Calmodulin kinase is a molecular switch for cardiac excitation–contraction coupling

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Edited by Clara Franzini-Armstrong, University of Pennsylvania School of Medicine, Philadelphia, PA, and approved January 2, 2001 (received for review September 19, 2000)

Signaling between cell membrane-bound L-type Ca^{2+} channels (LTCC) and ryanodine receptor Ca^{2+} release channels (RyR) on sarcoplasmic reticulum (SR) stores grades excitation–contraction coupling (ECC) in striated muscle. A physical connection regulates LTCC and RyR in skeletal muscle, but the molecular mechanism for coordinating LTCC and RyR in cardiomyocytes, where this physical link is absent, is unknown. Calmodulin kinase (CaMK) has characteristics suitable for an ECC coordinating molecule: it is activated by Ca^{2+}/calmodulin, it regulates LTCC and RyR, and it is enriched in the vicinity of LTCC and RyR. Intact cardiomyocytes were studied under conditions where CaMK activity could be controlled independently of intracellular Ca^{2+} by using an engineered Ca^{2+}-independent form of CaMK and a highly specific CaMK inhibitory peptide. CaMK reciprocally enhanced L-type Ca^{2+} current and reduced release of Ca^{2+} from the SR while increasing SR Ca^{2+} content. These findings support the hypothesis that CaMK is required to functionally couple LTCC and RyR during cardiac ECC.

Excitation–contraction coupling (ECC) in skeletal and cardiac muscle requires coordinated bidirectional interactions between cell membrane-bound L-type Ca^{2+} channels (LTCC) and ryanodine receptor Ca^{2+} release channel proteins (RyR) on intracellular sarcoplasmic reticulum (SR) Ca^{2+} stores (1). Substantial evidence indicates that a physical link between LTCC and RyR is essential for antegrade (2, 3) and retrograde (4) signaling in skeletal muscle type ECC. In contrast, cardiac ECC occurs in the apparent absence of a physical link (2) by a process termed Ca^{2+}-induced Ca^{2+} release (5), whereby L-type Ca^{2+} current (I_{Ca}) traverses a subcellular microdomain (6) to trigger release of intracellular Ca^{2+} ($[Ca^{2+}]_i$) by opening RyR on SR Ca^{2+} stores. In addition to antegrade signaling from LTCC to RyR, Ca^{2+} released from the SR can dynamically regulate I_{Ca} through dual mechanisms of inactivation (7) and facilitation (8). Both $[Ca^{2+}]_i$-dependent inactivation and facilitation are likely important for grading I_{Ca} to determine the strength of contraction, maintain $[Ca^{2+}]_i$ homeostasis, and to refill SR Ca^{2+} stores. Importantly, both RyR (9, 10) and LTCC (11, 12) are regulated by phosphorylation, raising the possibility that an appropriately positioned Ca^{2+}-activated kinase or phosphatase could translate Ca^{2+} activity changes in the LTCC-RyR microdomain into coordinating signals for these molecules during cardiac ECC.

Calmodulin (CaM) kinase (CaMK) is a multifunctional serine/threonine kinase that phosphorylates cardiac RyR (9, 13–16) and colocalizes with cardiac LTCC and RyR (8). CaMK activation reduces ryanodine binding to RyR (9, 13), can decrease RyR opening probability (9, 10, 16), and facilitates I_{Ca} (17–19) by inducing a modal gating shift in LTCC favoring long openings (12). Thus, CaMK has an appropriate subcellular localization pattern and operating characteristics to be a functional link between LTCC and RyR during cardiac ECC. Based on studies from isolated LTCC (12) and RyR (9), using rabbit cardiomyocytes, we hypothesized that CaMK activation functionally couples LTCC and RyR to reciprocally reduce SR Ca^{2+} release and facilitate I_{Ca} during cardiac ECC. An important experimental obstacle for unraveling Ca^{2+}-dependent signaling mechanisms during ECC has been the inability to independently control CaMK activity and $[Ca^{2+}]_i$. We circumvented these obstacles by using an engineered Ca^{2+}-independent, constitutively active form of CaMK and a specific CaMK inhibitory peptide to independently control CaMK activity, I_{Ca}, and SR Ca^{2+} content. Here, we show that CaMK activity is required to dynamically and reciprocally "link" I_{Ca} with SR Ca^{2+} release during cardiac ECC.

Methods

Electrophysiology. Electrophysiology with whole cell mode voltage clamp configuration using isolated rabbit ventricular myocytes was performed according to previously published methods (17). Briefly, cells were held at −80 mV for >5 min for adequate dialysis with pipette solution before initiating experiments. I_{Ca} was activated by stepping the cell membrane from −80 mV to +20 mV at 0.5 Hz for 300 ms. Experiments were performed at 24°C. Na^+ and K^+ currents were eliminated by adding Cs^+ and tetraethylammonium chloride (TEA) and reducing Na^+ and K^+ in the pipette and bath solutions. Elimination of the residual current by nifedipine (10 μM) or Cd^{2+} (100 μM) confirmed that the identity of active current was I_{Ca} (data not shown). The pipette (intracellular) solution was (in mM): CsCl 120.0, Hepes 10.0, TEA 10.0, phosphocreatine 5.0, MgATP 1.0, NaGTP 1.0, and pH was adjusted to 7.2 with 1.0 N CsOH. The bath (extracellular) solution was NMDG 137.0, CsCl 25.0, Hepes 10.0, glucose 10.0, $CaCl_2$ 1.8, $MgCl_2$ 0.5, and pH was adjusted to 7.4 with 12 N HCl.

Fluo 3 Fluorescence Measurements. Fluo 3 fluorescence measurements were used to reflect SR Ca^{2+} release by including the pentapotassium salt of the fluo 3 (Molecular Probes) in the pipette solution (100 μM) as previously described, with minor modifications (17). Under these conditions, elimination of SR Ca^{2+} release by incubation with ryanodine (10 μM) or thapsigargin (1 μM) eliminates >90% of the fluo 3 transient, indicating that the dynamic fluo 3 signal reflects Ca^{2+} release from the SR (data not shown). Voltage signals were low pass filtered at 50 Hz before analysis. Fluo 3 $[Ca^{2+}]_i$ transients were normalized by the ratio of the measured voltage (F) to the baseline voltage (F_o) by using PCLAMP 6.0.3.

SR Ca^{2+} Content. SR Ca^{2+} content was quantified as the integrated Na^+/Ca^{2+} exchanger current (20). We perfused the myocyte during a prolonged voltage command to −80 mV with a "spritz" of modified bath solution containing caffeine (20 mM) and equimolar substitution of NaCl for *N*-methyl-D-glucamine,

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: ECC, excitation–contraction coupling; CaM, calmodulin; CaMK, CaM kinase; LTCC, L-type Ca^{2+} channels; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; I_{Ca}, L-type Ca^{2+} current; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

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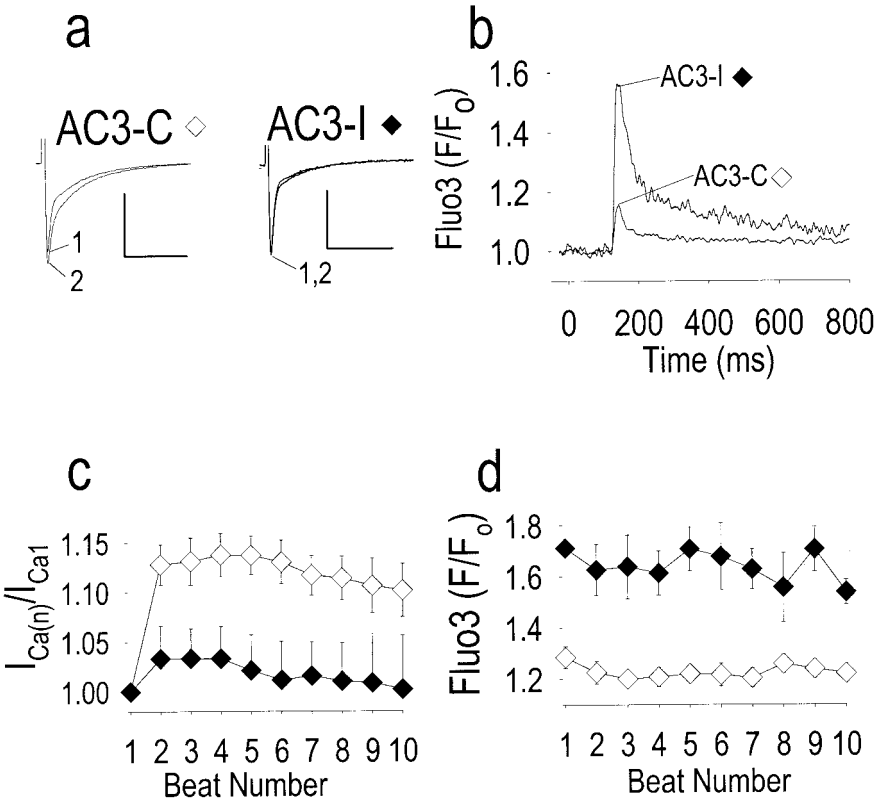

Fig. 1. CaMK inhibition prevents I_{Ca} facilitation and reciprocally increases release of Ca^{2+} from intracellular stores. (*a*) I_{Ca} facilitation is prominent in control cells dialyzed with an inactive peptide (AC3-C), but is markedly reduced after addition of the CaMK inhibitory peptide (AC3-I; see *Methods*). Numerals indicate the beat number, and the calibration bars represent 500 pA (vertical) and 100 ms (horizontal). (*b*) Averaged intracellular Ca^{2+} transients from $n = 3$ cells (AC3-I) and $n = 10$ cells (AC3-C) using the fluorescent indicator fluo 3 (see *Methods*) for beats 1–10 to show details of the sustained increase in release of SR Ca^{2+} following CaMK inhibition (AC3-I) compared with cells treated with the inactive control peptide (AC3-C). (*c*) Summary of data showing I_{Ca} facilitation expressed as the ratio of peak I_{Ca} during the first to the *n*th beat [$I_{Ca(n)}$]. I_{Ca} facilitation was significantly decreased by AC3-I ($n = 3$) compared with control ($P < 0.05$ for beats 2–8, $n = 10$). (*d*) Peak fluo 3 fluorescence signals for each stimulated beat, measured simultaneously with I_{Ca} reported in *c*. CaMK inhibition significantly increased peak fluo 3 fluorescence compared with control ($P < 0.005$ for all beats).

using a rapid solution exchanger (ALA, Westbury, NY). The resulting inward current was integrated by using PCLAMP 6.0.3 (Axon Instruments, Foster City, CA) and normalized for cell surface area. The rate of maximal SR Ca^{2+} release was estimated from the initial phase of inward Na^+/Ca^{2+} exchanger current fit to a single exponential equation.

Inhibitory Peptides. The CaMK inhibitory peptide AC3-I (KKALHRQEAVDCL, $IC_{50} \approx 0.5$ μM) was synthesized and isolated to >95% purity by RP-HPLC (Macromolecular Resources, Fort Collins, CO). The inactive control peptide AC3-C (KKALHAQERVDCL, $IC_{50} > 500$ μM) was a generous gift from Howard Schulman (Stanford University, Stanford, CA). AC3-I is a modified CaMK substrate, and the amino acid sequence HRQEAVDCL corresponds to the autophosphorylation site (T286/287) on CaMK, except T is modified to A to prevent phosphorylation.

Constitutively Active CaMK. Constitutively active CaMK (amino acid residues 1–380 of mouse type II, α isoform) was expressed in baculovirus, purified with a CaM affinity column as previously described (8), and used at a final concentration of 0.9 μM to approximate physiologic activity (21). The purified CaMK was made Ca^{2+}/CaM-independent by thiophosphorylation of Thr 286 in the presence of Ca^{2+}, CaM, and adenosine 5′-*O*-(3-thiotriphosphate) (8). The blank buffer does not support activation of I_{Ca} (8) (see Fig. 2) or LTCC (12) by endogenous CaMK, and was used as a control.

Results

CaMK Inhibition Disrupts Reciprocal Signaling Between LTCC and RyR. Facilitation of peak I_{Ca} occurs in ventricular myocytes, under control conditions (Fig. 1 *a*, *Left*, and *c*) (17–19), because of LTCC activation by CaMK (12). The $[Ca^{2+}]_i$ transient (Fig. 1*b*) is primarily the result of secondary Ca^{2+} release from the SR because >90% of the transient was eliminated by the SR-inactivating agents ryanodine or thapsigargin (data not shown) (22). CaMK inhibition prevented I_{Ca} facilitation (Fig. 1 *a*, *Right*, and *c*) and enhanced the $[Ca^{2+}]_i$ transient (Fig. 1 *b* and *d*). The enhanced SR Ca^{2+} release seen following CaMK inhibition (Fig. 1*b*) is consistent with the finding in isolated RyR that CaMK-catalyzed phosphorylation decreases RyR opening probability (9, 10). These findings strongly suggest that CaMK is required to maintain the normal relationship between I_{Ca} and SR Ca^{2+} release and implicate CaMK as a component of the molecular machinery for coordinating LTCC and RyR during cardiac ECC.

Constitutively Active CaMK Reconstitutes the Reciprocal Relationship of I_{Ca} to SR Ca^{2+} Release During ECC. To further probe the role of CaMK signaling during ECC, cardiomyocytes were dialyzed with constitutively active CaMK (8). The exogenous CaMK buffer blocked the effects of activation of endogenous CaMK (8, 12) (Fig. 2*a*, buffer control compare with Fig. 1*a*, AC3-I). The constitutively active, Ca^{2+}-independent CaMK (Fig. 2*a*, *Right*) reconstituted the control ECC phenotype (Fig. 1*a*, *Left*) by simultaneously enhancing I_{Ca} facilitation (Fig. 2 *a* and *c*) and

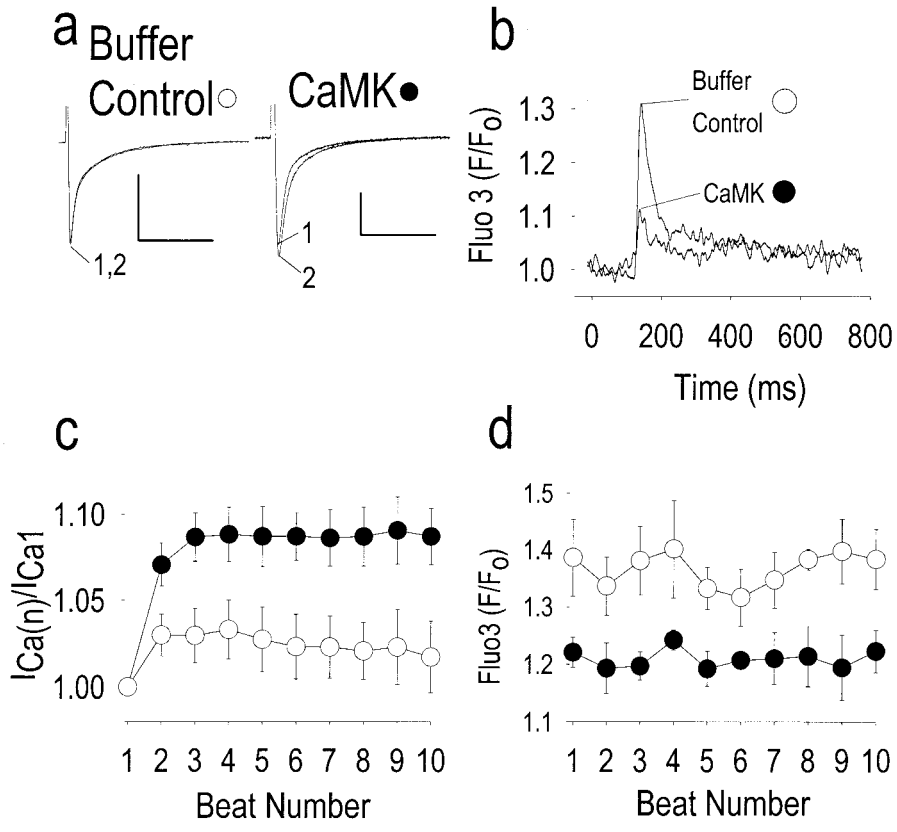

Fig. 2. Addition of constitutively active CaMK reconstitutes I_{Ca} facilitation and reciprocally reduces release of Ca^{2+} from intracellular stores. (*a*) I_{Ca} facilitation is absent in this cell dialyzed with buffer solution (Buffer Control), but is present after addition of constitutively active CaMK (CaMK). Numerals indicate the beat number as in Fig. 1, and the calibration bars represent 500 pA (vertical) and 100 ms (horizontal). (*b*) Averaged intracellular Ca^{2+} transients using the fluorescent indicator fluo 3 (see *Methods*), for beats 1–10 to show the sustained reduction in Ca^{2+} release from intracellular stores by CaMK ($n = 5$) compared with buffer alone ($n = 5$). (*c*) Summary of data showing I_{Ca} facilitation expressed as the ratio of peak I_{Ca} during the first to the *n*th beat [$I_{Ca(n)}$], as shown in Fig. 1. I_{Ca} facilitation was significantly increased by CaMK ($n = 5$) compared with control ($P < 0.05$ for beats 2 and 4–10, $n = 5$). (*d*) Peak fluo 3 fluorescence signals for each stimulated beat, measured simultaneously with I_{Ca} reported in *c*. CaMK significantly reduced peak fluo 3 fluorescence compared with control ($P < 0.05$ for beats 3, 5, 8–10).

reducing peak SR Ca^{2+} release (Fig. 2 *b* and *d*). Thus, CaMK supplementation reduced the apparent gain of the Ca^{2+}-induced Ca^{2+} release mechanism, a result that was directly opposite to the effect of CaMK inhibition (Fig. 1), confirming that CaMK activity is required for coordinated reciprocal signaling between LTCC and RyR.

CaMK Enhances SR Ca^{2+} Content and Lowers the Gain of Ca^{2+}-Induced Ca^{2+} Release. Changes in SR Ca^{2+} stores could contribute to the reduced $[Ca^{2+}]_i$ transient during CaMK activation, and apparent I_{Ca} facilitation could result from reduced Ca^{2+}-dependent I_{Ca} inactivation. To assess SR Ca^{2+} content under these conditions, we used a rapid solution exchange system to activate SR Ca^{2+} release with caffeine in the presence of extracellular Na^+, where the integral of the electrogenic Na^+/Ca^{2+} exchanger current reflects the content of SR Ca^{2+} (20). SR Ca^{2+} content was significantly enhanced by supplementation with constitutively active CaMK compared with cells dialyzed with control buffer (Fig. 3), likely because of activation of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (23, 24), and/or reduction in RyR opening (9, 10) by CaMK. In contrast, AC3-I did not significantly affect SR Ca^{2+} content compared with AC3-C. One possibility is that the greater $[Ca^{2+}]_i$ transient in AC3-I treated cells (Fig. 1 *b* and *d*) favors Ca^{2+}-dependent, phosphorylation-independent dissociation of phospholamban from SERCA (25) to increase SR Ca^{2+} uptake. Taken together, these data strongly suggest that CaMK directly regulates LTCC and RyR, reducing the gain of ECC.

We further estimated the maximal rate of SR Ca^{2+} release by measuring the initial rapid phase of inward Na^+/Ca^{2+} exchanger current. CaMK significantly slowed SR Ca^{2+} release compared with cells exposed to control buffer (Fig. 4 *a*, *b*, and *e*), further suggesting that CaMK directly reduced RyR opening during ECC, even with the increase in SR Ca^{2+} available for release. The finding that inward Na^+/Ca^{2+} exchanger current was larger (Fig. 3*e*) and slower (Fig. 4*e*) in CaMK-dialyzed cells indicates that the Na^+/Ca^{2+} exchanger was not likely saturated under these experimental conditions, and so could accurately report differences in SR Ca^{2+} content between experimental groups. Taken together, these findings strongly suggest that the mechanism of reciprocal regulation of I_{Ca} and SR Ca^{2+} release by CaMK involves reduction of RyR opening (9) and prolongation of LTCC openings (12).

Discussion

CaMK and Cardiac ECC. The present data show that CaMK can bridge the "communication gap" between LTCC and RyR, and provide a mechanism to explain coordinated LTCC-RyR activity in cardiac ECC. Ca^{2+}-induced Ca^{2+} release represents the better studied antegrade limb of cardiac ECC, but information is also communicated from the RyR to the L-type Ca^{2+} channel protein complex through a retrograde limb by a process termed crosstalk (7). Although inhibitory crosstalk occurs by $[Ca^{2+}]_i$-dependent inactivation of I_{Ca} (26), the present findings indicate that CaMK-dependent "facilitatory" crosstalk also occurs. It is likely that

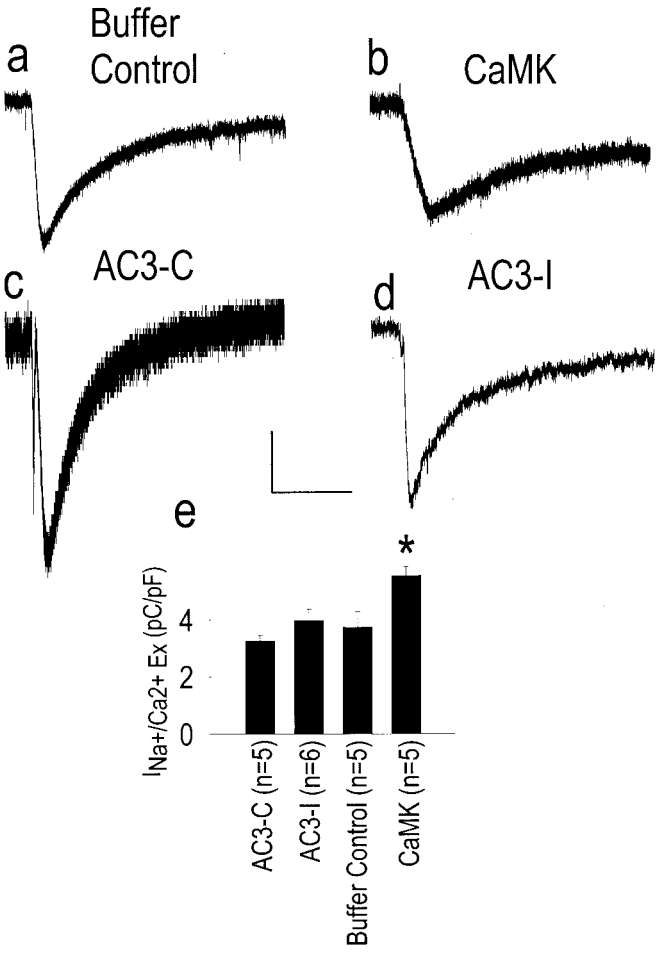

Fig. 3. CaMK increases SR Ca^{2+} content. Inward Na^+/Ca^{2+} exchanger current ($I_{Na+/Ca2+\ Ex}$) in response to perfusion with a caffeine and Na^+-containing solution in buffer control (*a*), CaMK (*b*), AC3-C (*c*), and AC3-I treated cardiomyocytes (*d*). Calibration bars indicate 50 pA (vertical) and 5 s (horizontal) for *a*–*d*. (*e*) Summary data for integrated I_{Na+}/Ca^{2+} Ex corrected for cell size from all experimental groups. *, $P = 0.005$, compared with cells treated with control buffer alone.

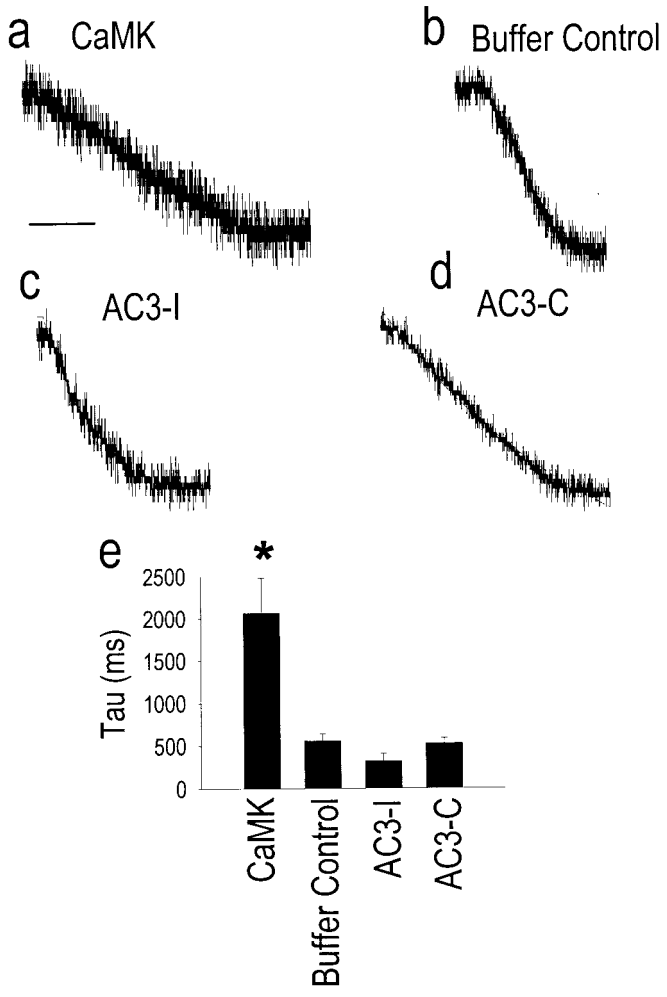

Fig. 4. CaMK slows SR Ca^{2+} release. (*a*–*d*) The initial rapid phase of SR Ca^{2+} release measured from the initial inward Na^+/Ca^{2+} exchanger current in response to rapid application of caffeine and Na^+ (see *Methods*). Dialysis of constitutively active CaMK (CaMK) (*a*) significantly slowed maximal SR Ca^{2+} release compared with cells treated with buffer control (*b*). Inhibition of endogenous CaMK with the inhibitory peptide AC3-I (*c*) tended to enhance the maximal rate of SR Ca^{2+} release compared cells treated with an inactive control peptide (*d*), AC3-C. (e) Summary data for the time constant of initial rapid SR Ca^{2+} release from the same cells in Fig. 3*e*. The calibration bar indicates 500 ms for *a* and *b*, and 200 ms for *c* and *d*. The record heights in *a*–*d* were normalized to 1.0. *, $P = 0.016$, compared with cells treated with buffer alone.

this facilitatory component of crosstalk is important for refilling SR Ca^{2+} stores by enhancing I_{Ca} while reducing SR Ca^{2+} release.

The present study of the role of CaMK in cardiac ECC uses a highly specific inhibitory peptide and constitutively active CaMK as experimental probes. Our finding that CaMK activity reduces the gain of cardiac ECC contrasts with a previous study where the cell membrane-permeant CaMK inhibitory compound KN-93 reduced the gain of ECC in ferret ventricular myocytes (27). In addition to inhibiting CaMK, KN-93 blocks multiple cell membrane ionic currents (28) and so could interfere with ECC independently of CaMK, for example, by inhibiting ion channel proteins on the SR that are thought to be important for balancing charges associated with Ca^{2+} flux during ECC. It is also possible that differences in experimental conditions may partially contribute to the discrepancy between these results. One strength of the present approach is that it employs both CaMK inhibitory and replacement strategies.

Reduction in the gain of ECC that follows addition of CaMK is similar to the ECC phenotype in experimental cardiac hypertrophy and heart failure (29). CaMK expression (30) and activity (31) are both increased in failing human hearts, raising the possibility that enhanced CaMK activity may explain the ECC phenotype in human heart failure (32), and so represent a therapeutic target. The functional properties of CaMK are partially governed by the proportion of different isoforms composing the heteromeric holoenzyme (33). Thus, changes in CaMK isoform populations that occur during cardiac development (34, 35) and disease (30) can provide a previously unrecognized dimension to regulation of cardiac ECC.

CaMK and RyR. Although it is clear that RyR is an important substrate for CaMK actions, the effect of CaMK-catalyzed RyR phosphorylation in isolated preparations remains controversial. Some authors have reported that exogenous CaMK increases (15, 16), whereas others have found that exogenous (9, 10) and endogenous (16) CaMK reduces RyR opening or ryanodine-RyR binding (13). Our results in intact cardiomyocytes where endogenous CaMK is inhibited or exogenous Ca^{2+}-independent CaMK is added are both consistent with the hypothesis that the net effect of CaMK activation is to reduce RyR opening.

We thank Martha Bass for expert technical assistance. We thank Dan Roden and Jeff Balser for helpful criticisms and suggestions. This work was supported by grants from the National Institutes of Health (National Heart, Lung, and Blood Institute, HL03727 and HL62494; to M.E.A.) and the American Heart Association SE affiliate (to M.E.A. and R.J.C.).

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