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Dual regulation of Ca^{2+}/calmodulin-dependent kinase II activity by membrane voltage and by calcium influx

(autophosphorylation/calcium channel facilitation/heart cells/confocal microscopy)

RUI-PING XIAO*, HEPING CHENG†, W. J. LEDERER†, TATSUO SUZUKI‡, AND EDWARD G. LAKATTA*§

*Laboratory of Cardiovascular Science, Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224; †Department of Physiology, University of Maryland School of Medicine, Baltimore, MD 21201; and ‡Department of Biochemistry School of Medicine, Nagoya City University Medical School, Mizuhocho, Mizuhoku, Nagoya 467, Japan

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ABSTRACT **Calcium entry through voltage-gated Ca^{2+} channels is critical in cardiac excitation–contraction coupling and calcium metabolism. In this report, we demonstrate both spatially resolved and temporally distinct effects of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) on L-type Ca^{2+} channel current (I_{Ca}) in rat cardiac myocytes. Either depolarization alone or calcium influx can increase the amplitude and slow the inactivation of I_{Ca}. The distinct voltage- and Ca^{2+}-dependent effects persist with time constants of ≈1.7 sec and 9 sec, respectively. Both effects are completely abolished by a specific peptide inhibitor of CaMKII. This CaMKII inhibitor also suppresses the prolongation of I_{Ca} induced by depolarizing holding potentials. Furthermore, using an antibody specific for the autophosphorylated (activated) CaMKII, we find that this kinase is localized close to sarcolemmal membranes and that the profile of CaMKII activation correlates qualitatively with the changes in I_{Ca} under various conditions. Therefore, we conclude that the action of CaMKII on I_{Ca} is dually regulated by membrane depolarization and by Ca^{2+} influx; the latter directly activates CaMKII, whereas the former likely promotes the interaction between constitutive CaMKII and the membrane-channel proteins. These regulatory mechanisms provide positive-feedback control of Ca^{2+} channels and are probably important in the regulation of cardiac contractility and other intracellular Ca^{2+}-regulated processes.**

The ubiquitous multifunctional protein kinase, Ca^{2+}/calmodulin-dependent kinase II (CaMKII), is a cytosolic enzyme activated by the signal-transduction pathways that elevate intracellular free Ca^{2+}. CaMKII is important in diverse cellular functions from modulating ionic channels in muscles (1–3) to encoding "memory" in neurons (4, 5). In heart muscle, ryanodine receptors (3) and phospholamban (6) (both of which are involved in intracellular Ca^{2+} homeostasis) have recently been shown to be regulated by CaMKII-mediated protein phosphorylation. Consequently CaMKII influences intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in heart muscle by regulating key proteins in the dominant intracellular Ca^{2+} store, the sarcoplasmic reticulum. However, an additional and possibly more important role that CaMKII may play in regulating the sarcolemmal Ca^{2+} current remains uncertain. In this study, we have investigated the effects of CaMKII on cardiac sarcolemmal Ca^{2+} channels using electrophysiological methods and confocal immunofluorescence microscopy. We report that the activated CaMKII localizes close to cardiac sarcolemmal membranes and mediates a convergent positive-feedback regulation of L-type Ca^{2+} channel current (I_{Ca}) by membrane depolarization and by Ca^{2+} influx. Our results also show a modulatory effect of membrane voltage on CaMKII action and provide an important regulatory mechanism for this widespread and multifunctional protein kinase. Because the CaMKII-mediated positive-feedback control of I_{Ca} occurs rapidly, it could contribute to the beat-to-beat regulation of cardiac contractility.

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METHODS

Preparation of Isolated Cardiac Myocytes. Single ventricular cardiac myocytes were isolated from 2- to 4-mo-old rat hearts by a standard enzymatic technique (7). Isolated cells were suspended in 1.0 mM $CaCl_2$/137 mM NaCl/5 mM KCl/15 mM dextrose/1.3 mM $MgCl_2$/1.2 mM NaH_2PO_4/20 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.4, adjusted with NaOH. Cells were stored at 37°C until use.

Electrophysiological Recording. I_{Ca} was measured with voltage-clamp technique in the whole-cell mode. The superfusion solution contained 1.0 mM $CaCl_2$, 117 mM *N*-methyl-D-glucamine, 20 mM tetraethylammonium chloride, 5 mM CsCl, 15 mM dextrose, 1.3 mM $MgCl_2$, 20 mM Hepes (pH 7.4, adjusted with HCl). Patch pipette electrodes (2–4 MΩ) were filled with the solution containing 120 mM CsCl, 20 mM Hepes, 1.5 mM $MgCl_2$, 3 mM Mg^{2+}-ATP, 3 mM 4-aminopyridine, 10 mM EGTA or bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) (pH 7.2, adjusted with CsOH). After the establishment of voltage clamp, 10 min was allowed to equilibrate the cell with the pipette solution. I_{Ca} was then recorded with a discontinuous-switch-clamp Axoclamp II amplifier (Axon Instruments, Foster City, CA). Peak value of I_{Ca} was measured as the difference between current levels at peak and at the end of the 200-msec pulse, and the I_{Ca} inactivation "rate" was indexed as the 63% decay time (7). All electrophysiological experiments were done at room temperature (23°C).

Immunocytochemical Methods. Isolated cells were pretreated with desired experimental reagents for 10 min before fixing and permeabilizing the cells with 100% ethanol at −20°C for another 10 min. After washing with Hepes buffer solution cells were further permeabilized by 1% Triton X-100 for 10 min. Nonspecific antibody binding was reduced by preincubation with 2% bovine serum albumin for 30 min. Cells were incubated with a primary antibody (anti-PY-66)

Abbreviations: CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; ICK, CaMKII fragment-(290–309); Ado*PP*[NH]*P*, adenosine 5′-[β,γ-imido]-triphosphate; BAPTA, bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; I_{Ca}, L-type Ca^{2+} channel current; PKC, protein kinase C; PKA, protein kinase A; $[K^+]_o$, extracellular K^+ concentration; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; P_1, depolarizing prepulse; P_2, second depolarizing pulse; I_{Ba}, Ba^{2+} current through L-type Ca^{2+} channels; V_H, holding potential; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

§To whom reprint requests should be addressed at: Gerontology Research Center, National Institute on Aging, 4940 Eastern Avenue, Baltimore, MD 21224.

(8) at 1:1000 dilution in Hepes buffer overnight at 4°C. The cells were then incubated for 2 hr with fluorescein-conjugated goat anti-rabbit IgG (Oncogene Science). All images were acquired with a Bio-Rad MRC 600 confocal microscope fitted with an Argon laser and processed using Interactive Data Language (IDL) software (Research Systems, Boulder, CO) (9).

Drugs. Calmodulin-dependent protein kinase II fragment (290–309) (ICK; a specific peptide inhibitor of CaMKII), EGTA, BAPTA, adenosine 5′-[β,γ-imido]triphosphate (Ado*PP*[NH]*P*), isoproterenol, norepinephrine, and thioridazine were purchased from Sigma. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) purchased from Sigma was dissolved in dimethyl sulfoxide. H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine] and phorbol 12-myristate 13-acetate were purchased from Calbiochem and dissolved in dimethyl sulfoxide.

Statistical Analysis. Data are presented as mean ± SEM. Student's *t* test or a paired *t* test was used to determine the significance of differences when appropriate. A value of $P < 0.05$ was considered significant.

RESULTS

Ca^{2+}-Dependent I_{Ca} Facilitation Mediated by CaMKII. Fig. 1 demonstrates the modulation of I_{Ca} in cardiac myocytes by sarcolemmal Ca^{2+} influx. After a Ca^{2+}-loading depolarizing prepulse to 0 mV (P_1), the peak level of I_{Ca} obtained during the second pulse (P_2) increased, and the decay time was prolonged. Average I_{Ca} amplitude of P_2 increased from 1.40 ± 0.13 nA to 1.53 ± 0.21 nA ($n = 9$, $P < 0.01$), and the 63% decay time of I_{Ca} was prolonged from 13.52 ± 1.42 msec to 24.32 ± 1.76 msec ($n = 9$, $P < 0.005$) (Fig. 1*A*). The Ca^{2+}-dependence of the I_{Ca} facilitation is demonstrated by the observations that inclusion of 10 mM BAPTA, a fast Ca^{2+} chelator (10), in the patch pipette solution (Fig. 1*B*) or replacement of Ca^{2+} in the bathing fluid with Ba^{2+} (Fig. 1*C*) blocks the potentiating effect of P_1. Although similar observations regarding the modulation of I_{Ca} by Ca^{2+} influx and by direct increase in intracellular Ca^{2+} have been reported

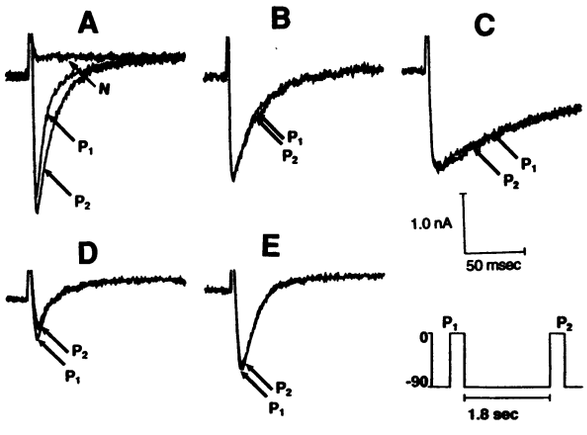

FIG. 1. Ca^{2+}-dependent I_{Ca} facilitation mediated by CaMKII in single cardiac cells. (*A*) Prepulse P_1 increases the peak level and prolongs the decay rate of I_{Ca}. Note that nifedipine (N), a specific L-type Ca^{2+}-channel blocker, at 1 μM blocks the I_{Ca}. (*B*) Effects of replacement EGTA in the pipette with 10 mM BAPTA. (*C*) Substitution of extracellular Ca^{2+} with equimolar Ba^{2+} abolishes the I_{Ca} facilitation ($n = 8$). (*D*) I_{Ca} traces with 10 mM Ado*PP*[NH]*P* included in the pipette ($n = 4$). (*E*) Effects of inhibition of CaMKII by a specific peptide blocker, ICK. The peptide corresponds to the fragment-(290–309) of α subunit of CaMKII (amino acid sequence: Leu-Lys-Lys-Lys-Asn-Ala-Arg-Arg-Lys-Leu-Lys-Gly-Ala-Ile-Leu-Thr-Thr-Met-Leu-Ala). Pairs of pulses of 200 msec (lower right) are applied every 30 sec.

(11–14), the molecular basis of these effects has not been identified. In additional experiments, we found that intracellular application of Ado*PP*[NH]*P*, a nonhydrolyzable ATP analogue, reversed the effect of the conditioning prepulse (Fig. 1*D*). Note that the peak of I_{Ca} during P_2 is decreased rather than increased as compared with P_1, and the decay rates are virtually identical. The currents were smaller when compared with control, probably due to the quick I_{Ca} run-down in the presence of Ado*PP*[NH]*P*. The blockade effect of Ado*PP*[NH]*P* on I_{Ca} facilitation suggests that a phosphorylation reaction may have been initiated by the Ca^{2+} influx during P_1, which, in turn, produced the facilitation of I_{Ca} during P_2. We therefore examined the role of three major protein kinase systems reported to modulate I_{Ca}: protein kinase C (PKC), CaMKII, and protein kinase A (PKA). Neither H-7 (10 μM, $n = 8$), a PKC blocker, nor down-regulation of PKC by incubation of cells with 10 μM phorbol 12-myristate 13-acetate for 24 hr ($n = 10$) altered I_{Ca} facilitation. However, the calmodulin inhibitors W-7 (10 μM, $n = 6$) and thioridazine (1 μM, $n = 5$) blocked the potentiation in I_{Ca} during P_2, suggesting possible involvement of a calmodulin-dependent protein kinase. Because these inhibitors lack high specificity, this initial observation was reexamined with the specific peptide inhibitor of CaMKII, ICK. This peptide corresponds to a portion of the "autoinhibitory" and calmodulin-binding domains of CaMKII (15) and when applied intracellularly (115 μM included in pipette) completely abolished the Ca^{2+}-dependent augmentation and prolongation of I_{Ca} (Fig. 1*E*). On average, the I_{Ca} peak amplitude in the presence and absence of the prepulse is 1.09 ± 0.10 nA and 1.18 ± 0.16 nA, respectively; the 63% decay time of I_{Ca} is 13.47 ± 1.11 msec and 13.67 ± 1.29 msec ($n = 9$), respectively. In the continued presence of ICK (used to block CaMKII), the β-adrenergic agonists isoproterenol (1 μM, $n = 4$) and norepinephrine (1 μM, $n = 3$) still enhanced I_{Ca} in a manner characteristic of PKA activation (7) but could not restore the pulse-dependent effects that had been blocked by the CaMKII inhibitor. Thus, PKA does not appear to provide an independent contribution to the I_{Ca} facilitation. These results indicate that the Ca^{2+}-dependent I_{Ca} potentiation in heart is due to CaMKII-dependent protein phosphorylation.

Voltage-Dependent Ca^{2+} Channel Facilitation. Further examination of the I_{Ca} facilitation shown in Fig. 1 led us to attempt to "separate" the purely voltage-dependent effects of P_1 from the action of P_1 to change $[Ca^{2+}]_i$ in cellular microdomains. To prevent Ca^{2+} entry during the prepulse, the depolarization level of P_1 was raised to +100 mV, well beyond the apparent reversal potential of the Ca^{2+} current (+50 to +60 mV in cardiac myocytes). Na^+/Ca^{2+} exchange didn't contribute the Ca^{2+} influx during these experiments because both internal and external Na^+ were absent. Qualitatively, the changes in I_{Ca} elicited by a single strong prepulse to +100 mV (Fig. 2*A*) were similar to those induced by a prepulse to 0 mV (Fig. 1). In six cells, the strong prepulse augmented the I_{Ca} peak from 1.41 ± 0.20 nA to 1.55 ± 0.22 nA ($P < 0.01$) and increased the 63% decay time from 14.20 ± 1.32 msec to 22.72 ± 1.90 msec ($P < 0.01$). Thus, the depolarization alone—i.e., in the absence of Ca^{2+} influx and an increase in $[Ca^{2+}]_i$—could affect the I_{Ca} facilitation. This result was further confirmed by the observation that BAPTA neither blocked nor attenuated this effect (Fig. 2*B*), in marked contrast to Fig. 1*B*. Importantly, the specific peptide CaMKII inhibitor also blocks the effects of the strong depolarizing prepulse (Fig. 2*C*), indicating that the same protein kinase, CaMKII, was involved in the voltage-dependent modulation of I_{Ca}.

Because Fig. 2 *A* and *B* provide evidence against the idea that Ca^{2+} influx during prepulse underlies the observed changes of I_{Ca} during these experiments, we suspected that the basal CaMKII activity present at resting $[Ca^{2+}]_i$ is in-

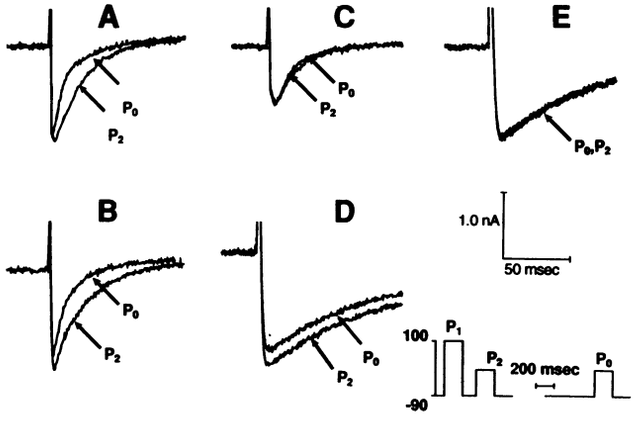

FIG. 2. Effects of a single strong depolarizing prepulse on subsequent I_{Ca}. (*A*) Representative current traces with and without the strong depolarizing prepulse. (*B*) BAPTA does not affect the potentiation effect of the voltage-conditioning prepulse (n = 10). (*C*) ICK, the specific peptide inhibitor of CaMKII, abolishes the I_{Ca} facilitation induced by the strong depolarizing prepulse within 20 min after breaking cell membrane (n = 6). (*D*) Facilitation of Ba^{2+} current (I_{Ba}) by the prepulse. The current traces were recorded within 1.0 min after the replacement of Ca^{2+} by Ba^{2+} in the bathing solution. (*E*) Blockade of the I_{Ba} facilitation in the same cell after 10-min exposure to Ba^{2+} solution. Voltage protocol (lower right): inset, the voltage-conditioning pulse P_1 is to +100 mV, and the interval between P_1 and test pulse P_2 is 150 msec. I_{Ca} during P_2 is compared with a control I_{Ca} obtained with a single test pulse (P_0) in the absence of a prepulse.

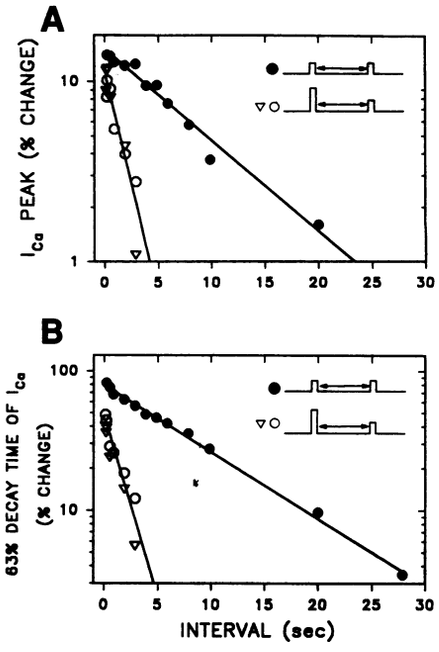

FIG. 3. Semilogarithmic plots of average time courses of the effects of prepulses on I_{Ca} amplitude (*A*) and kinetics (*B*). ○ and ●, Prepulse to +100 mV and to 0 mV, respectively, with 10 mM EGTA in the pipette (n = 6); △, prepulse to +100 mV with 10 mM BAPTA in the pipette (n = 6). Lines represent the exponential fittings of the data obtained with EGTA. Time constant (τ) for the persisting effect of prepulse to +100 mV is 1.7 sec, whereas τ for prepulse to 0 mV is ≈9 sec (8.6 sec in *A* and 9.1 sec in *B*). Ca^{2+}- and voltage-dependent inactivation of I_{Ca} by prepulse was also observed for brief interpulse intervals, consistent with previous reports (14, 17). The recovery from the inactivation followed a single-exponential process with a time constant of 43 msec for a prepulse to +100 mV and 41 msec for prepulse to 0 mV under experimental conditions. For brevity, the data shown in Fig. 3 are only those at interpulse intervals >120 ms, when the normal reactivation process is almost complete.

volved in this process. If this interpretation is correct, severe deprivation of intracellular Ca^{2+} should block the I_{Ca} alteration by reducing the basal CaMKII activity (16). This prediction was tested by replacing the bathing Ca^{2+} with Ba^{2+}. Immediately after Ba^{2+} application, the recorded current slows markedly, characteristic of Ba^{2+} current through the L-type Ca^{2+} channels (I_{Ba}) (Fig. 2*D*). Nevertheless, there is still a significant increase in the I_{Ba} amplitude associated with P_2 as compared with I_{Ba} of the test pulse in the absence of a prepulse (P_0) (Fig. 2*D*). This potentiating effect of prepulse on the I_{Ba} diminished after a longer period of exposure to the Ba^{2+} solution (Fig. 2*E*) because the I_{Ba} traces in Fig. 2*E* are almost identical to the I_{Ba} trace of P_0 in Fig. 2*D*. The time-dependent Ba^{2+} blockade shown here is consistent with the involvement of basal constitutive CaMKII activity maintained by the resting $[Ca^{2+}]_i$ (see *Discussion*).

Persistence of the Ca^{2+}- and Voltage-Dependent I_{Ca} Facilitation. The longevity of the voltage- and Ca^{2+}-dependent I_{Ca} potentiation differed markedly as shown in Fig. 3. The solid circles show that a prepulse to 0 mV that elicits Ca^{2+} influx produces a potentiation of the peak level of I_{Ca} during P_2 and a slowing of its inactivation, which persist with a time constant of ≈9 sec. In contrast, depolarization to +100 mV (the open circles) produces potentiation effects that persist with a time constant of ≈1.7 sec. Note that high concentration of BAPTA in pipette (the triangles) did not alter the persistence of the effects elicited by the large prepulse. The distinctive persistence further differentiates the voltage-dependent effect (Fig. 2) from the Ca^{2+}-dependent effect shown in Fig. 1.

Effect of Holding Potential on I_{Ca}. Not only does the activated CaMKII prolong the action of brief signals of membrane voltage and cytosolic Ca^{2+}, but it also integrates weak yet sustained signals, thereby making a cellular response possible. The effect of change in holding potential

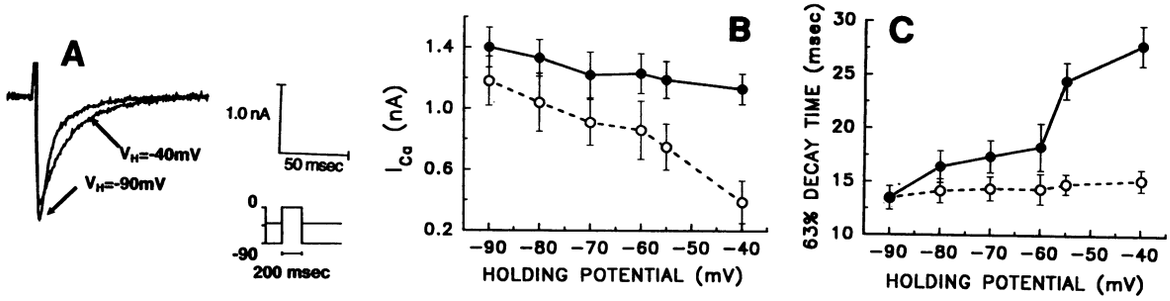

FIG. 4. Effect of V_H on I_{Ca} amplitude and 63% decay time of I_{Ca}. Membrane voltage is held at the desired level for 30 sec before a single 200-msec test pulse to 0 mV elicited from V_H. (*A*) Typical result of V_H-dependent changes of I_{Ca}. (*B*) Plots of I_{Ca} amplitudes in the absence (●) and presence (○) of CaMKII inhibitor ICK as a function of V_H (n = 6–9). (*C*) ICK blocks the V_H-dependent alteration in the inactivation kinetics of I_{Ca} (n = 6–9).

(V_H) on I_{Ca} is shown in Fig. 4*A*. Note that the decay time of I_{Ca} recorded at V_H of −40 mV is markedly prolonged as compared with that at V_H of −90 mV while the peak current amplitude is only 16% lower, presumably due to the voltage-dependent steady-state inactivation known to occur in cardiac cells (17). Furthermore, a CaMKII-inhibitor-sensitive component, both in the amplitude and in the kinetics of I_{Ca}, was observed at depolarizing holding potentials (Fig. 4 *B* and *C*). At V_H of −40 mV, two-thirds of the peak I_{Ca} depends on CaMKII activation (Fig. 4*B*) and concomitantly, the 63% decay time is reduced by ≈50% after ICK application (Fig. 4*C*). The V_H-dependent difference in the I_{Ca} kinetics is also suppressed by the CaMKII inhibitor (Fig. 4*C*). Therefore the transient CaMKII-specific modulation of I_{Ca} depicted in Figs. 1 and 2 has a tonic counterpart during steady-state moderate depolarization.

Intracellular Localization of Autophosphorylated CaMKII. Using the antibody anti-PY-66, which reacts specifically with the autophosphorylated (activated) CaMKII (8) and confocal microscopy, we examined the presence and the intracellular activation of CaMKII in cardiomyocytes. After high extracellular K^+ concentration ($[K^+]_o$) (30 mM) depolarization in the presence of 1 mM bathing Ca^{2+}, the area near surface sarcolemmal membrane has a significant enrichment of autophosphorylated enzyme (Fig. 5 *B* and *E*). Additionally, there appears a measurable amount of fluorescence on the T-tubular membranes, causing a slightly striated appearance (Fig. 5*B*). The highly fluorescent periphery has a thickness of ≈2 μm at half-maximal level and is approximately three times brighter than the interior (Fig. 5*E*). In cells at normal resting potential (5 mM $[K^+]_o$), a similar but weaker pattern is evident (Fig. 5*C*). On average, there is nearly a 2-fold increase of the quantitative fluorescence associated with 30 mM $[K^+]_o$-induced depolarization (data not shown). However, if $[Ca^{2+}]_i$ is forced to very low levels by removal of bathing Ca^{2+}, the immunofluorescence pattern is lost (Fig. 5*D*), rendering an image similar to that obtained without the primary antibody (Fig. 5*A*). Depolarization by high $[K^+]_o$ in the absence of Ca^{2+} in the bath fails to increase the amount of activated CaMKII (Fig. 5*D*). Finally, application of a calmodulin blocker, W-7 (10 μM), to the bathing solution can also block the anti-PY-66 specific fluorescence (data not shown).

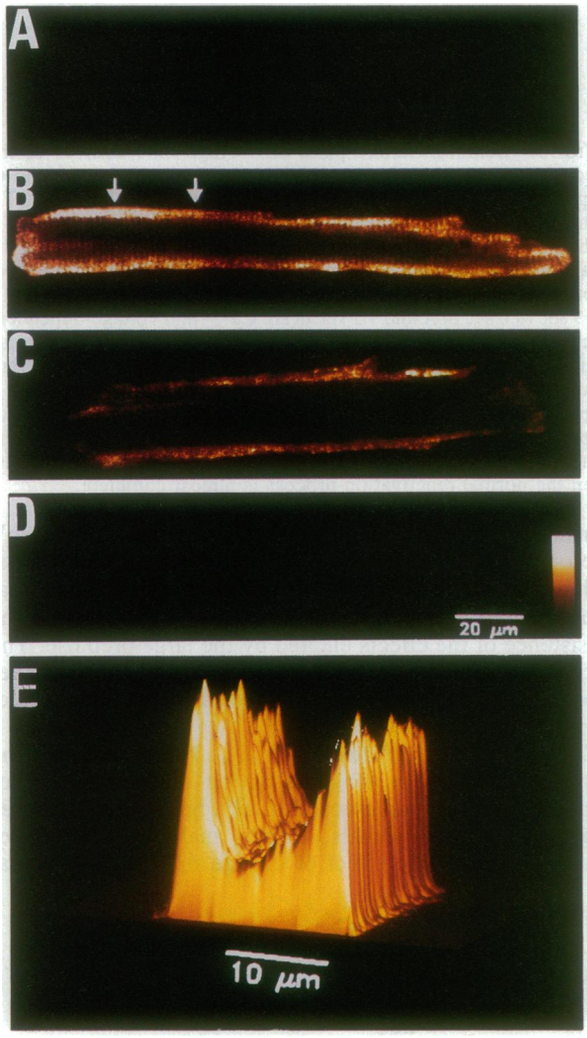

FIG. 5. Intracellular distribution of autophosphorylated CaMKII visualized by confocal fluorescence microscopy. (*A*) Nonspecific staining in the absence of the primary antibody. (*B*) Confocal section (thickness < 1 μm) image of a cell exposed to 30 mM $[K^+]_o$ and stained with the primary antibody Anti-PY-66. (*C*) Typical staining pattern of cells bathed in the standard Hepes buffer solution. (*D*) Removal of external Ca^{2+} diminishes the specific staining. Cells are bathed with 2 mM EGTA and depolarized with 30 mM $[K^+]_o$. The color bar for *A–D* represents fluorescence intensity of 0–130 on a linear scale. (*E*) Surface-plot of the segment in *B* indicated by the pair of arrows.

DISCUSSION

In the present study, we show that Ca^{2+}-dependent L-type Ca^{2+} channel facilitation coexists with voltage-dependent I_{Ca} facilitation in rat cardiomyocytes. Such prepulse-dependent modulation of I_{Ca} is independent of the well-documented Ca^{2+}- and voltage-dependent inactivation of I_{Ca} (14, 17). The Ca^{2+}-dependent facilitation depends on the Ca^{2+} influx (Fig. 1*C*) and its resultant change of $[Ca^{2+}]_i$ (Fig. 1*B*). In contrast, voltage-dependent I_{Ca} facilitation is elicited by a strong depolarizing prepulse that is not associated with Ca^{2+} influx. Although both Ca^{2+}- and voltage-dependent facilitation result in a similar increase in the I_{Ca} peak and a slowing of the I_{Ca} decay rate, they are distinct because the voltage-dependent effect and its Ca^{2+}-dependent counterpart have different time constants of decay (Fig. 3). Furthermore, the voltage-dependent facilitation is not affected by the high concentration of BAPTA used to buffer the $[Ca^{2+}]_i$ (Figs. 2*B* and 3) and is also manifested in facilitation of I_{Ba} immediately after Ba^{2+} application (Fig. 2*D*). These results suggest that the observed aftereffects of the strong, depolarizing prepulse are due to the depolarization *per se*, in contrast to the Ca^{2+}-entry dependence of the effects shown in Fig. 1. Because the selective CaMKII peptide inhibitor ICK completely abolished the augmentation in the peak and the prolongation in the I_{Ca} decay time elicited by either Ca^{2+} influx or the membrane depolarization, we conclude that both types of Ca^{2+} channel facilitation are mediated by the same kinase, CaMKII. The presence of CaMKII molecules in the rat cardiac myocytes, as revealed by the anti-PY-66-specific immunofluorescence (Fig. 5), provides the molecular basis for these functional observations. The Ca^{2+}-dependent CaMKII modulation of I_{Ca} is similar to that in the smooth muscle reported recently (1) and may also provide a mechanism for the potentiation of I_{Ca} by photo-release of caged Ca^{2+} (13). However, to our knowledge, no parallel to the voltage-dependent modulation on CaMKII action has been reported previously.

We further demonstrate that Ca^{2+}- and voltage-dependent CaMKII actions may be mediated by different signal-transduction cascades. As described above, these distinct actions have different lifetimes of persistence. The longer Ca^{2+}-dependent CaMKII effects may reflect the lifetime of the calmodulin-autophosphorylated CaMKII complex (18). Because 10 mM EGTA was normally present in the patch

pipette and resulted in a blockade of Ca^{2+} transients and contractions (data not shown), the observed Ca^{2+}-dependent CaMKII activation is inferred to occur in a microdomain near the sarcolemmal membranes. In contrast, the shorter persistence of the voltage-dependent CaMKII actions suggests a mechanism in which a direct activation of CaMKII is not involved. In support of this interpretation, tonic depolarization by high $[K^+]_o$ in the absence of Ca^{2+} failed to maintain CaMKII autophosphorylation (Fig. 5*D*) and biochemically measured CaMKII activity (16). However, membrane voltage could modulate the interaction between the mobile kinases and the membrane channel proteins. Specifically, depolarization may place the Ca^{2+} channel proteins into conformations that are more favorable for phosphorylation by the constitutive CaMKII.

In resting heart cells, there is significant basal activation of CaMKII, as shown in Fig. 5*C*. Tonic activation of CaMKII requires a certain level of $[Ca^{2+}]_i$. With 10 mM EGTA or BAPTA included in the patch pipette, the $[Ca^{2+}]_i$, as directly measured by Ca^{2+} indicator Indo-1, shows a transient decrease to <10 nM (data not shown). However, in the steady state, under which conditions our prepulse experiments were implemented, the resting $[Ca^{2+}]_i$ level (128 ± 10.2 nM, $n = 6$) is highly comparable to the value of unbuffered cells (135 nM, ref. 19). (This somewhat surprising normal steady level of $[Ca^{2+}]_i$ reflects a dynamic balance between Ca^{2+} homeostatic mechanisms in the rat heart cells and the loading of exogenous Ca^{2+} buffers.) Thus, a tonic CaMKII activation is likely to be presented under voltage-clamp conditions. The time-dependent blockade of the Ca^{2+}-channel facilitation by Ba^{2+} (Fig. 2 *D* and *E*) probably reflects different states of intracellular CaMKII activity and further supports the idea that membrane voltage plays an important role in the modulation of the function of constitutively active CaMKII. A similar mechanism has been proposed to explain the voltage-dependent PKA-mediated modulation of I_{Ca} in skeletal muscle (20).

The CaMKII-mediated potentiation of I_{Ca} demonstrated here provides a convergent positive-feedback control of Ca^{2+} influx in heart cells by cellular membrane depolarization states and Ca^{2+} signals. An important feature of the CaMKII action is probably the slow "relaxation" of these modulatory effects. This "memory" property of the voltage- and Ca^{2+}-dependent CaMKII action may enable the kinase to respond to repetitive signals on the basis of their frequency. Additionally, this property may also provide the cell a molecular means for integrating a weak yet enduring signal. As a manifestation of this point, Fig. 5*B* shows that membrane depolarization produced by high $[K^+]_o$ has been encoded into the increased activation of CaMKII. The increased CaMKII activation appears also partly responsible for the ICK-sensitive component in I_{Ca} amplitude and kinetics induced by depolarizing V_H (Fig. 4). Finally, data presented in Fig. 4*B* indicate that as the V_H is reduced from −90 to −40 mV, I_{Ca} inactivation is offset, in part, by a CaMKII effect. If the data in the presence of CaMKII peptide inhibitor better reflect the true voltage dependence of steady-state inactivation of I_{Ca}, the data obtained in the absence of CaMKII inhibitor would represent a combination of voltage-dependent steady-state inactivation and CaMKII-dependent potentiation of I_{Ca}.

To date, proper information on the intracellular distribution of CaMKII molecules in cardiac myocytes has not been available. Combined with an antibody specific to the autophosphorylated (activated) CaMKII and confocal microscopy, we have directly visualized the intracellular distribution of the activated enzyme and have examined the CaMKII activation in relation to the electrophysiological observations. The activated CaMKII molecules are mostly associated with sarcolemmal membranes, including the T-tubules. The unique spatial pattern of CaMKII activation fits nicely with the identified functional role of CaMKII in regulating the sarcolemmal Ca^{2+} channels that we demonstrated. Biochemical studies in other tissues also suggest that the intracellular CaMKII distribution may be related to its target proteins in a tissue-specific manner (21, 22). The profile of CaMKII activation under different experimental conditions is, as discussed above, qualitatively in agreement with the electrophysiological data on the CaMKII-mediated change in I_{Ca} and is generally consistent with the biochemical results obtained from neural tissues (23, 24).

In summary, we have found temporally separable Ca^{2+}- and voltage-dependent I_{Ca} facilitation with spatially resolved intracellular activation of CaMKII and a correlation between the changes in I_{Ca} and CaMKII activation. From these results we conclude that CaMKII mediates both Ca^{2+}- and voltage-dependent I_{Ca} facilitation in cardiac cells and that the action of CaMKII could be regulated by membrane potential *per se*, in a manner complementary to the more established regulation by cytosolic Ca^{2+}. These findings have important implications not only for the beat-to-beat regulation of cardiac function but also in response to small, tonic Ca^{2+} and membrane voltage signals under normal and pathophysiological circumstances.

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