Regulation of a voltage-sensitive release mechanism by Ca\(^{2+}\)-calmodulin-dependent kinase in cardiac myocytes

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Zhu, Jiequan, and Gregory R. Ferrier. Regulation of a voltage-sensitive release mechanism by Ca\(^{2+}\)-calmodulin-dependent kinase in cardiac myocytes. Am J Physiol Heart Circ Physiol 279: H2104–H2115, 2000.—A role for Ca\(^{2+}\)-calmodulin-dependent kinase (CamK) in regulation of the voltage-sensitive release mechanism (VSRM) was investigated in guinea pig ventricular myocytes. Voltage clamp was used to separate the VSRM from Ca\(^{2+}\) in regulation of the voltage-sensitive release mechanism (VSRM) was investigated in guinea pig ventricular myocytes. Voltage clamp was used to separate the VSRM from Ca\(^{2+}\)-induced release (CICR). VSRM contractions and Ca\(^{2+}\) transients were absent in cells dialyzed with standard pipette solution but present when 2–5 μM calmodulin was included. Effects of calmodulin were blocked by KN-62 (CamK inhibitor), but not H-89, a protein kinase A (PKA) inhibitor. Ca\(^{2+}\) current and caffeine contractions were not affected by calmodulin. Transient-voltage relations were bell-shaped without calmodulin, but they were sigmoidal and typical of the VSRM with calmodulin. Contractions with calmodulin exhibited inactivation typical of the VSRM. These contractions were inhibited by rapid application of 200 μM of tetracaine, but not 100 μM of Cd\(^{2+}\), whereas CICR was inhibited by Cd\(^{2+}\) but not tetracaine. In undialyzed myocytes (high-resistance microelectrodes), KN-62 or H-89 each reduced amplitudes of VSRM contractions by ~50%, but together they decreased VSRM contractions by 95%. Thus VSRM is facilitated by CamK or PKA, and both pathways regulate the VSRM in undialyzed cells.

excitation-contraction coupling; cardiac muscle; protein kinase

THE CONTRACTION of a mammalian heart is initiated by a rapid rise in intracellular free Ca\(^{2+}\) concentration, which is achieved primarily through release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) (2). Two mechanisms can release SR Ca\(^{2+}\) in cardiac muscle. Ca\(^{2+}\) entering the cardiac cell across the sarcolemma can bind to SR Ca\(^{2+}\) release channels (ryanodine receptors; RyRs) and cause them to open and release SR Ca\(^{2+}\) stores by a process called Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) (7). CICR can be initiated by Ca\(^{2+}\) entering through voltage-gated Ca\(^{2+}\) channels (1, 3, 5, 6, 21), reverse-mode Na\(^+\)/Ca\(^{2+}\) exchange (Na/Ca\(_{\text{EX}}\) ) (16, 18, 19, 25, 33, 34), or possibly Na\(^+\) channels when their selectivity for Na\(^+\) relative to Ca\(^{2+}\) has been altered pharmacologically (28). Recently, we have presented evidence that the release of SR Ca\(^{2+}\) also can be initiated by a voltage-sensitive release mechanism (VSRM), which operates independently of L-type Ca\(^{2+}\) current (I\(_{\text{Ca-L}}\), T-type Ca\(^{2+}\) current, or reverse-mode Na/Ca\(_{\text{EX}}\) (9–11, 14, 15, 22).

CICR and the VSRM have distinctly different characteristics. The VSRM is selectively inhibited by regulates, and by tetracaine at concentrations that do not inhibit I\(_{\text{Ca-L}}\) or by CICR coupled to I\(_{\text{Ca-L}}\) (22, 23). Similarly, I\(_{\text{Ca-L}}\) and the contractions triggered by I\(_{\text{Ca-L}}\) are inhibited by verapamil, nifedipine, or Ca\(^{2+}\) at concentrations that do not block the VSRM (9–11, 14, 15, 22).

The VSRM also is insensitive to Na\(^+\) channel blockade with tetrodotoxin (TTX) or lidocaine (9–11, 14, 15, 22), and functions in the absence of Na\(^+\) in the extracellular medium (9) and in cells dialyzed with 0 mM Na\(^+\) (10, 11, 13). Furthermore, the VSRM exhibits voltage-dependent activation and inactivation properties that are distinctly different from those of I\(_{\text{Ca-L}}\) and Na/Ca\(_{\text{EX}}\) (9–11, 14, 15, 22).

The amplitudes of contractions initiated by the VSRM are not proportional to Ca\(^{2+}\) current but instead show a sigmoidal dependence on membrane depolarization (9–11, 14, 15, 22). This dependence on membrane depolarization implies the existence of voltage sensors located in the sarcolemma. It is not yet known whether activation of voltage sensors in cardiac muscle is communicated to RyRs physically through connections between these two proteins or by some intermediate signal such as phosphorylation.

Cardiac RyRs have phosphorylation sites for cAMP-dependent protein kinase A (PKA) and for Ca\(^{2+}\)-calmodulin-dependent kinase II (CamK) (12, 20, 31). It is possible that the VSRM requires phosphorylation by one or both of these pathways, but that dialysis with patch pipettes reduces the intracellular concentrations of diffusible intermediates required for phosphorylation. Indeed, VSRM contractions are inhibited in voltage-clamp experiments conducted with patch pipettes in the whole cell configuration (11, 14). However, we have demonstrated that contractions (11, 14) and Ca\(^{2+}\) transients (10, 13) with activation and inactivation characteristics of the VSRM can be elicited with patch pipettes when 50 μM cAMP is added to the pipette solutions. Furthermore, the ability of cAMP to restore availability of the VSRM is abolished by H-89 (11), a

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specific inhibitor of PKA (4). These observations suggest that phosphorylation by PKA can increase the availability of the VSRM for activation.

It is not known whether CamK also may modulate availability of the VSRM, or whether phosphorylation by either or both pathways regulates activation of the VSRM in intact, undialyzed cardiac myocytes. Therefore, the objectives of the present study were to determine the following: 1) whether phosphorylation by CamK facilitates activation of the VSRM; 2) if voltage clamp with patch pipettes disrupts phosphorylation of the VSRM by CamK; and 3) whether the PKA and/or CamK phosphorylation paths are essential for activation of the VSRM in undialyzed ventricular myocytes.

MATERIALS AND METHODS

Cell isolation. Studies were conducted within the guidelines published by the Canadian Council on Animal Care and approved by the Dalhousie University Committee on Animal Care. Methods for cell isolation have been published previously (9).

Male (~90%) and female Charles River guinea pigs (250–350 g) were anesthetized with pentobarbital sodium (80 mg/kg ip). Hearts were removed and perfused retrogradely through the aorta (10–12 ml/min) at 37°C with use of oxygenated Ca2+-free solution of one of two compositions (in mM): solution 1) 120 NaCl, 3.8 KCl, 1.2 KH2PO4, 1.2 MgSO4, 10 HEPES, 11 glucose, pH 7.4 with NaOH, bubbled with 100% O2, or solution 2) 120 NaCl, 4 KCl, 4 NaH2PO4, 22 NaHCO3, 5.5 glucose, 1 MgSO4 (bubbled with 95% O2-5% CO2). Myocytes were disaggregated enzymatically with BMC collagenase A (Boehringer Mannheim) and protease (type 14, Sigma, St. Louis, MO) in combination with solution 1, whereas collagenase 1A (Sigma) and protease type 14 were used with solution 2. After 5–12 min of exposure to enzymes, the ventricles were minced and stored in a high K+ solution of the following composition (in mM): 80 KOH, 50 glutamic acid, 30 KCl, 30 KH2PO4, 20 taurine, 10 HEPS, 10 glucose, 3 MgSO4, 0.5 EGTA (pH 7.4 with KOH). For experimentation, isolated myocytes were transferred to an experimental chamber on an inverted microscope and superfused at 3 ml/min with oxygenated (100% O2) heated (37°C) solution containing (in mM) 45 NaCl, 100 choline Cl, 2 CaCl2, 4 KCl, 1 MgCl2, 10 glucose, 10 HEPS (pH 7.4 with NaOH), and 250 μM lidocaine or 250 μM lidocaine + 50 μM TTX to block Na+ current.

Experimental methods. Continuous whole cell voltage-clamp recordings were made with 1–3 MΩ fire-polished patch pipettes (A-M Systems, Everett, WA) coupled to an Axopatch 200A amplifier with CV202A head stage (Axon Instruments, Foster City, CA). Patch pipettes contained (in mM) 70 KCl, 70 K aspartate, 4 MgATP, 1 MgCl2, 2.5 KH2PO4, 0.12 CaCl2, 0.5 EGTA, 10 HEPS, pH 7.2 with KOH, with or without 2–5 μM bovine brain calmodulin, or 50 μM 8-bromo-cAMP. Na+ was omitted from the pipette solution to inhibit Na/CaEX. Liquid junction potentials were compensated before data acquisition. In other experiments, recordings were made with high-resistance microelectrodes (16–25 MΩ, filled with 2.7 M KCl) and switch clamp (sample rate 7–12 kHz) with an Axoclamp 2A voltage-clamp amplifier (Axon Instruments). pCLAMP 6 software (Axon Instruments) was used for data acquisition and measurement. Unloaded cell shortening was measured with a video edge detector (120 Hz sampling, Crescent Electronics, Sandy, UT) (9). Current, voltage, and contractions were digitized with a Labmaster A/D interface at sample rates up to 50 kHz (TL1–125, Axon Instruments) and stored on a computer. Activation steps were preceded by 10 conditioning pulses from a holding potential of −80 to 0 mV, to maintain Ca2+ stores, followed by repolarization to a postconditioning potential (Vpc). In some experiments, a computer-triggered device was used to change solutions bathing a single cell within 300 ms at 37°C (11, 13, 22). This device permitted rapid application of drugs, after conditioning pulses but before activation steps, to minimize changes on SR loading.

In some experiments, cell fluorescence was measured using DeltaRAM system software (Photon Technology International, Monmouth Junction, NJ). Cells were loaded with 1.0 μM fura 2-acetoxymethyl ester (fura 2-AM) for 20 min at room temperature. After loading, extracellular dye was eliminated by superfusion of the cells with physiological buffer solution for 20 min. The emission field was limited to slightly smaller than the size of individual myocytes with an adjustable window. The cells were alternately excited at 340 and 380 nm and fluorescence emitted by the cell was recorded at 510 nm. Background fluorescence was not subtracted. Changes in intracellular Ca2+ were expressed as changes in the 340/380 ratio of fluorescence between the peak of fluorescence transients and the immediately preceding baselines. Excitation and fluorescence data acquisition were accomplished by using Felix software (Photon Technology International). Recorded voltage steps were used to align fluorescence and current recordings in data recordings from Felix and pCLAMP.

Sources of drugs and chemicals. Ryanodine, H-89, and KN-62 were purchased from Calbiochem (La Jolla, CA), pentobarbital sodium from MTC Pharmaceuticals (Cambridge, Ontario), fura-2AM and DMSO from Molecular Probes (Eugene, OR), and TTX from Alomone Labs (Jerusalem, Israel). All other chemicals were purchased from Sigma Chemical. All drugs were dissolved in deionized water. Kinase inhibitors H-89 and KN-62 were added to the pipette solution or to the superfusate as indicated. Fura 2-AM was dissolved in DMSO and diluted in physiological solution.

Data analyses. Ionic currents, voltage, and contraction were measured with pCLAMP 6 software. Peak inward currents were measured as the difference between maximum inward (downward) deflection of the current trace and a reference point at the end of the depolarizing step (usually 200 ms). Potassium currents were not blocked in the present study because previous reports have indicated that some potassium channel blocking agents (e.g., Cs+) alter excitation contraction-coupling substantially (17, 34). Amplitudes of contractions were measured as the difference between maximum cell shortening and a point immediately before the onset of cell shortening. For protocols in which two contractions were initiated by two sequential activation steps, the amplitude of the second contraction was measured with respect to a point immediately before onset of the second phasic contraction. Significance of differences between population means was tested with a Student’s t-test or one-way ANOVA with a Bonferroni correction for multiple comparisons. Differences between current-voltage or contraction-voltage relationships were analyzed with a two-way repeated measures analysis of variance. Post hoc comparisons were made with a Bonferroni test. Statistical analyses were performed by using Sigma Stat 1.02 (Jandel) or SAS 5.0 (SAS Institute) software. Measured data are presented as means ± SE. The number of replicates (n) is equal to the number of myocytes from which data were collected; no more than two replicates (myocytes) were collected from the same heart.
RESULTS

To investigate a possible role for CamK in activation of the VSRM, cell shortening, and transmembrane currents were recorded from isolated guinea pig ventricular myocytes at 37°C. We have previously shown that VSRM and CICR contractions can be activated separately with sequential test steps to −40 and 0 mV, respectively, in undialyzed myocytes or myocytes dialyzed with cAMP (9–11, 15, 22). The same protocol was used in the present study (Fig. 1A). When myocytes were dialyzed with patch pipettes containing a standard intracellular solution without cAMP, the activation step to −40 mV did not elicit a VSRM contraction (Fig. 1B). However, the step from −40 to 0 mV elicited a phasic contraction coupled to Ca2+ entry through I\textsubscript{Ca-L} (9–11, 15, 22).

CamK is activated by Ca\textsuperscript{2+} and by calmodulin. Under the conditions used in the experiments illustrated by Fig. 1B, Ca\textsuperscript{2+} is provided by I\textsubscript{Ca-L}. However, it is possible that dialysis decreases intracellular calmodulin concentration below levels needed for activation of the VSRM. Therefore, we tested the effects of adding calmodulin to the intracellular pipette solution. Figure 1C shows that addition of 2 \mu M calmodulin to the pipette solution resulted in initiation of a prominent VSRM contraction by the test step to −40 mV. To determine whether this effect of calmodulin was mediated through activation of CamK, similar experiments were conducted with pipette solution containing calmodulin and 5 \mu M KN-62, a specific blocker of CamK (32). Under these conditions, the step to −40 mV no longer activated the VSRM (Fig. 1D), however, the step to 0 mV still elicited I\textsubscript{Ca-L} and a phasic contraction.

Figure 2A shows mean data for amplitudes of contractions and currents in experiments with and without calmodulin added to the pipette solutions. VSRM contractions were virtually absent with the step to −40 mV in myocytes without calmodulin in the pipette, but increased significantly when calmodulin was present. Furthermore, KN-62 dramatically reduced the mean amplitudes of VSRM contractions to levels similar to those in the absence of calmodulin. Figure 2A also shows that the effects of KN-62 were specific. KN-62 did not prevent activation of the VSRM by cAMP, and conversely H-89, a specific inhibitor of PKA (4), did not prevent activation of the VSRM with calmodulin. Minimal inward current was observed with the step to −40 mV with all combinations of kinase activators and inhibitors (Fig. 2B). In contrast to VSRM contractions, contractions initiated by I\textsubscript{Ca-L} were present in the absence of calmodulin in the pipette solution (Fig. 2A). Addition of calmodulin to the pipette solution caused a modest but significant increase in the mean amplitude of I\textsubscript{Ca-L}-induced contractions elicited by the step to 0 mV. This effect was reversed by KN-62 but not H-89. Effects of calmodulin with and without KN-62 or H-89 on the amplitude of I\textsubscript{Ca-L} initiated by the step to 0 mV approximately paralleled effects on the corresponding contraction initiated by this step (Fig. 2B).

**Fig. 1.** Representative recordings showing effects of calmodulin (CaM) and of an inhibitor of Ca\textsuperscript{2+} calmodulin-dependent protein kinase (CamK) on activation of the voltage-sensitive release mechanism (VSRM) contractions in ventricular myocytes. A: Voltage-clamp protocol. Test steps were preceded by ten 200-ms conditioning pulses to 0 mV from a holding potential of −80, at 2 Hz. After repolarization to a postconditioning potential (\textit{V}\textsubscript{rc}) of −65 mV, sequential test steps to −40 and 0 mV were used to initiate VSRM and Ca\textsuperscript{2+}-induced CICR contractions, respectively. Na+ current was blocked with 250 \mu M lidocaine. B–D: representative records of currents (top) and contractions (bottom) from different cells and traces recorded during the test steps only. In the absence of CaM or cAMP in the pipette, the step to −40 mV did not elicit a VSRM contraction, but the step to 0 mV initiated a contraction coupled to \textit{L}-type Ca\textsuperscript{2+} current (I\textsubscript{Ca-L}) (B). When 2 \mu M CaM was included in the pipette solution, a large VSRM contraction was initiated by the step to −40 mV (C). Inclusion of 5 \mu M KN-62, a specific blocker of CamK, and CaM in the pipette prevented activation of the VSRM contraction, but did not abolish the contraction and current initiated by the step to 0 mV (D).
used as a measure of SR Ca$^{2+}$ load (2, 26). Figure 3B (top) shows a representative recording of a caffeine contracture induced in a myocyte dialyzed without calmodulin in the pipette. Figure 3B (bottom) shows that caffeine induced a contracture with a similar magnitude in a myocyte studied with a patch pipette containing 2 μM calmodulin. Figure 3C illustrates mean data for peak amplitudes of caffeine contractures and for amplitudes of VSRM and CICR contractions measured in the same myocytes. Again, VSRM contractions were virtually absent without calmodulin but were present with calmodulin. However, there was no significant difference in the mean amplitudes of caffeine contractures elicited in myocytes dialyzed with and without calmodulin. These data indicate that addition of calmodulin to the patch pipette did not affect SR Ca$^{2+}$ significantly. Figure 3D shows that addition of calmodulin to the patch pipette also had no effect on inward currents initiated by the test steps in these experiments.

To confirm that contractions elicited by steps to −40 mV in the presence of calmodulin represent facilitation of Ca$^{2+}$ release rather than an increase in myofilament Ca$^{2+}$ sensitivity, we measured Ca$^{2+}$ transients in cells loaded with fura 2. Ca$^{2+}$ transients initiated by sequential steps to −40 and 0 mV were measured in cells with or without 5 μM calmodulin in the patch pipettes (Fig. 4). Figure 4A shows representative recordings of currents and transients in the absence of calmodulin. The step to −40 mV initiated little change in fluorescence ratio; however, the step to 0 mV was accompanied by a rapidly rising Ca$^{2+}$ transient. In contrast, in experiments in which calmodulin was added to the pipette solution, both steps initiated Ca$^{2+}$ transients (Fig. 4B). Mean data for Ca$^{2+}$ transients and currents are presented in Fig. 4C. In the absence of calmodulin, Ca$^{2+}$ transients were essentially absent with steps to −40 mV but were present with the step to 0 mV. However, in the presence of calmodulin, steps to −40 and 0 mV initiated transients of similar magnitudes. Calmodulin had no effect on inward currents.

If Ca$^{2+}$ transients and contractions that appeared when calmodulin was included in the pipette solution were caused by the VSRM, they should exhibit characteristics described for VSRM contractions in undialyzed myocytes. The voltage dependence of CICR is bell-shaped, whereas the voltage dependence of the VSRM is sigmoidal. Therefore, we compared the voltage dependence of Ca$^{2+}$ transients in the presence and absence of calmodulin, by applying test steps to different membrane potentials after a train of 10 conditioning potentials (Fig. 5, top). Figure 5A shows representative recordings of inward currents and Ca$^{2+}$ transients initiated by three different voltage steps in the absence of calmodulin. A step to −40 mV initiated little if any inward current or Ca$^{2+}$ transient. However, a step to 0 mV initiated inward Ca$^{2+}$ current, which was accompanied by a rapidly rising Ca$^{2+}$ transient. In the absence of calmodulin, both the inward current and the amplitude of the Ca$^{2+}$ transient decreased markedly when the test step was increased to

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**Fig. 2.** Mean data demonstrating that CaM facilitates initiation of VSRM contractions by an action involving CamK. A: mean amplitudes of contractions initiated by voltage steps to −40 and 0 mV. VSRM contractions were virtually absent without CaM (Control, n = 32) but were present with 2 μM CaM in the pipette (n = 22). With CaM, VSRM contractions were abolished by KN-62 (n = 12) but not H-89 (n = 30). However, KN-62 did not prevent activation of VSRM contractions with 50 μM cAMP (n = 15). Contractions coupled to ICa,L (step to 0 mV) occurred in the absence of CaM but increased in amplitude with CaM in the pipette. Effects of CaM on ICa,L contractions were prevented by KN-62 but not H-89. B: mean amplitudes of inward currents initiated by voltage steps to −40 mV were small and did not change significantly with the pipette solutions tested. Magnitudes of ICa,L paralleled those of the corresponding contractions in A. **P < 0.01 with respect to 0 μM CaM; ††P < 0.01, with respect to 2 μM CaM.

To determine whether calmodulin facilitated the VSRM by increasing SR Ca$^{2+}$, we assessed SR Ca$^{2+}$ content in control and calmodulin-treated myocytes with caffeine. First, the amplitudes of VSRM and CICR contractions were assessed with the same voltage-clamp protocol shown in Fig. 1. Then the protocol was repeated, except that a single rapid application of 10 mM caffeine was substituted for the activation steps to −40 and 0 mV (Fig. 3A). The peak magnitudes of contractures induced by application of caffeine were

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+60 mV. When 3 μM calmodulin was included in the patch pipette, similar changes in current magnitude were seen with steps to the same potentials (Fig. 5B). However, in the presence of calmodulin the Ca2+ transients no longer followed the voltage dependence of Ca2+ current. A large transient was observed with the test step to ~40 mV and 0 mV and was still present with the step to +60 mV (Fig. 5B). Mean data showing the voltage dependence of transients are shown in Fig. 5C. In the absence of calmodulin, Ca2+ transients...
showed a bell-shaped voltage dependence typical of CICR. Inclusion of calmodulin in the pipette solution caused an increase in the amplitude of the Ca^{2+} transients, and the voltage dependence became clearly sigmoidal. Calmodulin had no effect on the amplitudes or bell-shaped voltage dependence of inward Ca^{2+} current (Fig. 5D).

The VSRM also can be differentiated from other mechanisms of excitation-contraction coupling by its pharmacological characteristics. Therefore, we determined the effects of Cd^{2+}, TTX, and tetracaine on contractions initiated by sequential steps to −40 and 0 mV. Figure 6A shows contractions and currents elicited in a myocyte voltage clamped with a patch pipette containing 2 μM calmodulin. Lidocaine was present throughout the experiment. Figure 6B shows currents and contractions recorded after a rapid switch to extracellular solution containing 100 μM of Cd^{2+} + 50 μM of TTX, 3 s before the test steps. The phasic contraction initiated by the step to −40 mV remained. However, the inward current and contraction triggered by the step to 0 mV were virtually abolished. Switches to Cd^{2+} and TTX also inhibited L-current and contraction with the step to 0 mV when the step from −40 to 0 mV was omitted to control for any effects of sequence of activation (not illustrated). Figure 6C presents mean data demonstrating that exposure to Cd^{2+} in the presence of TTX strongly inhibited I_{Ca,L} and contractions.
initiated by the step to 0 mV, but Cd\textsuperscript{2+} did not significantly affect the mean amplitude of VSRM contractions or the small inward current initiated by the step to -40 mV. Thus VSRM contractions elicited in the presence of calmodulin were independent of CICR coupled to I\textsubscript{Ca-L} or Na\textsuperscript{+} current. It is highly unlikely that the VSRM contraction was triggered by Na/Ca EX, because the pipette contained 0 mM Na to inhibit reverse Na/Ca EX.

The VSRM is selectively inhibited by 200 μM tetracaine in undialyzed myocytes (22). Figure 7, A and B, shows contractions and currents elicited in a representative experiment with a patch pipette containing calmodulin. Tetracaine (200 μM) and TTX (50 μM) were applied with the rapid switching device 3 s before and during the test steps. Tetracaine abolished the VSRM contraction elicited by the step to -40 mV, but had little or no effect on the contraction and current initiated by the step to 0 mV. Figure 7C shows mean data indicating that tetracaine significantly inhibited VSRM contractions by ~75% but did not affect I\textsubscript{Ca-L} or contractions accompanying activation of I\textsubscript{Ca-L}. Thus VSRM and I\textsubscript{Ca-L} contractions in myocytes dialyzed with calmodulin exhibited the same differential blockade with tetracaine and Cd\textsuperscript{2+}, respectively, as documented previously in undialyzed myocytes (21).

Phasic VSRM contractions also can be identified by their steady-state inactivation characteristics (10, 11, 15). We determined the voltage dependence of inactivation for contractions supported by calmodulin with a voltage protocol in which a step to -40 mV, to activate the VSRM, was preceded by conditioning steps to different potentials (V\textsubscript{PC}) (Fig. 8A). A switch to 100 μM Cd\textsuperscript{2+} and 50 μM TTX was made 3 s before each test step to -40 mV. Representative recordings of currents and contractions preceded by different V\textsubscript{PC} are shown in Fig. 8B. Inward current was absent for all test steps. Contraction was absent when the V\textsubscript{PC} was -30 mV, but it appeared and became larger with more negative V\textsubscript{PC}. Figure 8, C and D, respectively, shows mean contractions, normalized to maximum contraction, as a function of V\textsubscript{PC} in the presence of calmodulin and in the presence of calmodulin and H-89, to eliminate any role for PKA. The lines are Boltzmann functions fitted to the data. VSRM contractions were completely unavailable at V\textsubscript{PC} more positive than -40 mV and fully available near -70 mV. In the presence of calmodulin alone, half-inactivation voltage (V\textsubscript{1/2}) was -55.9 mV and k was 3.9 mV. The relationship determined in the presence of 5 μM H-89 was identical (V\textsubscript{1/2} = 56.2 mV, k = 4.3 mV). These steady-state inactivation parameters are similar to those for VSRM contractions in undialyzed myocytes (15) and myocytes dialyzed with cAMP (11).

The VSRM is readily available for activation in myocytes voltage clamped with high-resistance microelectrodes, without addition of exogenous agents to activate the adenyl cyclase-PKA or CamK phosphorylation pathways (9, 14, 15, 22). To determine whether phosphorylation via one or both of these pathways also

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Fig. 6. Differential blockade with Cd\textsuperscript{2+} separates I\textsubscript{Ca-L} contractions from VSRM contractions supported by CaM. A: currents and contractions elicited by sequential test steps to -40 and 0 mV were recorded with patch pipettes containing CaM and extracellular solution containing lidocaine. B: currents and contractions recorded with 100 μM Cd\textsuperscript{2+} + 50 μM TTX applied 3 s before and during test steps. Drug application inhibited I\textsubscript{Ca-L} and abolished contraction coupled to I\textsubscript{Ca-L} but did not inhibit the VSRM contraction. C: mean data from 26 cells. Application of Cd\textsuperscript{2+} and TTX did not significantly affect the amplitudes of VSRM contractions or currents initiated by the step to -40 mV but abolished I\textsubscript{Ca-L} current and contractions initiated by the test step to 0 mV. **P < 0.01.
is essential for the availability of the VSRM in undia-
lyzed myocytes, we examined the effects of adding H-89
and KN-62 in experiments with high-resistance micro-
electrodes to minimize intracellular dialysis. The top
panels in Fig. 9 show recordings of currents and con-
tractions elicited by sequential activation steps to
\(-40\) and 0 mV before addition of kinase inhibitors. In all
three examples, the step to \(-40\) elicited a phasic VSRM
contraction, and the step to 0 mV activated a contrac-
tion coupled to \(I_{\text{ca,L}}\). The bottom panels of Fig. 9,
A and B, respectively, show that exposure of myocytes either
to H-89 or KN-62 individually, reduced the amplitudes
but did not prevent activation of VSRM contractions.

Fig. 7. VSRM contractions supported by CaM are selectively inhibited by tetracaine. A: currents and contrac-
tions elicited by sequential test steps to \(-40\) and 0 mV were recorded with patch pipettes containing CaM and
extracellular solution containing lidocaine. B: currents and contractions recorded with 200 \(\mu\)M tetracaine + 50 \(\mu\)M
TTX applied 3 s before and during test steps. Tetracaine abolished the VSRM contraction with no effect on \(I_{\text{ca,L}}\)
or contraction coupled to \(I_{\text{ca,L}}\). C: mean data for 12 experiments. Tetracaine significantly inhibited VSRM
contractions with no significant effect on \(I_{\text{ca,L}}\) contractions or currents. **P < 0.01.

**DISCUSSION**

Our observations suggest that activation of the
VSRM is facilitated by the CamK and adenylyl cyclase-
PKA phosphorylation pathways in cardiac ventricular
myocytes. Both paths appear to contribute signifi-
cantly to the availability of the VSRM in undialyzed
myocytes, because inhibition of either path signifi-
cantly reduced the amplitudes of VSRM contractions,
and simultaneous inhibition of both pathways essen-
tially abolished VSRM contractions. Also, activation of
the VSRM was inhibited when myocytes were voltage
clamped with patch pipettes, but addition of either
calmodulin or cAMP to the intracellular solution re-
stored activation. These observations suggest that
intracellular dialysis with patch pipettes must disrupt
both pathways sufficiently to prevent activation of the
VSRM. Clearly, in studies conducted with patch pi-
pettes, it is important to consider that intracellular
dialysis can disrupt regulatory pathways, which in-
volve diffusible second messengers.
Effects of H-89 and KN-62 were very specific. In experiments with patch pipettes, H-89 did not inhibit calmodulin-supported contractions, and KN-62 did not prevent activation of contraction supported by cAMP. Thus in undialyzed myocytes, it is unlikely that the enhanced effect of simultaneous exposure to H-89 and KN-62 can be attributed to a simple increase in total inhibitor concentration. Therefore, the observation that each inhibitor by itself inhibited activation of the VSRM by about half indicates it is likely that both phosphorylation pathways regulate activation of the VSRM in undialyzed cells. H-89 and KN-62 in combination almost completely prevented activation of the VSRM in undialyzed myocytes. This suggests it is unlikely that phosphorylation pathways other than adenylyl cyclase-PKA and CamK contribute significantly to the availability of the VSRM under basal conditions in intact myocytes.

Fig. 8. CaM-supported contractions exhibit steady-state inactivation with characteristics of the VSRM. A: availability of the VSRM was assessed with test steps to −40 mV preceded by different V_{pc}. Cd^{2+} (100 μM) and TTX (50 μM) were applied 3 s in advance and during all test steps. B: representative recordings show absence of inward currents (top) in response to the test steps, with any preceding V_{pc}. However, contractions (bottom) increased in amplitude as V_{pc} were made more negative. C: mean data normalized to maximum contraction and fitted to Boltzmann functions, for myocytes dialyzed with CaM (n = 6). D: an identical steady-state inactivation curve was obtained from cells dialyzed with CaM + H-89 (n = 4).
tials. In addition, experiments with fura 2 show that Ca^{2+} release was modified over a wide range of membrane potentials. In the absence of calmodulin in the patch pipette solution, Ca^{2+} transients followed a bell-shaped voltage dependence, typical of CICR coupled to L-type Ca^{2+} current. However, inclusion of calmodulin in the pipette solution caused the voltage dependence to become clearly sigmoidal, as predicted when the VSRM is activated (9–11, 14, 15, 22). Calmodulin also caused a marked increase in the magnitudes of Ca^{2+} transients initiated at all test potentials. Thus the VSRM contributed substantially to initiation of SR Ca^{2+} release over its entire activation range.

Our experiments also demonstrate that the component of excitation-contraction coupling facilitated by calmodulin is most likely the same mechanism identified as the VSRM in undialyzed myocytes and cells dialyzed with cAMP, because it shares the same electrophysiological and pharmacological characteristics (9–11, 14, 15, 22). Calmodulin-supported VSRM contractions persisted when contractions triggered by Ca^{2+} influx via \( I_{Ca-L} \) were inhibited by Ca^{2+} channel blockade, but were selectively abolished by tetracaine, which inhibits the VSRM. The phasic VSRM contractions supported by calmodulin also showed steady-state inactivation relations virtually identical to those

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**Fig. 9.** Effects of inhibitors of PKA and CamK on contractions and currents in undialyzed myocytes. A-C show representative recordings of currents and contractions from cells voltage clamped with 18–24 MΩ microelectrodes, to minimize intracellular dialysis. In each panel, the top pair of traces were recorded under control conditions, and the bottom pair were recorded after 10 min exposure to H-89 (A), KN-62 (B), or both H-89 and KN-62 (C) added to the extracellular solution. Each kinase inhibitor by itself partially reduced the amplitude of VSRM contractions initiated by the step to −40 mV. When both inhibitors were applied simultaneously, VSRM contractions were virtually abolished.

**Fig. 10.** Mean data showing that activation of the VSRM is influenced by both PKA and CamK in undialyzed myocytes. In each panel, mean amplitudes of VSRM and \( I_{Ca-L} \) contractions elicited by steps to −40 and 0 mV, respectively, are shown, top. Mean amplitudes of inward current elicited by the same voltage steps are shown at bottom. Data collected before exposure to kinase inhibitor are designated as control in each panel. H-89 (\( n = 11 \)) (A) and KN-62 (\( n = 11 \)) (B) only partially inhibited VSRM contractions when used individually. H-89 and KN-62 in combination (\( n = 8 \)) (C) virtually abolished VSRM contractions, but only reduced \( I_{Ca-L} \) contractions to one-third of control amplitude. Both inhibitors partially inhibited \( I_{Ca-L} \) but had no significant effect on currents on the step to −40 mV. *\( P < 0.05; **P < 0.01.\)
described for VSRM contractions in undialyzed guinea pig or rat ventricular myocytes (10, 15) and for myocytes dialyzed with cAMP (11). In all cases, the inactivation parameters were clearly different from those of L-type Ca\(^{2+}\) channels (15) and clearly different from Na/Ca\(_{\text{EX}}\), which is not subject to steady-state inactivation nor inhibited by tetracaine (22, 27).

The effects of adding calmodulin to the patch pipette were different from those of cAMP. Calmodulin facilitated initiation of Ca\(^{2+}\) release and contraction without affecting the magnitude of \(I_{\text{Ca-L}}\) or the amplitudes of caffeine contractures. In contrast, the inclusion of 8-bromo-cAMP in patch pipettes increased the peak amplitude of \(I_{\text{Ca-L}}\) current (11). Furthermore, cAMP is known to stimulate SR Ca\(^{2+}\) uptake (2). These additional actions of cAMP have led to the suggestion that cAMP might result in a “hair trigger” for CICR by causing Ca\(^{2+}\) overload (28, 35). Under these conditions, a very small \(I_{\text{Ca-L}}\) at negative or positive potentials might initiate a large Ca\(^{2+}\) release and be mistaken as the VSRM (28, 35). However, this is not a tenable explanation, because CICR continued to be graded by the magnitude \(I_{\text{Ca-L}}\) in the presence of cAMP, and currents of similar magnitudes with and without cAMP in the pipette, induced contractions of similar amplitudes (11). In addition, the present study with calmodulin provides direct evidence that activation of the VSRM can occur without elevation of SR Ca\(^{2+}\) load or stimulation of \(I_{\text{Ca-L}}\).

Phosphorylation of the VSRM by two separate pathways suggests that the contribution of the VSRM to cardiac contraction is highly regulated. The adenylyl cyclase-PKA and CamK pathways represent components of two different regulatory systems. The two regulatory pathways may not function in complete independence because cross talk occurs between the two, and regulatory agents may affect the two paths oppositely. For example, elevation of intracellular Ca\(^{2+}\) stimulates CamK but inhibits most adenylyl cyclase isozymes which are active in cardiac tissues (30).

It is possible that changes in SR Ca\(^{2+}\) content, as well as Ca\(^{2+}\) release, might contribute to inhibition of VSRM contractions by when phosphorylation pathways are disrupted (12, 20, 24, 31). However, CICR coupled to \(I_{\text{Ca-L}}\) persisted at a basal level in the same myocytes in which intracellular dialysis without cAMP or calmodulin, or exposure to kinase inhibitors, abolished VSRM contractions. This observation is important because it indicates that SR stores of Ca\(^{2+}\) were still sufficient to allow CICR to initiate contraction. Furthermore, caffeine contractures were not significantly affected by dialysis with or without calmodulin. These observations indicate that the abolition of VSRM contractions cannot be explained by depletion of SR Ca\(^{2+}\) and must largely reflect the effects of phosphorylation on SR Ca\(^{2+}\) release.

The VSRM is remarkable in that either the CaM kinase or adenylyl cyclase and/or PKA phosphorylation pathways must be available in order for the VSRM to be activated significantly. In contrast, other mechanisms that are modulated by phosphorylation, such as \(I_{\text{Ca-L}}\) and CICR coupled to \(I_{\text{Ca-L}}\), exhibit a basal level of activity after phosphorylation is inhibited. This raises the possibility that phosphorylation of the VSRM may not be simply regulatory in nature but actually might be an essential step linking depolarization to SR Ca\(^{2+}\) release. At least, these observations suggest fundamental differences in regulation of the VSRM and CICR. Additional investigation will be required to determine how regulation of these two mechanisms contributes to modulation of cardiac contraction.

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