{-}L}$ responses to CaMK are voltage dependent and suggest a new model of L-type Ca^{2+} channel regulation where voltage-dependent changes control $I_{Ca\text{-}L}$ responses to Ca^{2+}–CaM and CaMK signalling.

(Resubmitted 12 August 2003; accepted after revision 27 October 2003; first published online 31 October 2003)
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L-type Ca^{2+} current ($I_{Ca\text{-}L}$) is a prominent feature of the cardiac action potential plateau where it triggers release of Ca^{2+} from the sarcoplasmic reticulum (SR) to modulate contraction (Tanabe *et al.* 1990), Ca^{2+}-dependent gene transcription (Song *et al.* 2002) and is a source of inward current for arrhythmia-initiating afterdepolarizations (January *et al.* 1988; Wu *et al.* 2002). $I_{Ca\text{-}L}$ is regulated by cytoplasmic Ca^{2+} (Ca_i^{2+}) through binding to the Ca_i^{2+}-sensing protein calmodulin (Ca^{2+}–CaM) (Peterson *et al.* 1999; Zuhlke *et al.* 1999), and Ca^{2+}–CaM can bind to the L-type Ca^{2+} channel C terminus and inactivate $I_{Ca\text{-}L}$ (Peterson *et al.* 1999; Zuhlke *et al.* 1999). However, Ca^{2+}–CaM can also activate the Ca^{2+}–CaM-dependent protein kinase II (CaMK) (Hudmon & Schulman, 2002), and CaMK increases $I_{Ca\text{-}L}$ by shifting L-type Ca^{2+} channels into a high opening probability gating mode, through a phosphorylation-dependent mechanism (McCarron *et al.* 1992; Dzhura *et al.* 2000). The site of CaMK action for increasing $I_{Ca\text{-}L}$ is uncertain, but CaMK can bind to the L-type Ca^{2+} channel C terminus in a region that overlaps with known Ca^{2+}–CaM binding domains (Hudmon *et al.* 2002). Thus, the L-type Ca^{2+} channel C terminus appears to play a critical role for transducing $I_{Ca\text{-}L}$ responses to Ca_i^{2+}. In this regard, it is of interest that the Ca_i^{2+}-sensing L-type Ca^{2+} channel C terminus is mobilized by cell membrane depolarization (Kobrinsky *et al.* 2003), suggesting the possibility that $I_{Ca\text{-}L}$ regulation by Ca_i^{2+} may also be voltage dependent.

Ca_i^{2+} can increase as a direct consequence of $I_{Ca\text{-}L}$, or through secondary release of Ca^{2+} from the SR in heart. Some studies suggest that SR Ca^{2+} release is more important than $I_{Ca\text{-}L}$ for activating CaMK, because CaMK-dependent phosphorylation of the SR regulatory protein phospholamban is reduced 50–90% when SR Ca^{2+} release is prevented by ryanodine (Kuschel *et al.* 1999; Bartel *et al.* 2000). On the other hand, Ca_i^{2+} from

 DOI: 10.1113/jphysiol.2003.053314

$I_{Ca\text{-}L}$ is sufficient for engaging Ca^{2+}–CaM-dependent $I_{Ca\text{-}L}$ inactivation mechanisms in non-cardiac cells (Zuhlke *et al.* 1999; Peterson *et al.* 1999) where SR Ca^{2+} release is not anticipated to contribute to Ca_i^{2+}. Furthermore, it remains unknown whether Ca_i^{2+} for Ca^{2+}–CaM-dependent $I_{Ca\text{-}L}$ inactivation and for activating CaMK is primarily from $I_{Ca\text{-}L}$ or the SR in cardiomyocytes during dynamic changes in cell membrane potential, as occur in the working heart.

We controlled CaMK activity with a Ca^{2+}–CaM-independent form of CaMK and a CaMK inhibitory buffer (IB), previously shown to prevent $I_{Ca\text{-}L}$ increases by endogenous CaMK (Wu *et al.* 2001*a*), and inhibited Ca^{2+}–CaM with an inhibitory peptide (Wu *et al.* 2001*b*) or Ba^{2+} substitution to separately control CaMK and Ca^{2+}–CaM activity in ventricular myocytes, in order to differentially test the effects of Ca_i^{2+} from $I_{Ca\text{-}L}$ and SR for regulating $I_{Ca\text{-}L}$ availability. $I_{Ca\text{-}L}$ was measured in cardiomyocytes using a non-steady-state inactivation protocol that mimicked time and voltage conditions present during the cardiac action potential. Our findings support the novel concept that CaMK regulation of $I_{Ca\text{-}L}$ in cardiomyocytes depends upon cell membrane potential. Both Ca_i^{2+} from $I_{Ca\text{-}L}$ and the SR can recruit Ca^{2+}–CaM for $I_{Ca\text{-}L}$ inactivation, but SR Ca^{2+} release is required for CaMK effects, while SR Ca^{2+} release also predominates for Ca^{2+}–CaM-dependent inactivation at strong depolarizations. A new model of voltage- and Ca_i^{2+}-dependent $I_{Ca\text{-}L}$ regulation is proposed.

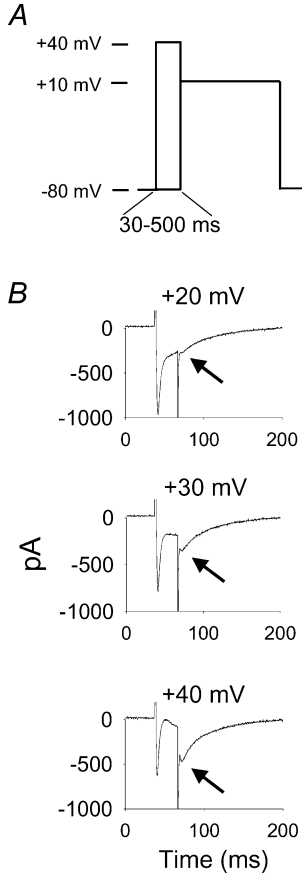

Figure 1. Non-steady-state inactivation voltage clamp protocol reveals increases in relative L-type Ca^{2+} current ($I_{Ca\text{-}L}$) after brief, positive conditioning steps
A, a schematic representation of the voltage clamp protocol used for this study. Peak $I_{Ca\text{-}L}$ was measured at a +10 mV test pulse, after conditioning steps from –80 to +40 mV in 10 mV steps, lasting from 30 to 500 ms. *B*, conditioning steps (30 ms) from +20 to +40 mV progressively increase available $I_{Ca\text{-}L}$ at the test pulse of +10 mV (indicated by arrows). The conditioning prepulse potential is labelled above each panel. A transient outward Ca^{2+}-activated Cl^- current is superimposed on $I_{Ca\text{-}L}$ during some conditioning steps positive to +10 mV (see Methods for details) (Wu *et al.* 1999*b*).

Methods

Electrophysiology

Whole-cell mode configuration was used for voltage clamping isolated rabbit ventricular myocytes according to previously published methods (Wu *et al.* 1999*a*). Ventricular myocytes were isolated from New Zealand White rabbits killed by pentobarbital (50 mg kg^{-1}, I.V.) overdose prior to excising the heart. The Vanderbilt University Animal Care Committee approved all experiments. Cells were held at –80 mV for >5 min for adequate dialysis with pipette solution before initiating experiments. $I_{Ca\text{-}L}$ was conditioned by stepping the cell membrane from –80 mV to +40 mV in 10 mV increments from 30 to 500 ms at 0.1 Hz, and peak $I_{Ca\text{-}L}$ was measured at a test potential of +10 mV (Fig. 1) and expressed as relative current. In some experiments $I_{Ca\text{-}L}$ inactivation was quantified as the fraction of residual inward current present at the end of 30 ms (R_{30}) and 80 ms (R_{80}) conditioning pulses. All experiments were performed at 24°C. Adding Cs^+ and TEA and reducing Na^+ and K^+ in the pipette and bath solutions eliminated Na^+ and K^+ currents. A Ca^{2+}-activated Cl^- conductance ($I_{Cl,Ca}$), known to be activated by SR Ca^{2+} release in rabbit ventricular myocytes (Wu & Anderson, 2000) at cell membrane potentials more positive than +20 mV (Wu *et al.* 1999*b*), was most clearly seen as a transient outward current in response to voltage command steps to +30 and +40 mV. $I_{Cl,Ca}$ was eliminated by niflumic acid (10–20 μM, data not shown) and thapsigargin (Fig. 6*A*), and was not present at the test command potential of +10 mV. Thus, $I_{Cl,Ca}$ was not likely to have significantly contributed to

$I_{Ca\text{-}L}$ measurements because the relationship of relative current (see below) to conditioning potential was not affected by niflumic acid (data not shown), and the relationship between relative current and R_{30} and R_{80} was not altered during positive voltage commands in IB (see below, Fig. 5*A* and *B*) or IB and thapsigargin (Fig. 7*A* and *B*). However, we cannot rule out the possibility that $I_{Cl,Ca}$ did contribute to CaMK and Ca^{2+}–CaM effects, especially at voltage command steps to +30 and +40 mV (Wu *et al.* 1999*b*). Elimination of the residual current by nifedipine (10 μM) or Cd^{2+} (100 μM) confirmed that the identity of active inward current was $I_{Ca\text{-}L}$ (data not shown). The control pipette (intracellular) solution was (mM): CsCl 120.0, EGTA 10.0, Hepes 10.0, tetraethylammonium chloride (TEA) 10.0, phosphocreatine 5.0, $CaCl_2$ 3.0, MgATP 1.0, NaGTP 1.0, and pH was adjusted to 7.2 with 1.0 N CsOH. The ability of endogenous CaMK to facilitate $I_{Ca\text{-}L}$ was eliminated by addition of an inhibitory buffer (IB) solution (see below) (Wu *et al.* 1999*a*, 2001*a*). The bath (extracellular) solution was NMDG 137.0, CsCl 25.0, Hepes 10.0, glucose 10.0, $CaCl_2$ (or $BaCl_2$) 1.8, $MgCl_2$ 0.5, and pH was adjusted to 7.4 with 12 N HCl. Ryanodine (10 μM) or thapsigargin (1 μM) were added to the bath solution for some experiments. Myocyte contraction was eliminated under these conditions (data not shown).

Approaches to controlling CaM and CaMK activity

A recombinant monomeric truncation mutant of the mouse CaMK α isoform (amino acid residues 1–380) was expressed using baculovirus and then purified using CaM agarose affinity chromatography (Brickey *et al.* 1990). This CaMK was stored in IB (50 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol, 10% (v/v) ethylene glycol) and activated by autophosphorylation in a 100 μl reaction containing 50 mM Hepes, pH 7.5, 2 mM magnesium acetate, 1.5 mM $CaCl_2$, 18 μM CaM, 2 mM DTT and 100 μM ATPγ S. The reaction was initiated by addition of the (1–380) CaMK (9 μmol l^{-1} final subunit concentration), incubated at 30°C for 10 min, and stopped by the addition of EDTA (10 mM). IB was used without added CaMK, or after inactivation of enzymatically active CaMK by heating, to observe CaMK-independent effects of manipulating Ca^{2+}–CaM and SR Ca^{2+} release. IB prevents $I_{Ca\text{-}L}$ facilitation by endogenous CaMK (Wu *et al.* 2001*a*) and was used to separate CaMK from Ca^{2+}–CaM activity and SR Ca^{2+} release. Ca^{2+}–CaM-dependent autophosphorylation of the CaMK produces a constitutively active species that can phosphorylate substrates in the absence of Ca^{2+}–CaM. Ca^{2+}-independent activity was typically 35–50% of total activity in the presence of Ca^{2+}–CaM using the peptide substrates syntide-2 or autocamtide. Constitutively active CaMK and IB were diluted 10-fold in the pipette solution (0.9 μM final) for use in voltage clamp studies and its activity confirmed *in vitro*, as described (Wu *et al.* 1999*a*). This dilution was chosen to approximate the physiological CaM kinase activity in heart (~1–2 μM) derived from percentage yield calculations during purification (Iwasa *et al.* 1986; Gupta & Kranias, 1989).

The CaMK inhibitory peptide AC3-I (KKALHRQEAVDCL, IC_{50} ~3 μM) (Braun & Schulman, 1995) (Macromolecular Resources, Fort Collins, CO, USA) is a modified CaMK substrate, which inhibits endogenous and thiophosphorylated constitutively active CaMK. AC3-I was included in the pipette solution at a final concentration of 20 μM. The CaM binding peptide 290–309 (Calbiochem) is modelled on the CaM binding domain of CaMK, and inhibits Ca^{2+}–CaM signalling generally, and was added to the pipette solution at a final concentration of 50 μM. CaMK, 290–309 and AC3-I were dialysed into cells for 5–10 min prior to initiating experiments.

Statistics

The null hypothesis was evaluated with Student's *t* test or ANOVA, as appropriate. Bonferroni's correction was applied for multiple comparisons.

Results

Enhanced $I_{Ca\text{-}L}$ availability following brief, positive conditioning pulses

Brief, positive conditioning prepulses progressively increased relative $I_{Ca\text{-}L}$ availability (Fig. 1), while IB significantly reduced relative $I_{Ca\text{-}L}$ availability (Fig. 2*A*–*C*), compared to control pipette solution, suggesting that endogenous CaMK can increase $I_{Ca\text{-}L}$ under action potential plateau conditions. The increase in $I_{Ca\text{-}L}$ in response to brief (30–130 ms), positive conditioning prepulses was lost at longer (500 ms) prepulse durations when Ca^{2+} was the charge carrier (Fig. 2*D*), but persisted when Ba^{2+} was substituted for Ca^{2+} (Fig. 2*E*), in cells dialysed with IB. The persistence of Ca^{2+}-dependent reduction in $I_{Ca\text{-}L}$ availability in IB indicates that Ca^{2+}–CaM-dependent inactivation is operative under

these experimental conditions. These findings show that Ca^{2+} critically determines $I_{Ca\text{-}L}$ availability during time and voltage conditions present during the ventricular action potential plateau, and serve as a starting point for dissecting the role of Ca^{2+}–CaM, CaMK and SR Ca^{2+} release in determining $I_{Ca\text{-}L}$ availability in cardiac myocytes.

CaMK increases $I_{Ca\text{-}L}$ at action potential plateau conditions

Previous work has shown that IB prevented CaMK mediated I_{Ca} increases in cardiac myocytes (Wu *et al.* 1999*a*, 2001*a*), strongly suggesting that CaMK inhibition was a critical feature of IB actions at $I_{Ca\text{-}L}$. On the

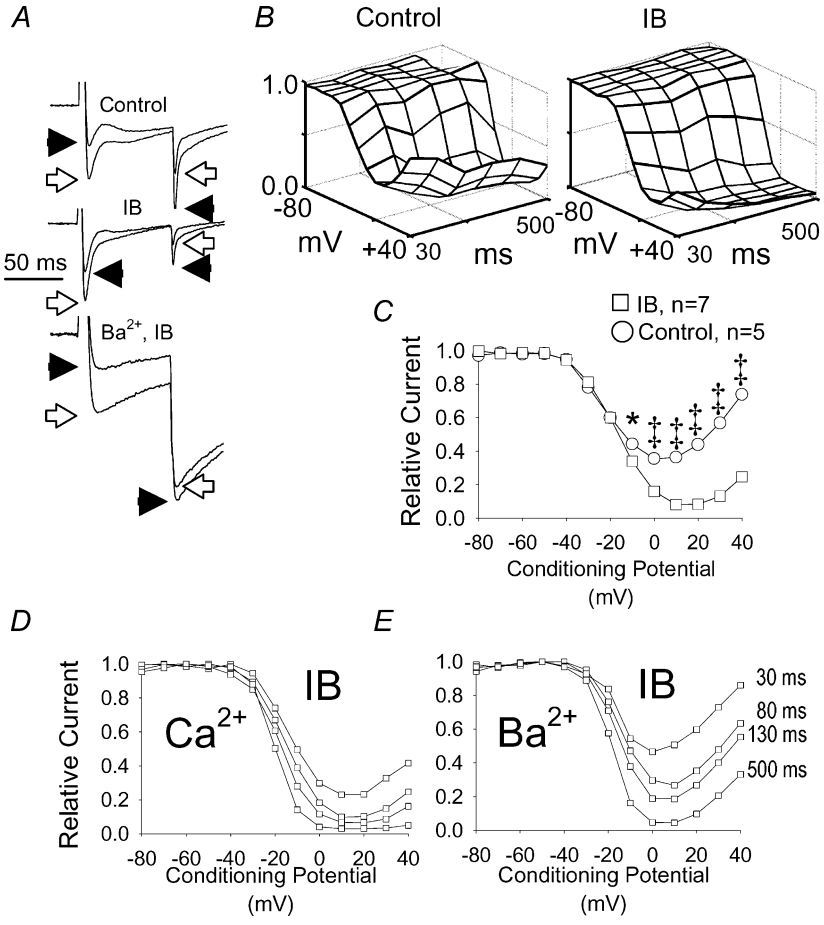

Figure 2. A CaMK inhibitory buffer (IB) reduces $I_{Ca\text{-}L}$ at action potential plateau potentials
A, current tracings recorded in control pipette solution with Ca^{2+} as charge carrier (Control), IB pipette solution with Ca^{2+} as charge carrier (IB) and IB pipette solution with Ba^{2+} as charge carrier (Ba^{2+}, IB) are shown. Currents in response to +20 mV (open arrows) and +30 mV (filled arrow heads) conditioning prepulses are superimposed and normalized to the peak $I_{Ca\text{-}L}$ at +20 mV for comparison. *B*, topology plots showing the combined effects of conditioning prepulse potentials (–80 to +40 mV) and conditioning prepulse durations (30–500 ms) on relative peak Ca^{2+} current (I_{Ca}) recorded during the test potential step to +10 mV in cells ($n = 3$ for each group) dialysed with control solution (Control) or IB solution (IB). The grid lines on the topology plots indicate conditioning potentials in +10 mV increments (evenly spaced from –80 to +40 mV) and conditioning prepulse durations (evenly spaced at 30, 80, 130, 190, 300 and 500 ms). *C*, peak $I_{Ca\text{-}L}$ after 80 ms conditioning prepulses from –80 to +40 mV (abscissa) in cells dialysed with control buffer ($n = 5$) that permits activation of endogenous CaMK and with IB ($n = 7$, see Methods for details) that prevents activation of endogenous CaMK. This IB data set is also used in Figs 3–6 and this Control data set is used in Figs 4 and 5 for other comparisons. *D* and *E* show a family of non-steady-state inactivation relationships for a single cardiomyocyte dialysed with IB using (*D*) Ca^{2+} or (*E*) Ba^{2+} as charge carrier. The duration of the conditioning prepulse is indicated for each of the isochrones in *C* and *D*. Peak $I_{Ca\text{-}L}$ is normalized to the maximum value in all panels. $^{*}P < 0.05$, $^{\ddagger}P < 0.001$.

other hand, the persistence of Ca^{2+}-dependent $I_{Ca\text{-}L}$ inactivation (Fig. 2*A*, *D* and *E*) suggested IB did not disrupt Ca^{2+}–CaM signalling, generally. We created a topographical surface plot of relative $I_{Ca\text{-}L}$ availability to better illustrate the effects of IB over a wide range of conditioning times and voltages (Fig. 2*B*). These plots reveal the functional targeting of significant IB actions to time and voltage durations relevant to the action potential plateau ($P < 0.05$ for all intergroup comparisons from conditioning potentials between 0 and +40 mV and conditioning pulse durations from 30 to 130 ms). In order to more thoroughly test the concept that IB was selective for CaMK and that reduction in $I_{Ca\text{-}L}$ at action potential plateau potentials in IB was due to CaMK, we dialysed a Ca^{2+} independent form of CaMK into myocytes in the presence of IB (Fig. 3*B*). Ca^{2+}–CaM-independent CaMK significantly restored reduced $I_{Ca\text{-}L}$ availability in IB (Fig. 3*B*), indicating that inhibition of endogenous CaMK was the critical IB effect. The effects of Ca^{2+}–CaM-independent CaMK on $I_{Ca\text{-}L}$ were specifically due to the enzymatic activity of the exogenous kinase, as they were significantly reduced by coadministration of the CaMK inhibitory peptide AC3-I (data not shown), and eliminated by heat inactivation of the added CaMK (Fig. 3*B*). CaMK effects were confined to brief conditioning pulses (30–130 ms), and were lost after longer conditioning prepulses (190–500 ms, Fig. 4), confirming that CaMK actions on $I_{Ca\text{-}L}$ were targeted to time and voltage domains relevant to the cardiac action potential plateau.

We next considered the relationship between $I_{Ca\text{-}L}$ inactivation during brief conditioning voltage pulses (R_{30} and R_{80}) and relative $I_{Ca\text{-}L}$ availability (relative current) in the subsequent test pulse (Fig. 1), in order to better understand the apparent voltage dependence of CaMK signalling to L-type Ca^{2+} channels (Dzhura *et al.* 2000). At a positive conditioning potential (+20 mV) R_{30} and R_{80} accurately predicted relative current under control conditions, in IB and in IB after CaMK replacement (Fig. 5*A* and *B*). CaMK replacement significantly restored

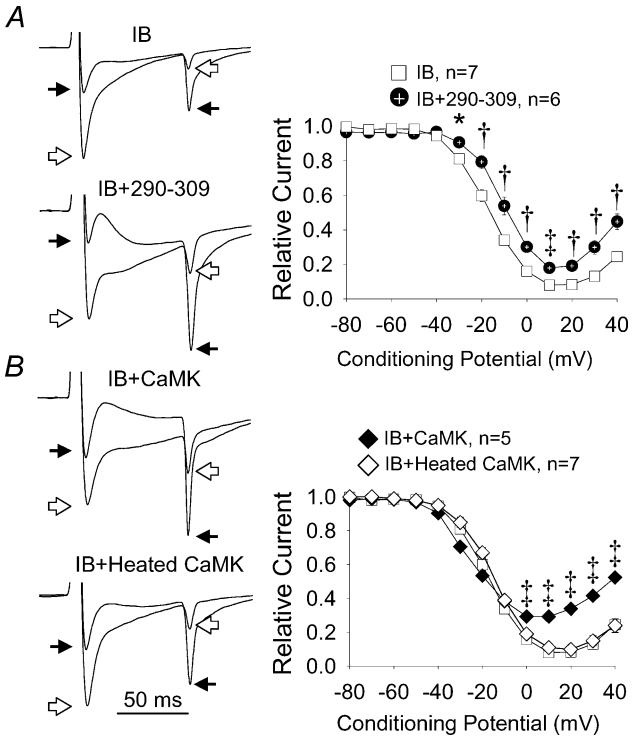

Figure 3. The effects of Ca^{2+}–CaM and CaMK on $I_{Ca\text{-}L}$
A, the left panels show superimposed current tracings (as in Fig. 2*A*) in response to conditioning prepulses to +20 mV (open arrows) and +40 mV (filled arrows) in cells dialysed with IB (IB) or a combination of IB and the Ca^{2+}–CaM inhibitory peptide 290–309 (IB + 290–309). The right panel shows summary findings for peak $I_{Ca\text{-}L}$ after 80 ms conditioning prepulses from –80 to +40 mV (abscissa) in cells treated as described in the left panels. *B,* the left panels show superimposed current tracings labelled as in *A* (above), but these cells were dialysed with IB and a Ca^{2+}-independent form of CaMK (IB + CaMK) or IB and heat-inactivated CaMK (IB + heated CaMK). The right panel shows summary findings for peak $I_{Ca\text{-}L}$ (as in *A*), but from cells treated as in the left panels. Dialysis with a Ca^{2+}–CaM-independent form of CaMK that is resistant to IB increases relative $I_{Ca\text{-}L}$ (80 ms prepulses) compared to IB alone after positive conditioning pulses. CaMK activity is ablated by heat inactivation and peak $I_{Ca\text{-}L}$ is normalized to the maximum value. $^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$ for *A* and *B*.

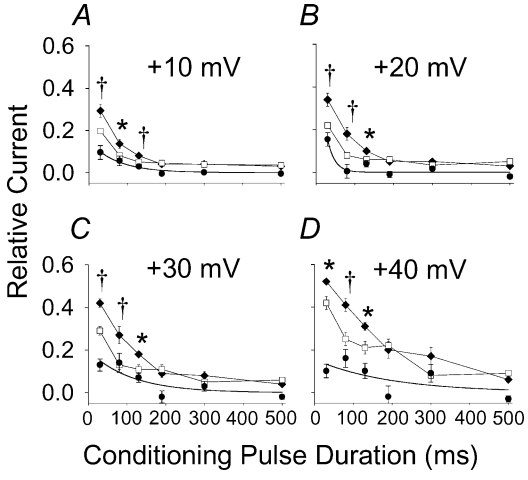

Figure 4. The effect of exogenous Ca^{2+}–CaM-independent CaMK on $I_{Ca\text{-}L}$ availability after positive conditioning prepulses
A–D, each shows peak $I_{Ca\text{-}L}$ responses to conditioning prepulses of varying durations (abscissa) at four positive membrane potentials (indicated above each plot). Cells were dialysed with IB to inhibit endogenous CaMK activity in the presence (filled diamonds, $n = 5$) or absence (open squares, $n = 7$) of Ca^{2+}-independent CaMK that is resistant to IB. Filled circles represent the difference in relative $I_{Ca\text{-}L}$ in IB + CaMK and IB alone and the contiuous lines are exponential fits of these differences. $^{*}P < 0.05$, $^{\dagger}P < 0.01$.

R_{30}, R_{80} and relative current under these conditions. In contrast, IB significantly reduced R_{30} compared to relative current after a weakly depolarizing conditioning step to –20 mV, but CaMK replacement failed to equalize R_{30} and relative current (Fig. 5*C*). IB significantly reduced both R_{80} and relative current after a –20 mV conditioning potential, but R_{80} was significantly less than relative current even under control conditions (Fig. 5*D*). These comparisons between $I_{Ca\text{-}L}$ inactivation and relative ($I_{Ca\text{-}L}$) current suggest that a greater fraction of L-type Ca^{2+} channels were available to open at the +10 mV test voltage following inactivation of $I_{Ca\text{-}L}$ during the –20 mV than the +20 mV conditioning step. Taken together, results from experiments using both CaMK inhibition and replacement strategies indicate that CaMK can significantly increase $I_{Ca\text{-}L}$ by a time- and voltage-dependent mechanism in cardiac myocytes.

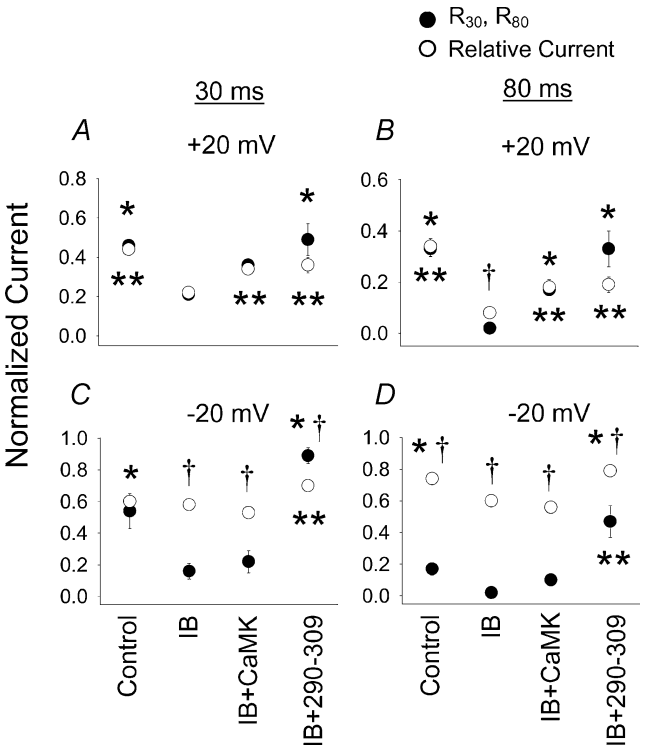

Figure 5. The effect of CaMK on relative and residual $I_{Ca\text{-}L}$
The effect of CaMK on relative $I_{Ca\text{-}L}$ (recorded during test pulses to +10 mV as in Fig. 1, shown as open circles) after conditioning prepulses to +20 mV (*A* and *B*) and –20 mV (*C* and *D*) and the residual $I_{Ca\text{-}L}$ at the end of 30 ms (R_{30}, *A* and *C*) and 80 ms (R_{80}, *B* and *D*) conditioning prepulses (filled circles). The experimental pipette solutions are labelled below *C* and *D*, but are also valid for *A* and *B*. Cells were dialysed with control pipette solution (Control), IB solution (IB), IB containing a Ca^{2+}-independent form of CaMK (IB + CaMK) or IB containing the CaM binding peptide 290–309 (IB + 290–309) and all data are from Figs 2 and 3. Significant differences in residual current (R_{30} and R_{80}, $^{*}P < 0.05$) or relative current ($^{**}P < 0.05$) between the IB condition and Control, IB + CaMK or IB + 290–309 are indicated. Significant differences between residual currents and relative current measurements for each experimental condition are shown ($^{\dagger}P < 0.05$).

Ca^{2+}–CaM reduces $I_{Ca\text{-}L}$ availability at action potential plateau potentials

The finding that cells dialysed with IB retained Ca^{2+}-dependent inactivation (Figs 2*A*, *D* and *E*) suggested that Ca^{2+}–CaM remained operative under these experimental conditions. Cellular dialysis with the Ca^{2+}–CaM binding peptide 290–309 did significantly increase relative $I_{Ca\text{-}L}$ availability. In contrast to CaMK that was only effective after positive conditioning potentials (Figs 3*B* and 5), 290–309 was effective after weakly and strongly depolarizing conditioning potentials (Figs 3*A* and 5). These data show that endogenous Ca^{2+}–CaM was a significant signal transduction element for grading $I_{Ca\text{-}L}$ in the presence of IB. However, 290–309 did not fully increase $I_{Ca\text{-}L}$ availability to levels present with Ba^{2+} as charge carrier (Fig. 2*E*), perhaps indicating that a constitutively bound pool of CaM (Erickson *et al.* 2001; Pitt *et al.* 2001) was inaccessible to the peptide.

In contrast to the effects of 290–309 on $I_{Ca\text{-}L}$ availability, peak $I_{Ca\text{-}L}$ during the conditioning pulse in IB + 290–309 (4.1 ± 0.4 pA pF^{-1}, $n = 5$) was significantly less than peak $I_{Ca\text{-}L}$ recorded in IB alone (7.1 ± 0.9 pA pF^{-1}, $n = 7$) or in IB + CaMK (8.1 ± 0.5 pA pF^{-1}, $n = 5$), whereas IB + 290–309 did not decrease peak $I_{Ca\text{-}L}$ compared to control solution (6.2 ± 0.5 pA pF^{-1}, $n = 5$).

SR Ca^{2+} release selectively reduces $I_{Ca\text{-}L}$ after brief, positive conditioning pulses

The results of experiments so far show that the CaMK-dependent component of Ca_i^{2+} signalling to L-type Ca^{2+} channels is voltage dependent (Figs 3*B* and 4); however, they do not distinguish between $I_{Ca\text{-}L}$ and SR Ca^{2+} release as dominant sources of signalling Ca_i^{2+} for grading $I_{Ca\text{-}L}$. Both ryanodine and thapsigargin significantly increased $I_{Ca\text{-}L}$ after positive conditioning potentials in the presence of IB, indicating that Ca^{2+}-induced Ca^{2+} release is present under these experimental conditions (Fig. 6*A*–*C*). Reduction in SR Ca^{2+} release by either ryanodine or thapsigargin targeted $I_{Ca\text{-}L}$ increases over similar cell membrane potential (Fig. 6*B* and *C*) and temporal domains (Fig. 6*D*) as CaMK (Figs 3*B* and 4), suggesting the possibility that activation of endogenous CaMK is predominately due to SR Ca^{2+} release, and that Ca^{2+}–CaM-dependent inactivation relies on SR Ca_i^{2+} under action potential plateau conditions.

CaMK actions at $I_{Ca\text{-}L}$ are determined by SR Ca^{2+} release

Previous studies suggest SR Ca^{2+} is important for activating endogenous CaMK in cardiac myocytes (Kuschel *et al.* 1999; Wu *et al.* 1999*a*, 2001*b*; Bartel *et al.* 2000), so we reasoned that Ca^{2+}-independent CaMK would circumvent the requirement for activation of endogenous CaMK by SR Ca^{2+} release. Surprisingly, thapsigargin-treated cells did not show increases in $I_{Ca\text{-}L}$ availability at action potential plateau potentials in response to Ca^{2+}-independent CaMK, but did show a significant depolarizing shift in $I_{Ca\text{-}L}$ availability in response to weaker depolarizations (Fig. 6*E*). This result was in striking contrast to the significant increases in $I_{Ca\text{-}L}$ after positive conditioning pulses when myocytes with intact SR Ca^{2+} release were supplemented with Ca^{2+}-independent CaMK (Figs 3*B* and 4). In contrast to CaMK-dependent increases in R_{30}, R_{80} and relative $I_{Ca\text{-}L}$ at +20 mV (Fig. 5*A* and *B*), CaMK replacement was ineffective for increasing these parameters after thapsigargin at +20 mV (Fig. 7*A* and *B*). CaMK replacement also failed to evoke a consistent response in

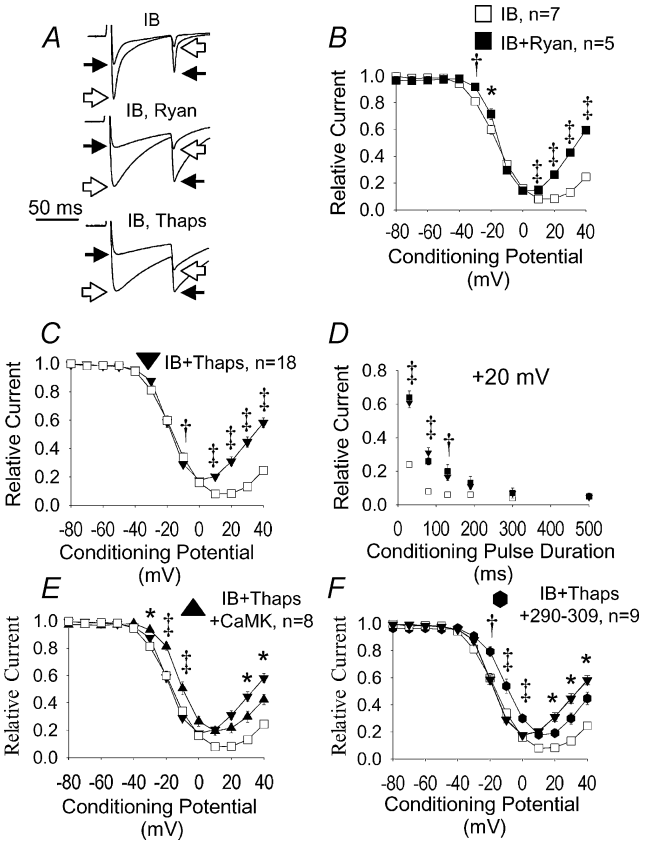

Figure 6. CaM and CaMK require SR Ca_i^{2+} after positive conditioning pulses

A, superimposed current tracings displayed as in Fig. 3*A*. Cells were dialysed with IB in all panels (IB), but ryanodine (IB, Ryan) or thapsigargin (IB, Thaps) were added to the bath solution to inactivate SR Ca^{2+} release in some experiments. Both ryanodine (*B*, Ryan) and thapsigargin (*C*, Thaps) significantly increased available $I_{Ca\text{-}L}$ after 80 ms conditioning prepulses in cells dialysed with IB to inhibit endogenous CaMK. Pre-pulse potentials (abscissa) are plotted against peak $I_{Ca\text{-}L}$ normalized to the maximum value (ordinate). *D,* normalized peak $I_{Ca\text{-}L}$ values from *B* and *C* are plotted against conditioning prepulses to +20 mV for a range of prepulse durations (abscissa). No significant differences in peak $I_{Ca\text{-}L}$ were present between ryanodine- and thapsigargin-treated cells. $^*P < 0.05$, $^\dagger P < 0.01$ and $^\ddagger P < 0.001$ for panels *B–D*. *E*, addition of Ca^{2+}-independent CaMK failed to increase relative $I_{Ca\text{-}L}$ in cells dialysed with IB and treated with thapsigargin after positive conditioning prepulses (IB + CaMK + Thaps). *F*, the Ca^{2+}–CaM inhibitory peptide 290–309 failed to increase relative $I_{Ca\text{-}L}$ in cells dialysed with IB and treated with thapsigargin (IB + Thaps + 290–309) after positive conditioning prepulses. $^\ddagger P < 0.001$, $^\dagger P < 0.01$, $^*P < 0.05$ for comparisons in panels *E* and *F*.

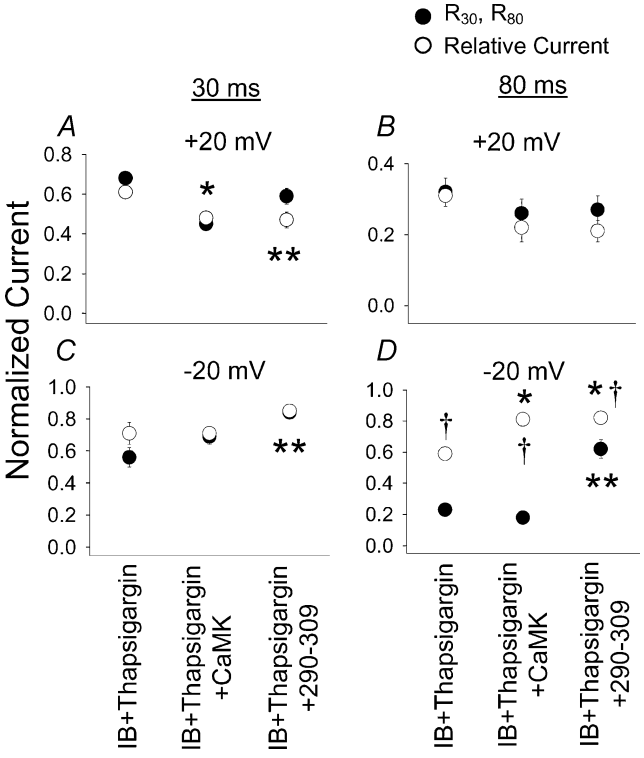

Figure 7. The effect of CaMK and Ca^{2+}–CaM on relative $I_{Ca\text{-}L}$ and R_{30} and R_{80} in cells treated with thapsigargin

The data are displayed as in Fig. 5 except thapsigargin was included in the bath solution and cells were dialysed with IB (IB + thapsigargin), IB and Ca^{2+}-independent CaMK (IB + thapsigargin + CaMK) or the Ca^{2+}–CaM inhibitory peptide 290–309 (IB + thapsigargin + 290–309). The experimental conditions are labelled below *C* and *D*, but are also valid for *A* and *B*. The data sets were previously displayed for relative current in Fig. 6*E* and *F*. Significant differences between the IB + thapsigargin and other groups for residual current (R_{30} and R_{80}, $^*P < 0.05$) and relative current ($^{**}P < 0.05$) are indicated. Significant differences between residual currents and relative currents for each experimental condition are shown ($^\dagger P < 0.01$).

R_{80} and relative current after a –20 mV conditioning step (Fig. 7*D*). These data underscore the close relationship between SR Ca^{2+} release and L-type Ca^{2+} channel function, and suggest the possibility that the Ca^{2+}-independent form of CaMK acts to increase $I_{Ca\text{-}L}$ availability through a SR-dependent mechanism.

SR Ca^{2+} release significantly determines Ca^{2+}–CaM responses after strong depolarizations

In contrast to CaMK (Fig. 3*B*), Ca^{2+}–CaM effects on $I_{Ca\text{-}L}$ appear to be voltage independent because they operate over a broad range of physiological conditioning potentials (Fig. 3*A*). SR Ca^{2+} release does contribute to Ca^{2+}-dependent $I_{Ca\text{-}L}$ inactivation (Balke & Wier, 1991; Wu *et al.* 2001*b*), and reduction of SR Ca^{2+} release by ryanodine (Fig. 6*B*) or thapsigargin (Fig. 6*C*) significantly increased relative $I_{Ca\text{-}L}$ in cells dialysed with IB only after positive conditioning steps, suggesting that the source of activator Ca_i^{2+} for Ca^{2+}–CaM-dependent inactivation may vary in a voltage-dependent manner in cardiac myocytes. In order to better understand the contribution of $I_{Ca\text{-}L}$ and SR to Ca^{2+}–CaM for regulating $I_{Ca\text{-}L}$ responses, we dialysed 290–309 into myocytes after thapsigargin. Ca^{2+}–CaM inhibition with 290–309 significantly increased relative $I_{Ca\text{-}L}$ (Fig. 6*F*) and R_{30} (Fig. 7*C*) and R_{80} (Fig. 7*D*) at –20 mV, but not at +20 mV (Fig. 7*A* and *B*), supporting previous findings that $I_{Ca\text{-}L}$ is sufficient for Ca^{2+}–CaM-dependent inactivation, but suggesting that normal SR Ca^{2+} release significantly determines Ca^{2+}–CaM signalling to L-type Ca^{2+} channels at strongly depolarized conditioning potentials, present during the action potential plateau.

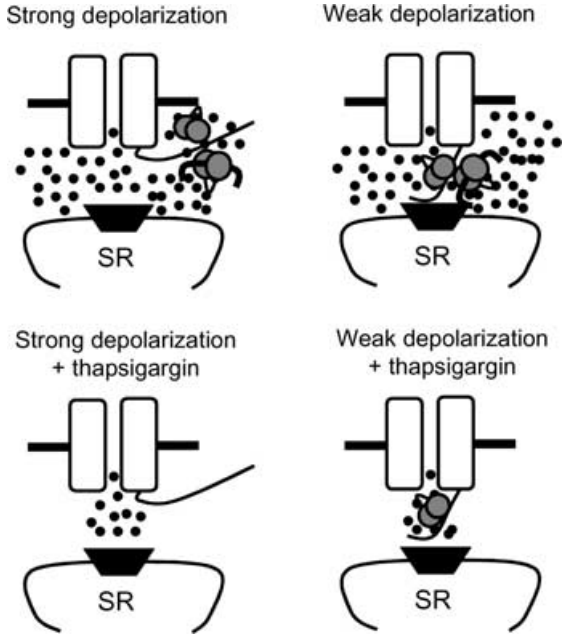

Figure 8. Schematic depiction of the hypothesized relationship between cell membrane potential, L-type Ca^{2+} channel C terminus motion and Ca_i^{2+} 'sensing' by CaM and CaMK
The L-type Ca^{2+} channel pore forming subunit (α_{1C}) is shown as a pair of open rectangles with a central pore. The C terminus protrudes into the cytoplasmic space from the right rectangle and the pore region and SR Ca^{2+} release channel (ryanodine receptor, dark trapezoid) are *en face*. Ca^{2+}–CaM is depicted as a pair of stippled circles linked by a curved segment and activated CaMK is shown as a thick bar with curved ends bound to Ca^{2+}–CaM. According to the hypothesized model, strong depolarizations motivate the C terminus to move away from the α_{1C} pore so that Ca_i^{2+} sensing is primarily from the SR. Weak depolarizations leave the C terminus in the vicinity of the pore where sensed Ca_i^{2+} is directly from $I_{Ca\text{-}L}$. Inactivation of SR Ca^{2+} release (by thapsigargin) results in significant impairment of Ca_i^{2+} sensing through CaM and CaMK during strong depolarizations, while Ca_i^{2+} from $I_{Ca\text{-}L}$ is sufficient for Ca^{2+}–CaM at weak depolarizations in the absence of SR Ca^{2+} release.

Discussion

CaMK, $I_{Ca\text{-}L}$ and SR Ca^{2+} release

The present studies use a combination of approaches to inhibit endogenous CaMK-dependent $I_{Ca\text{-}L}$ increases (with IB) or control CaMK activity (with exogenous Ca^{2+}–CaM independent CaMK) while SR Ca^{2+} release is preserved or eliminated (with ryanodine or thapsigargin) in cardiomyocytes. The central finding of these experiments is that CaMK increases in $I_{Ca\text{-}L}$ are functionally targeted over time and voltage domains that are directly relevant to the cardiac action potential plateau. This finding suggests CaMK actions are analogous to protein kinase A, which can also regulate L-type Ca^{2+} channels by a voltage-dependent mechanism (Sculptoreanu *et al.* 1993). Previous investigations established that CaMK can directly increase L-type Ca^{2+} channel openings in excised membrane patches from ventricular myocytes (Dzhura *et al.* 2000), but these experiments did not test for temporal- or voltage-dependent features of CaMK signalling and could not measure effects of SR Ca^{2+} release. The dramatic loss of CaMK signalling effects after elimination of SR Ca^{2+} release (Figs 6*E* and *F* and 7) shows those CaMK actions are reliant upon SR Ca^{2+} release. One possible unifying explanation for these observations is that an important CaMK action on $I_{Ca\text{-}L}$ in intact myocytes may be indirect, via modulation of SR Ca^{2+} release (Li *et al.* 1997; Wu *et al.* 2001*a*). The concept that CaMK exerts important actions on $I_{Ca\text{-}L}$ by influencing SR Ca^{2+} release may reconcile earlier reports showing that $I_{Ca\text{-}L}$ increases were linked to dynamic reduction in SR Ca^{2+} release (Delgado *et al.* 1999), but were eliminated by inactivation of SR Ca release

(Wu *et al.* 1999*a*, 2001*b*). Interestingly CaMK slightly, but significantly, increased relative $I_{Ca\text{-}L}$ availability at weakly depolarized potentials in the absence of SR Ca^{2+} release (Figs 6*E* and 7*D*), raising the possibility that CaMK may be capable of regulating $I_{Ca\text{-}L}$ by a SR-independent pathway under these voltage clamp conditions. The finding that $I_{Ca\text{-}L}$ availability responses to CaMK (Fig. 6*E*) and 290–309 (Fig. 6*F*) are very similar in thapsigargin, after weak and strong depolarizations, suggests that in the absence of SR Ca^{2+} release, CaMK and Ca^{2+}–CaM may compete for shared molecular machinery, such as the L-type Ca^{2+} channel C terminus. Thus, the present studies add important new information to our understanding of how CaMK may contribute to $I_{Ca\text{-}L}$ regulation in the working heart.

Ca^{2+}–CaM, $I_{Ca\text{-}L}$ and SR Ca^{2+} release

CaM is a ubiquitous Ca_i^{2+}-sensing protein that is required for Ca_i^{2+}-dependent $I_{Ca\text{-}L}$ inactivation in cardiac L-type Ca^{2+} channels (Peterson *et al.* 1999; Zuhlke *et al.* 1999). CaMK and CaM colocalize with L-type Ca^{2+} channels and ryanodine receptors (Wu *et al.* 1999*a*; Pate *et al.* 2000; Balshaw *et al.* 2001; Pitt *et al.* 2001; Dzhura *et al.* 2002; Erickson *et al.* 2003), and are capable of regulating both of these proteins. Ca^{2+}–CaM also activates other proteins, including CaMK, so that myriad effects potentially complicate interpretation of Ca^{2+}–CaM inhibition experiments. The present experiments used IB dialysis and a Ca^{2+}–CaM inhibitory peptide (290–309) to separately control Ca^{2+}–CaM and CaMK signalling. These findings support the concept that Ca^{2+}–CaM reduces available $I_{Ca\text{-}L}$ in a voltage-independent manner (Fig. 3*A*). However, the critical source of Ca_i^{2+} for Ca^{2+}–CaM is determined by cell membrane voltage because ryanodine (Fig. 6*B*) and thapsigargin (Fig. 6*C*) only increased $I_{Ca\text{-}L}$ at plateau potentials and because 290–309 was ineffective at increasing $I_{Ca\text{-}L}$ at plateau potentials in the absence of SR Ca^{2+} release (Figs 3*A* and 6*F*). In contrast, CaM sequestration with the 290–309 peptide enhanced I_{Ca} availability after weakly depolarizing prepulses, independent of SR Ca^{2+} release (Figs 6*F* and 7*D* and *E*), suggesting that $I_{Ca\text{-}L}$ alone is a sufficient source of Ca_i^{2+} for Ca^{2+}–CaM-dependent inactivation of $I_{Ca\text{-}L}$ at weakly depolarized cell membrane potentials. The finding that Ca^{2+}–CaM competition by 290–309 significantly increased relative currents and slowed $I_{Ca\text{-}L}$ inactivation in IB (Fig. 5) is consistent with a recent report showing marked slowing of $I_{Ca\text{-}L}$ inactivation and action potential prolongation in cardiomyocytes transfected with Ca^{2+}-binding deficient, dominant-negative CaM mutants (Alseikhan *et al.* 2002). A potential limitation to studies with 290–309 is highlighted by the finding that peak $I_{Ca\text{-}L}$ during the conditioning pulse was reduced in IB + 290–309 compared to IB alone, raising the possibility that an outward current, such as $I_{Cl,Ca}$, may be activated by 290–309 and complicate $I_{Ca\text{-}L}$ measurements during this experimental condition. Taken together, these results reveal the interdependence of CaM, SR Ca^{2+} release and cell membrane potential and add to other recent work highlighting the importance of CaM as a Ca_i^{2+}-driven signalling element for regulating I_{Ca} in heart.

The relationship between $I_{Ca\text{-}L}$ inactivation and availability

The present experiments show that it is possible to distinguish between SR Ca^{2+}, CaM and CaMK signalling effects on relative $I_{Ca\text{-}L}$ availability in cardiac myocytes. Both CaM (Fig. 3*A*) and CaMK (Fig. 3*B*) can separately regulate $I_{Ca\text{-}L}$ availability under non-steady-state conditions. Relative $I_{Ca\text{-}L}$ availability is closely related to $I_{Ca\text{-}L}$ inactivation after positive conditioning pulses (Figs 5*A* and *B*, and 7*A* and *B*). This relationship is consistent with the concept that $I_{Ca\text{-}L}$ inactivation (R_{30} and R_{80}) during the conditioning pulse directly determines $I_{Ca\text{-}L}$ availability during the subsequent test pulse. Experiments to inhibit or replace CaMK (Fig. 5), eliminate SR Ca^{2+} release, or reduce Ca^{2+}–CaM (Fig. 7) did not alter this relationship at +20 mV. In contrast, R_{30} was significantly less than relative $I_{Ca\text{-}L}$ after CaMK inhibition with IB (Fig. 5*C*), suggesting that CaMK could reduce this measure of $I_{Ca\text{-}L}$ inactivation at –20 mV without altering the pool of L-type Ca^{2+} channels available for opening in response to the +10 mV test pulse. SR Ca^{2+} also reduced R_{30} and R_{80} during CaMK inhibition at –20 mV (compare IB in Fig. 5*C* and *D* with IB + thapsigargin in Fig. 7*C* and *D*). However, Ca_i^{2+} from $I_{Ca\text{-}L}$ was sufficient for significant Ca^{2+}–CaM actions on R_{30} and R_{80} at –20 mV, because these measures were both increased by 290–309 in the combined presence of IB and thapsigargin (Fig. 7*D*).

A model for voltage- and Ca_i^{2+}-dependent regulation of $I_{Ca\text{-}L}$ in heart

The recent finding that the L-type Ca^{2+} channel C terminus undergoes significant voltage-dependent movement (Kobrinsky *et al.* 2003) provides a potentially important context for understanding our findings. The C terminus is now accepted to be richly endowed with Ca_i^{2+} sensing machinery, and three distinct Ca^{2+}–CaM binding domains have been identified (Zuhlke *et al.* 1999; Pate *et al.*

2000; Pitt *et al.* 2001). On the other hand, the C terminus is also capable of binding activated CaMK (Hudmon *et al.* 2002). Given that both Ca^{2+}–CaM and CaMK can converge upon the C terminus and given that the C terminus is significantly mobile over the cell membrane potential ranges used in our study, it is possible that the C terminus could be variably positioned to differentially respond to Ca_i^{2+} from $I_{Ca\text{-}L}$ (when positioned near the pore region) or the SR (when positioned outside of the pore region). Based upon these considerations and upon our finding that SR Ca^{2+} release was required for complete Ca^{2+}–CaM and CaMK actions at +20 mV (Figs 5 and 7), we hypothesize that the C terminus moves away from the pore region during strong depolarizations. Because Ca_i^{2+} from $I_{Ca\text{-}L}$ was sufficient for Ca^{2+}–CaM at –20 mV, we further hypothesize that the C terminus is close enough to the pore region during weak depolarizations to sense Ca_i^{2+} directly from $I_{Ca\text{-}L}$ (Fig. 8).

Our findings show that CaMK responses are very different during non-steady-state time and voltage domains that approximate action potential plateau conditions than during steady-state conditions. While most studies have understandably focused on the effects of prolonged voltage clamp command pulses, in order to measure steady-state responses, steady-state behaviours may not always be relevant to the physiology of the action potential. These experiments highlight the importance of considering non-steady-state measurements for understanding the effects of cellular signals on ionic current responses.

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Acknowledgements

This work was supported in part by grants from the National Institutes of Health (NHLBI, HL62494, HL70250, HL46881). M.E.A. is an Established Investigator of the American Heart Association. J.T.K. is partially funded by a NHI training grant. We thank Ms Martha Bass and Ms Jinying Yang for expert technical assistance, and Dr Lou DeFelice for criticisms and insightful comments.