Contribution of a voltage-sensitive calcium release mechanism to contraction in cardiac ventricular myocytes

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Howlett, Susan E., Jie-Quan Zhu, and Gregory R. Ferrier. Contribution of a voltage-sensitive calcium release mechanism to contraction in cardiac ventricular myocytes. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H155–H170, 1998.—The contribution of a voltage-sensitive release mechanism (VSRM) for sarcoplasmic reticulum (SR) Ca$^{2+}$ to contraction was investigated in voltage-damped ventricular myocytes at 37°C. Na$^+$ current was blocked with lidocaine. The VSRM exhibited steady-state inactivation (half-inactivation voltage: −47.6 mV; slope factor: 4.37 mV). When the VSRM was inactivated, contraction-voltage relationships were proportional to L-type Ca$^{2+}$ current ($I_{Ca,L}$). When the VSRM was available, the relationship was sigmoidal, with contractions independent of voltage positive to −20 mV. VSRM and $I_{Ca,L}$ contractions could be separated by activation-inactivation properties. VSRM contractions were extremely sensitive to ryanodine, thapsigargin, and conditioning protocols to reduce SR Ca$^{2+}$ load. $I_{Ca,L}$ contractions were less sensitive. When both VSRM and $I_{Ca,L}$ were available, sigmoidal contraction-voltage relationships became bell-shaped with protocols to reduce SR Ca$^{2+}$ load. Myocytes demonstrated restitution of contraction that was slower than restitution of $I_{Ca,L}$. Restitution was a property of the VSRM. Thus activation and recovery of the VSRM are important in coupling cardiac contraction to membrane potential; SR Ca$^{2+}$ load, and activation interval.

excitation-contraction coupling; calcium current; cardiac muscle; sarcoplasmic reticulum; ryanodine

IT IS WELL ESTABLISHED that contraction in heart is initiated by an increase in free intracellular Ca$^{2+}$. This increase in activator Ca$^{2+}$ is derived from two sources, Ca$^{2+}$ influx and Ca$^{2+}$ release from intracellular stores. It is generally believed that the main routes for Ca$^{2+}$ entry are voltage-gated Ca$^{2+}$ channels and reverse-mode Na$^+$/Ca$^{2+}$ exchange, whereas the main source for Ca$^{2+}$ release is the sarcoplasmic reticulum (SR) (3, 15, 19). Fabiato (11) has shown that a rapid rise in intracellular free Ca$^{2+}$ can cause Ca$^{2+}$ release from cardiac SR. This phenomenon has been called Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) (11).

Identification of the trigger or triggers for SR Ca$^{2+}$ release continues to be a subject of intense research. Evidence has been presented for at least three mechanisms that trigger CICR. These are 1) Ca$^{2+}$ influx via L-type Ca$^{2+}$ channels (4, 7, 22), 2) elevation of intracellular Ca$^{2+}$ via reverse Na$^+$/Ca$^{2+}$ exchange in response to a rapid rise in intracellular Na$^+$ during the upstroke of the action potential (15, 19), and 3) elevation of intracellular Ca$^{2+}$ via reverse Na$^+$/Ca$^{2+}$ exchange in response to depolarization, although this last mechanism may depend on intracellular Na$^+$ concentration and temperature (6, 16, 17, 29, 30).

Recently we reported a study of excitation-contraction (EC) coupling in guinea pig ventricular myocytes at 37°C that used high-resistance microelectrodes to prevent intracellular dialysis (13). Under these conditions, the threshold for activation of contractions is much more negative than the threshold for activation of L-type Ca$^{2+}$ current ($I_{Ca,L}$). In addition, the magnitude of contraction is not proportional to the magnitude of inward current, and contractions remain maximal at membrane potentials near or beyond the reversal potential for $I_{Ca,L}$ (13). In that study we were able to separate a new component of contraction that was abolished by a low concentration of ryanodine, but not by L-type Ca$^{2+}$ channel blockers or Na$^+$ channel blockers, from contraction initiated by $I_{Ca,L}$ (13). The new component of contraction exhibits a sigmoidal contraction-voltage relationship in contrast to the bell-shaped relationship observed for contractions initiated by $I_{Ca,L}$. It is also unlikely that this component of contraction is initiated by reverse Na$^+$/Ca$^{2+}$ exchange, because its voltage dependence is not affected by large changes in concentrations of extracellular Na$^+$ or Ca$^{2+}$ (12, 32). Because activation of this new component of contraction clearly is dependent on membrane potential but is not proportional to macroscopic transmembrane current, we have called this component a voltage-sensitive release mechanism (VSRM) for SR Ca$^{2+}$ (13). The present study examines the contribution of this voltage-activated Ca$^{2+}$ release mechanism to cardiac EC coupling.

Many previous studies have reported bell-shaped contraction-voltage relationships in which the amplitudes of contractions or Ca$^{2+}$ transients were proportional to the magnitude of $I_{Ca,L}$ (2, 3, 4, 9, 10, 20). However, many of these earlier studies of cardiac EC coupling in isolated myocytes have been conducted with conditions different from those in our study, including use of holding potentials near −40 mV (2, 3, 9, 10, 20). The absence of the VSRM in those studies might be explained if the VSRM has steady-state inactivation properties and is inactivated at −40 mV. Therefore, one of the goals of this study was to determine whether the VSRM exhibits steady-state inactivation and to determine the voltage range over which inactivation occurs.

Contractions initiated by the VSRM are abolished by 30 nM ryanodine, an agent that disrupts EC coupling at the level of SR release of Ca$^{2+}$ (13). Thus SR Ca$^{2+}$ stores are likely essential for initiation of contraction by the VSRM. Repetitive activation of $I_{Ca,L}$ serves to load or maintain SR stores of Ca$^{2+}$ (3), and altering the voltage of repetitive depolarizations can be used to manipulate SR Ca$^{2+}$ load and the amount of releasable Ca$^{2+}$ (14). Therefore, a second goal of the present study...
It was to determine and compare the effects of protocols designed to alter SR Ca\(^{2+}\) load on the components of contraction initiated by the VSRM or by \(I_{\text{Ca,L}}\). SR Ca\(^{2+}\) loading and recovery of SR Ca\(^{2+}\) release also are believed to be important components in restitution of contractility, which plays a major role in adjusting the magnitude of contraction in response to changes in the interval between contractions (3, 5). Therefore, the final goal of this study was to evaluate the role of the VSRM in restitution.

**METHODS**

Cell isolation. All experiments were performed in accordance with the guidelines published by the Canadian Council on Animal Care, and this investigation was approved by the Dalhousie University Committee on Animal Care. Male guinea pigs (350–400 g, Charles River) were injected with heparin (3.3 IU/g) 30 min before anesthesia with pentobarbital sodium (80 mg/kg). The chest was opened, and the heart was rapidly cannulated in situ and immediately perfused retrogradely through the aorta (10–12 ml/min) with oxygenated (100% \(O_2\), 36°C) Ca\(^{2+}\)-free solution of the following composition (in mM): 120 NaCl, 3.8 KCl, 1.2 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 10 N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES), and 11 glucose (pH 7.4 with NaOH). The heart was then removed from the chest during perfusion with Ca\(^{2+}\)-free solution for 7–8 min. Collagenase A (35–40 mg, Boehringer Mannheim) and protease (4.8–6 mg, Sigma type XIV; Sigma, St. Louis, MO) were then included in 65 ml of this Ca\(^{2+}\)-free solution, and the heart was perfused for an additional 5–8 min. After enzymatic dissociation, the ventricles were minced and washed in a substrate-enriched solution of the following composition (in mM): 80 KOH, 50 glutamic acid, 30 KCl, 30 KH\(_2\)PO\(_4\), 20 taurine, 10 HEPES, 10 glucose, 3 MgSO\(_4\), and 0.5 ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid (pH 7.4 with KOH). In some experiments, rat ventricular myocytes were utilized. The isolation procedure was similar to that for guinea pig myocytes except that dissociation was accomplished with collagenase (20 mg, Worthington type 2) and trypsin (2 mg, Sigma). After 1–2 h of incubation at room temperature, myocytes were placed in a modified culture dish (approximate volume = 0.75 ml) in an open-perfusion microincubator (model PDMI-2, Medical Systems) on the stage of an inverted microscope.

**Cell images** were monitored with a closed-circuit television camera with interlace defeat and partial scan capability (model 1-GP-CD60, Panasonic) and were displayed on a video monitor (model VM-1220C, Hitachi Densi). Unloaded cell shortening was sampled at 120 Hz with a video edge detector (Crescent Electronics, Sandy, UT) coupled to the television camera. Details of specific voltage-clamp protocols are provided in the appropriate sections in RESULTS. In most experiments, voltage-clamp protocols were repeated two to three times and the data were averaged. Current, voltage, and contractions were digitized with a Labmaster A/D interface at 125 kHz (TL1–125, Axon Instruments) and stored on hard disk for subsequent analysis.

Experimental methods. Discontinuous single-electrode voltage-clamp recordings (sample rate 10–14 kHz) were made with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA). Recordings were made with high-resistance microelectrodes (18–25 M\(\Omega\)), filled with 2.7 M KCl to reduce cell dialysis. A 2.7 M KCl-agar bridge was used as a bath ground to minimize liquid junction potential changes. Voltage-clamp protocols were generated with pCLAMP software (Axon Instruments); pCLAMP software also was used to acquire and analyze data on computer. Recordings were only made from rod-shaped myocytes with clear, well-organized striations and with resting potentials more negative than −85 mV. In all experiments, both current and transmembrane voltage were recorded. During discontinuous single-electrode voltage clamp, we continuously monitored the output of the switching circuit to ensure that adequate settling time for accurate voltage measurement was maintained.

Inward and delayed rectifier potassium currents were not blocked in this study because of the effects of blocking agents on the VSRM are not known. Therefore, changes in steady currents at the ends of the activation steps represent steady-state current-voltage (I-V) relations. After trains of conditioning pulses, the membrane potential was usually repolarized to a postconditioning potential (\(V_{\text{PC}}\)) more positive than the holding potential. Thus, background currents during the \(V_{\text{PC}}\) period also reflect steady-state I-V relations.

\(I_{\text{Ca,L}}\) was measured as the difference between the peak inward current and a reference point at the end of the voltage step (normally 250 ms). Figure 1 demonstrates that current measured in this way is abolished by 2 µM verapamil, an established L-type Ca\(^{2+}\)-channel blocker. The top trace in Fig. 1A shows the membrane potential response to a voltage-clamp step from −65 to −5 mV measured by the current-passing electrode. In a previous study, we demonstrated that the voltage measured by the current-passing electrode is an accurate measurement of the membrane potential by monitoring membrane potential with a second independent electrode (13). The next two traces show the corresponding current records before and after exposure of the cell to 2 µM verapamil. The inward current deflection following the capacitive transient was completely blocked by verapamil. The bottom trace is a difference trace derived by subtracting the current trace in the presence of verapamil from the control trace. Figure 1B shows I-V relationships derived from voltage steps to different potentials before and after exposure to verapamil. Figure 1C shows the I-V relationship derived from the difference currents. The results indicate that verapamil completely blocked the current identified as \(I_{\text{Ca,L}}\) and that this current was identical to the difference in current before and after drug treatment.

Data analyses. Ionic current, voltage, and contraction were measured with pCLAMP analysis software. Significance of differences between population means was tested with a Student's \(t\)-test with a Bonferroni correction for multiple comparisons. I-V relationships, contraction-voltage relationships, and time courses were analyzed with a two-way repeated-measures analysis of variance. Post hoc comparisons were made with a Bonferroni test. All statistical analyses were performed with SigmaStat (SAS Institute, version 1.02) or with SAS (SAS Institute). Nonlinear curve-fitting procedures
were conducted with SigmaPlot (Jandel, version 2.0). Data are presented as means ± SE. The value of \( n \) represents the number of myocytes sampled; no more than two replicates (myocytes) were collected from the same heart.

Sources of drugs and chemicals. Lidocaine was purchased from Sigma, and ryanodine was purchased from Calbiochem (San Diego, CA). All drugs were dissolved in distilled water, except nifedipine stock, which was prepared in ethanol. Choline chloride was purchased from Fisher Scientific (Fairlawn, NJ).

RESULTS

Effects of \( v_{PC} \) on contraction-voltage and I-V relations. Figure 2 (A and B) shows representative original recordings of membrane currents and contractions determined in the presence of 200 \( \mu \)M lidocaine to inhibit inward \( Na^+ \) current. A schematic of the voltage-clamp protocol is illustrated at the top of each panel. The complete voltage-clamp protocol was repeated every 7 s. Each test step was preceded by a train of 10 200-ms conditioning steps to 0 mV to activate \( I_{Ca-L} \) and maintain SR \( Ca^{2+} \) loading. With this conditioning protocol, signs of \( Ca^{2+} \) overload were not observed. In Fig. 2A, the test step was separated from the last conditioning step by a 300-ms \( v_{PC} \) of −40 mV. When the \( v_{PC} \) was −40 mV, a test step to −10 mV initiated both inward current and a large contraction, whereas a test step to +80 mV activated no inward current and only a very small contraction. Traces in Fig. 2B were recorded with a similar voltage-clamp protocol, except that the \( v_{PC} \) was −70 mV. Here a test step to −40 mV activated a small inward current and a large contraction, and a test step to −10 mV activated a larger inward current and contraction (Fig. 2B). However, in contrast to the results shown in Fig. 2A, a depolarizing step to +80 mV initiated a large contraction but did not activate inward current when the \( v_{PC} \) was −70 mV.

The effect of \( v_{PC} \) on contractions and inward currents is clearly seen when contraction-voltage and I-V relations are plotted as shown in Fig. 2, C and D, respectively. These curves represent mean values ± SE for 12 cells studied with a \( v_{PC} \) of −70 mV and 7 cells studied with a \( v_{PC} \) of −40 mV. When the \( v_{PC} \) was −40 mV, a bell-shaped contraction-voltage relation was observed (Fig. 2C). The magnitude of contractions increased to a peak at 0 mV and then declined at more positive potentials. These changes in the magnitudes of contractions were proportional to changes in \( Ca^{2+} \) current, as shown in Fig. 2D. Figure 2C also shows contraction-voltage relations determined by voltage steps from a \( v_{PC} \) of −70 mV. The threshold for activation of these contractions was about −60 mV, and contractions increased to a plateau at approximately −20 mV (Fig. 2C). Contractions remained large at very positive potentials, even though peak inward current decreased (Fig. 2D). We previously suggested that the additional component of contraction activated when the \( v_{PC} \) is more negative than −40 mV may represent activation of a VSRM (13).

Steady-state inactivation properties of the VSRM. Our observation that the VSRM component of contraction was inhibited when test steps were made from a \( v_{PC} \) of −40 mV suggests that the VSRM might show...
voltage-dependent inactivation. To determine whether the VSRM exhibits the property of steady-state voltage inactivation, we examined the effects of systematically changing the $V_{PC}$ on the magnitude of contractions activated by voltage steps to $-240$ mV. We have shown previously that contractions initiated by an activation step to $-240$ mV are entirely attributable to the VSRM (13). The schematic in Fig. 3A depicts the voltage-clamp protocol used in these studies. Each test step was preceded by 10 conditioning steps to 0 mV followed by repolarization to the $V_{PC}$ for 700 ms. The $V_{PC}$ was changed in 5-mV steps, from $-35$ to $-70$ mV, with each repetition of the voltage-clamp protocol. Each 700-ms $V_{PC}$ was followed by a very brief (10 ms) step to $-65$ mV. The complete voltage-clamp protocol was repeated every 9 s. Representative original records of contraction and current also are shown in Fig. 3B. When the $V_{PC}$ was $-35$ mV, no contraction was observed with the step to $-40$ mV. However, when the $V_{PC}$ was changed to $-45$ mV, a small contraction was elicited by the activation step. Successively larger contractions were observed with $V_{PC}$ values of $-55$ and $-65$ mV (Fig. 3B). The activation step elicited very little inward current with any of the $V_{PC}$ values tested. Figure 3C shows a plot of mean magnitudes of peak contraction ($\pm$SE) as a function of $V_{PC}$ for eight myocytes. These data were fitted with a Boltzmann equation of the following form: $y = (a - b)/(1 + \exp[(V_{PC} - v_{h})/k]) + b$, where $a$ is the maximum contraction, $b$ is the minimum contraction, $v_{h}$ is the half-inactivation voltage, and $k$ is the slope factor. Mean data also were normalized to the maximum contraction and plotted as a function of $V_{PC}$ in Fig.
3D. The line in Fig. 3D represents a Boltzmann function fitted to the normalized data using the following equation: \( y = \frac{1}{1 + \exp((v_{PC} - v_h)/k)} \). \( v_h \) and \( k \) were calculated for each myocyte (\( n = 8 \)). Mean \( v_h \) was \(-47.6 \pm 1.0 \) mV; mean \( k \) was \( 4.37 \pm 0.65 \) mV. These observations clearly show that the contractions initiated by the activation step to \(-40 \) mV exhibit the property of steady-state inactivation.

Separation of contractions induced by the VSRM and \( I_{Ca,L} \) by voltage. The observation that contractions initiated by the VSRM exhibit the property of steady-state inactivation suggests that the activation and inactivation properties of contraction can be used to separate contractions initiated by the VSRM from those initiated by \( I_{Ca,L} \). Indeed, we previously have shown that contractions initiated by the VSRM can be separated from contractions initiated by \( I_{Ca,L} \) by sequential steps to \(-40 \) and \( 0 \) mV (13). Figure 4 shows records in which the VSRM was activated by a 250-ms step to \(-40 \) mV from a \( v_{PC} \) of \(-65 \) mV, and \( I_{Ca,L} \) was activated by the second step to \( 0 \) mV. Figure 4A shows contractions and currents recorded under control conditions. The step to \(-40 \) mV activated a large contraction and little if any inward current. The second step to \( 0 \) mV activated an additional contraction and \( I_{Ca,L} \). Figure 4C shows the effects of exposing the same cell to 2.5 \( \mu \)M nifedipine. Nifedipine selectively blocked \( I_{Ca,L} \) and the corresponding contraction. The VSRM contraction was only slightly reduced in amplitude. Mean results for five myocytes exposed to nifedipine are presented in Fig. 5. Nifedipine significantly inhibited \( I_{Ca,L} \) and the contraction elicited by the step to \( 0 \) mV (Fig. 5, D and B, respectively) but had no significant effect on the current or contraction elicited by the step to \(-40 \) mV (Fig. 5, C and A, respectively). Similar results were observed in an additional five cells exposed to verapamil (2 \( \mu \)M) (not illustrated).

In contrast to \( Ca^{2+} \) channel blockers, ryanodine (30 nM) strongly inhibited the VSRM contraction, rather than the \( I_{Ca,L} \) contraction. Figure 4B shows control recordings of currents and contractions initiated by sequential steps to \(-40 \) and \( 0 \) mV. Fig. 4D shows that exposure of the same cell to ryanodine abolished the contraction initiated by the step to \(-40 \) mV but had little effect on current and contraction initiated by the step to \( 0 \) mV. Mean data for ryanodine also are presented in Fig. 5. Ryanodine virtually abolished contractions initiated by the VSRM (from \( 2.2 \pm 0.2 \) to \( 0.10 \pm 0.03 \), \( P < 0.001 \)). In contrast, contractions initiated by the second step to \( 0 \) mV were only moderately decreased by exposure to ryanodine (from \( 1.64 \pm 0.2 \) to \( 1.06 \pm 0.14 \), \( P < 0.05 \)). Treatment with ryanodine did not affect inward currents associated with steps to either \(-40 \) or \( 0 \) mV (Fig. 5, C and D).

These observations indicate that sequential steps to \(-40 \) and \( 0 \) mV separate two types of contractions with...
clearly different pharmacological sensitivities. These observations also suggest that VSRM contractions should be very sensitive to manipulations designed to alter SR Ca\textsuperscript{2+} load, whereas contractions initiated by I\textsubscript{Ca-L} should be much less sensitive to these manipulations.

Effects of conditioning pulses designed to alter SR load on contractions initiated by L-current and VSRM. To increase SR loading, we used trains of conditioning pulses to 0 mV to repetitively activate I\textsubscript{Ca-L}. Figure 6A shows representative recordings of voltages and contractions with conditioning pulses to 0 mV, which is close to the peak of the I-V relation for I\textsubscript{Ca-L}. After the first contraction, which is a rest contraction, a positive staircase was observed for the next nine conditioning pulses. In this example, the train of conditioning pulses was followed by repolarization to a v\textsubscript{PC} of -70 mV without a test step. To reduce SR load, trains of conditioning pulses to -40 mV were used to activate release of SR Ca\textsuperscript{2+} with minimal activation of I\textsubscript{Ca-L} (Fig. 6B). With this protocol, the rest contraction had the same magnitude as the corresponding rest contraction in Fig. 6A, but there was no positive staircase. Clearly, conditioning pulses to 0 or -40 mV had very different effects on magnitude of contraction during the conditioning trains.

Next we determined the effects of conditioning pulses to 0 and -40 mV on contractions initiated by sequential steps to -40 and 0 mV. Figure 7A was recorded when the activation steps were preceded by a series of 10 conditioning pulses to 0 mV. With this protocol the step to -40 mV activated a large contraction and little if any inward current, whereas the second step to 0 mV activated an additional contraction and I\textsubscript{Ca-L} (A). C: 2.5 µM nifedipine selectively blocked I\textsubscript{Ca-L} and the corresponding contraction, with little effect on the VSRM contraction. To maintain SR Ca\textsuperscript{2+} stores in the presence of Ca\textsuperscript{2+} channel blockade, conditioning pulses to positive potentials (e.g., +80 mV) were used to cause Ca\textsuperscript{2+} entry via Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange. B: control recordings of currents and contractions initiated by sequential steps to -40 and 0 mV in a separate cell. Exposure of this cell to 30 nM ryanodine inhibited VSRM contraction but had little effect on current and contraction initiated by the step to 0 mV (D).
associated with the steps to −40 and 0 mV were not affected by changing the conditioning-pulse voltage in this example. Mean data for the effects of conditioning-pulse voltage are shown in Fig. 5. Contractions initiated by the VSRM were significantly reduced in amplitude (from 2.2 ± 0.2 to 0.3 ± 0.1 µm, P < 0.001, Fig. 5A), whereas the amplitude of I_{Ca-L} contractions was not significantly decreased by conditioning pulses to −40 mV (from 1.6 ± 0.2 to 1.5 ± 0.3 µm, NS, Fig. 5B). The small inward current initiated by the step to −40 mV was not significantly affected (Fig. 5C); however, there was a significant increase in peak I_{Ca-L} when conditioning-pulse voltage was changed from 0 to −40 mV (Fig. 5D, P < 0.05). The effects of changing condi-

tioning-pulse voltage to −40 mV on the VSRM contraction were very similar to those of ryanodine.

Comparison of the effects of conditioning pulses to −40 mV with effects of ryanodine on contraction-voltage and I-V relationships. The results of the preceding experiments (Fig. 5) show that the VSRM is affected much more than I_{Ca-L}-induced contractions by conditioning steps to −40 mV or by exposure to ryanodine. Thus one would predict that changing conditioning-pulse voltage might also affect the contribution of the VSRM to contractions initiated when both mechanisms are available. Therefore, we determined the effects of conditioning-pulse amplitude on contraction-voltage relationships determined with voltage steps to a wide range of potentials. First, we determined the effects of conditioning-pulse voltage on I-V and contraction-voltage relationships when the VSRM was inactivated by a v_{PC} of −40 mV (Fig. 8, A and B). I-V and contraction-voltage relationships determined from a v_{PC} of −40 mV were both bell shaped. When conditioning-pulse voltage was changed from 0 to −40 mV, I-V relationships were not affected; however, the magnitudes of the contractions were significantly decreased at all voltages (P < 0.05). Figure 8C shows the difference between contraction-

Fig. 5. Mean effects of nifedipine, ryanodine, and conditioning pulses (CP) to −40 mV on contractions and currents initiated by sequential activation steps to −40 and 0 mV. Shown are histograms of mean data collected with the same voltage-clamp protocol as the examples shown in Fig. 4. Control data were collected when the conditioning pulses were to 0 mV. Nifedipine (2.5 µM) had no significant effect on contractions or currents initiated by voltage steps from −65 to −40 mV (A and C). However, nifedipine significantly inhibited both contractions and currents initiated by steps to 0 mV (B and D). In contrast, either ryanodine (30 nM) or conditioning pulses to −40 mV caused significant decreases in the magnitudes of contractions initiated by the VSRM (A). Magnitudes of contractions initiated by I_{Ca-L} were only moderately reduced by ryanodine and were not affected by conditioning pulses to −40 mV (B). Ryanodine had no effect on peak inward current recorded in response to steps to −40 or 0 mV (C and D, respectively). However, conditioning pulses to −40 mV significantly increased Ca^{2+} current associated with the step to 0 mV (D). n = 25 cells for control, 5 cells for nifedipine, 20 cells for ryanodine, and 14 cells for conditioning pulses to −40 mV. *Significantly different from control.

Fig. 6. Effects of changing conditioning-pulse voltage from 0 to −40 mV on contraction amplitude. A: representative original recordings of voltage and contractions during trains of conditioning pulses to 0 mV, followed by return to a v_{PC} of −70 mV. After the first contraction of the train, contractions exhibited a positive staircase. B: similar sequence recorded when the voltage of conditioning pulses was −40 mV. Contractions during the trains were greatly decreased in amplitude.
We also determined the effects of 30 nM ryanodine on contraction-voltage and I-V relations initiated by activation steps from \( V_{PC} \) values of −40 and −70 mV. In these experiments, all conditioning pulses were to 0 mV (Fig. 9). Ryanodine significantly reduced (\( P < 0.05 \)) inward current determined with either \( V_{PC} \) but did not shift the voltage dependence (Fig. 9A and D). Figure 9B shows that ryanodine significantly decreased (\( P < 0.05 \)) the amplitudes of contractions initiated from a \( V_{PC} \) of −40 mV. The contraction-voltage relationship in the presence of ryanodine remained bell shaped. Figure 9C shows the difference between the contraction-voltage relationships determined in the absence and presence of ryanodine. When contraction-voltage relationships were determined from a \( V_{PC} \) of −70 mV (Fig. 9E), ryanodine changed the shape of the contraction-voltage relationship from sigmoidal to bell shaped. Ryanodine also significantly decreased (\( P < 0.05 \)) the amplitudes of contractions initiated at virtually all test voltages (Fig. 9E). The component of contraction inhibited by ryanodine showed a sigmoidal voltage dependence (Fig. 9F). These effects of ryanodine on contraction-voltage relationships were very similar to those of changing conditioning-pulse voltage from 0 to −40 mV.

The effects of thapsigargin on contraction-voltage and I-V relationships determined from a \( V_{PC} \) of −70 mV in rat ventricular myocytes. An alternate method of evaluating the contribution of SR Ca\(^{2+}\) release to contraction is to inhibit SR Ca\(^{2+}\) uptake with thapsigargin, an agent that blocks the SR Ca\(^{2+}\)-adenosinetriphosphatase. Figure 10, A and B, shows representative recordings from a rat ventricular myocyte before and after exposure to 0.2 \( \mu \)M thapsigargin. Contractions and currents initiated by sequential steps to −40 and 0 mV from a \( V_{PC} \) of −65 mV were very similar to those observed with guinea pig ventricular myocytes. Thapsigargin strongly inhibited the contraction initiated by the VSRM but only partially inhibited the contraction accompanying \( I_{Ca-L} \). Figure 10, C and D, shows mean contraction-voltage and I-V relationships recorded with voltage steps from a \( V_{PC} \) of −70 mV (\( n = 3 \)). Thapsigargin had no effect on \( I_{Ca-L} \) but significantly reduced the amplitudes of contractions (\( P < 0.05 \)). In addition, thapsigargin, like ryanodine or conditioning pulses to −40 mV, caused the contraction-voltage relationship to become bell shaped.

In rat myocytes there is clear separation between the threshold for activation of contraction and the threshold for activation of inward current (Fig. 10, C and D), probably because of the absence of T-type Ca\(^{2+}\) current in this species (28). Because rat myocytes only have L-type Ca\(^{2+}\) current, we were able to compare the steady-state inactivation properties of the VSRM to those of \( I_{Ca-L} \). The voltage-clamp protocol used in these experiments is similar to that shown in Fig. 3. The \( V_{PC} \) was changed in 5-mV steps with each repetition of the voltage-clamp protocol. Each 700-ms \( V_{PC} \) was followed by a very brief (10 ms) step to −65 mV, followed by a step to −35 mV to activate the VSRM, or a brief step to −50 mV followed by a step to 0 mV to activate \( I_{Ca-L} \). Figure 10E shows mean normalized steady-state inacti-
vation curves for the VSRM and $I_{\text{Ca-L}}$ determined in nine myocytes. The VSRM had a $v_h$ of $-53.2 \pm 0.4$ mV and a $k$ of $4.6 \pm 0.2$ mV, whereas the corresponding values for $I_{\text{Ca-L}}$ were $-25.3 \pm 0.9$ mV and $6.0 \pm 0.2$ mV, respectively. The $v_h$ of the VSRM was 28 mV negative to that of $I_{\text{Ca-L}}$ and was significantly different from $v_h$ of $I_{\text{Ca-L}}$ ($P < 0.001$). The values of $k$ for the VSRM and $I_{\text{Ca-L}}$ also were significantly different ($P < 0.001$). Thus, when the $v_{PC}$ was $-40$ mV, the VSRM was completely inactivated but $I_{\text{Ca-L}}$ was still fully available.

Comparison of the effects of conditioning pulses to $-40$ mV with effects of ryanodine on restitution of contraction. Contractions initiated by an early test stimulus following a previous activation are small, but show recovery or restitution when the test interval increases (5). We determined the time course of restitution of contraction in isolated myocytes as well as restitution of $I_{\text{Ca-L}}$. The voltage-clamp protocol used in these experiments is shown at the top of Fig. 11A. Test steps to 0 mV from a $v_{PC}$ of $-65$ mV were used to activate both VSRM and $I_{\text{Ca-L}}$ components of contraction (total contraction). The interval ($\Delta t$) between the last conditioning pulse and the test step was increased progressively from 6 to 246 ms, in 20-ms increments. Figure 11A shows representative original recordings of current and contraction for three selected test intervals (26, 106, and 186 ms). Contraction increased progressively as the interval was lengthened. In contrast, $\text{Ca}^{2+}$ current increased when the test interval was lengthened from 26 to 106 ms, but remained relatively constant with further increases in test interval. Mean data for restitution of contraction are shown in Fig. 11B. The line represents a single exponential fit to the mean data. The exponential function for restitution of contraction had a time constant of 57.8 ms.

Steps to 0 mV from a $v_{PC}$ of $-65$ mV were used to activate both VSRM and $I_{\text{Ca-L}}$ components of contraction (total contraction)
We next determined the effects of 30 nM ryanodine on restitution of total contraction determined with test step to 0 mV from a \( v_{PC} \) of either −40 or −70 mV (Fig. 12). The voltage-clamp protocols are shown at the far right of Fig. 12. For the ryanodine experiments, the conditioning pulses were to 0 mV. Ryanodine significantly decreased the magnitude of contractions initiated by activation steps from \( v_{PC} \) = −70 to 0 mV (total contraction), and the plateau of the curve was reached at a shorter test interval than in control (Fig. 12A). Ryanodine significantly decreased the magnitude of contraction and made the contraction-voltage relationship bell shaped (E, \( P < 0.05 \)).

We then determined whether contractions initiated by the VSRM exhibited restitution. VSRM contractions were elicited by a test step to −30 mV from a \( v_{PC} \) of −65 mV. Figure 12B shows changes in magnitude of VSRM contractions with progressive increase in test interval. In the absence of ryanodine, restitution of VSRM contractions (Fig. 12B) followed a time course similar to that for total contractions (Fig. 12A). Ryanodine had virtually the same effect on restitution of VSRM contractions as on total contraction (Fig. 12B, \( P < 0.05 \)). Thus restitution of total contraction could be accounted for almost entirely by restitution of the VSRM component of contraction. We also evaluated restitution of contractions initiated at different test intervals by test steps to 0 mV from a \( v_{PC} \) of −35 mV to inactivate the VSRM. With this protocol, contractions were smaller and exhibited very little restitution (Fig. 12C). In addition, with this protocol, contractions at all test intervals were insensitive to 30 nM ryanodine.

We also examined the effects of conditioning-pulse voltage on restitution of contraction. Figure 12D shows the effects of changing the voltage of conditioning pulses from 0 to −40 mV on total contraction. When test steps were preceded by conditioning pulses to −40 mV, restitution was inhibited (\( P < 0.05 \)). Figure 12E shows the effects of changing conditioning-pulse amplitude on contractions initiated by the VSRM. The magnitudes of contractions also were decreased, although the
Fig. 10. Currents and contractions in rat ventricular myocytes. A and B: representative recordings of currents (top) and contractions (bottom) elicited by sequential voltage steps to -40 and 0 mV (shown schematically above A). Thapsigargin (0.2 µM) almost abolished the contraction initiated by the step to -40 mV but only partially inhibited the contraction initiated by the step to 0 mV. C: mean contraction-voltage relationships determined from a $V_{PC}$ of -70 mV. Thapsigargin significantly reduced the amplitudes of contraction and converted the contraction-voltage relationship from sigmoidal to bell shaped. Current was unaffected by thapsigargin (D). $^*P < 0.05$ with respect to control. E: mean steady-state inactivation curves for the VSRM contraction and $I_{Ca,L}$ determined in 9 rat myocytes. Curves were normalized to maximum contraction or inward current. Voltage-clamp protocols were similar to that shown in Fig. 3. Specific voltage steps are given in RESULTS. $V_h$ for VSRM (-53.2 ± 0.4 mV) was significantly different from that of $I_{Ca,L}$ (-25.3 ± 0.9 mV, $P < 0.001$). Values for $k$ also were significantly different (4.6 ± 0.2 mV for VSRM and 6.0 ± 0.2 mV for $I_{Ca,L}$, $P < 0.001$).
difference was not statistically significant. Figure 12F shows that contractions initiated by steps from −35 to 0 mV were virtually unaffected by changing conditioning-pulse voltage. Thus conditioning pulses to −40 mV and treatment with ryanodine virtually eliminate restitution of contraction, and this effect was mediated primarily through actions on the VSRM.

DISCUSSION

The objectives of this study were 1) to determine whether the VSRM exhibits steady-state inactivation and to determine the voltage range over which inactivation occurs, 2) to determine and compare the effects of protocols designed to alter SR Ca2+ load on components of contraction initiated by the VSRM or by ICa,L, and 3) to evaluate the role of the VSRM in restitution. Our observations demonstrate that the VSRM does show steady-state inactivation, is exquisitely sensitive to conditioning-pulse voltage and drugs that disrupt SR function, and is an important determinant of restitution of cardiac contraction.

One of the central observations of this study was that the VSRM exhibits steady-state inactivation properties. The VSRM was fully available when activation steps were made from membrane potentials more negative than −60 mV. The V, of the VSRM was found to be approximately −48 mV in guinea pig and −53 mV in rat. Complete inactivation occurred near −35 and −40 mV in the two species, respectively. Clearly, earlier studies of cardiac EC coupling, which utilized holding or conditioning voltages near −40 mV, would have almost completely inactivated the VSRM (2, 3, 9, 10, 20). Indeed, the bell-shaped contraction-voltage or Ca2+ transient-voltage relationships reported in those studies likely reflects activation of contraction by ICa,L in the absence of the VSRM.

The steady-state inactivation properties of the VSRM help distinguish it from CICR coupled to ICa,L. The steady-state inactivation curve for the VSRM was found to be significantly different statistically from that for ICa,L, with respect to both V, and k values. The steady-state inactivation curves for the VSRM and ICa,L were separated by 28 mV at V,. Because of this wide separation, the VSRM was completely inactivated when VPC was −40 mV but ICa,L was still fully available. Because the inactivation properties of the VSRM and ICa,L are widely divergent, it is very unlikely that the VSRM represents CICR initiated by ICa,L.

The steady-state inactivation properties of the VSRM allowed us to utilize two sequential activation steps to −40 and 0 mV to separate VSRM and ICa,L-induced contractions within a single voltage-clamp protocol. The ability of nifedipine to selectively inhibit ICa,L and the ICa,L-induced contraction with minimal effect on the VSRM contraction confirmed this separation. This selective effect of nifedipine also indicates that initiation of the VSRM contraction cannot be attributed to a very small influx of Ca2+ through L-type Ca2+ channels on the step to −40 mV, because inhibition of the large Ca2+ current on the step to 0 mV resulted in strong inhibition of the ICa,L-induced contraction.

The steady-state inactivation properties of the VSRM also serve to distinguish it from contractions triggered by reverse Na+Ca2+ exchange. Contractions attributed to reverse Na+Ca2+ exchange can be elicited by activation steps from either −70 or −40 mV (30). This indicates that contractions initiated by Na+Ca2+ ex-
change do not show inactivation at −40 mV. Indeed, Na⁺/Ca²⁺ exchange is not known to exhibit voltage-dependent inactivation. Furthermore, contractions attributed to reverse Na⁺/Ca²⁺ exchange demonstrate sigmoidal, N-shaped, or progressively increasing contraction-voltage relations with activation steps from −40 mV, depending on the intracellular concentration of Na⁺ (16, 23, 29, 30). The present study was conducted with high-resistance microelectrodes, which would minimize intracellular dialysis and which did not contain Na⁺. Under these conditions the contraction-voltage relationships were always bell shaped when the VPC was −40 mV.

Steady-state inactivation is generally described as a property of voltage-gated ion channels. In skeletal muscle, which also shows voltage-dependent release of SR Ca²⁺, the voltage sensor for EC coupling is believed to be the sarcolemmal L-type Ca²⁺ channel (24). The L-type Ca²⁺ channel is believed to be linked physically to the Ca²⁺ release channel (ryanodine receptor) in junctional SR (24). Interestingly, the release mechanism in skeletal muscle exhibits voltage-dependent inactivation (8, 24). Thus demonstration that the VSRM in cardiac myocytes also exhibits steady-state inactivation suggests that the voltage sensor for the VSRM may be a voltage-gated ion channel. The identity of the voltage sensor in cardiac myocytes is unknown. It would seem unlikely that the L-channel serves as the voltage sensor because the VPC of the cardiac L-channel is near −25 mV under our conditions as well as in other
studies (21). However, we cannot completely exclude this possibility because the voltage sensitivity of L-type Ca\(^{2+}\) channels might change when they are coupled to another protein such as Ca\(^{2+}\) release channels in the SR. Of the known voltage-sensitive channels in the cardiac cell membrane, only the T-type Ca\(^{2+}\) channel has a steady-state inactivation curve that closely matches that of the VSRM. The \(v_0\) of the T-type Ca\(^{2+}\) channel has been reported to be near −50 mV, with a \(k\) value of −5 mV (27, 31). These values correspond closely to the \(v_0\) and \(k\) values determined for the VSRM in the present study. However, in the present study we also were able to demonstrate the VSRM in rat ventricular myocytes, which have been reported not to have T-type Ca\(^{2+}\) myocytes, which have been reported not to have VSRM in rat ventricular myocytes, which have been reported not to have VSRM in rat ventricular myocytes, which have been reported not to have VSRM in rat ventricular myocytes, which have been reported not to have VSRM in rat ventricular myocytes, which have been reported not to have VSRM in rat ventricular myocytes, which have been reported not to have VSRM in rat ventricular myocytes, which have been reported not to have VSRM in rat ventricular myocytes, which have been reported not to have VSRM in rat ventricular myocytes, which have been reported not to have.

The intracellular Ca\(^{2+}\) transient that initiates cardiac contraction is derived from release of SR Ca\(^{2+}\) as well as influx of Ca\(^{2+}\) through the sarcolemma. The magnitude of Ca\(^{2+}\) released from the SR varies with SR load (14). Han et al. (14) demonstrated that test pulses to 0 mV activate only a small Ca\(^{2+}\) transient when preceded by conditioning pulses to −30 mV, to provide a low SR Ca\(^{2+}\) load. However, the same test pulse initiated a much larger Ca\(^{2+}\) transient, when preceded by conditioning pulses to 0 mV, to increase SR Ca\(^{2+}\) load. In the present study, contractions initiated by the VSRM and \(I_{Ca-L}\) were affected differentially by similar changes in conditioning-pulse voltage. VSRM contractions were greatly reduced in amplitude by conditioning-pulse protocols designed to reduce SR Ca\(^{2+}\) loading. This is compatible with the VSRM contraction, depending on release of SR Ca\(^{2+}\). In contrast, contractions initiated by activation of \(I_{Ca-L}\) were much less affected by conditioning-pulse voltage. These observations suggest that the \(I_{Ca-L}\) contraction, unlike the VSRM contraction, depends on both SR Ca\(^{2+}\) release and Ca\(^{2+}\) influx. This interpretation is also supported by the effects of ryanodine and thapsigargin, which interfere with SR function. Both agents strongly inhibited VSRM contractions but had much less effect on \(I_{Ca-L}\) contractions. Our observations with 30 nM ryanodine imply that, in undialyzed guinea pig cells at 37°C, part of the \(I_{Ca-L}\) contraction may be mediated by direct activation of myofilaments by Ca\(^{2+}\) influx. There are conflicting reports as to whether influx of Ca\(^{2+}\) via \(I_{Ca-L}\) is sufficient to initiate contraction directly (3). Effects of ryanodine vary widely depending on temperature, duration of exposure, concentration, stimulation interval, atrial versus ventricular tissue, and species (3). Thus in some species virtually all contraction can be eliminated by ryanodine (e.g., adult rat ventricle), whereas in others a ryanodine-resistant component has been observed (e.g., guinea pig ventricle) (3). For example, in field-stimulated guinea pig ventricular myocytes (18) and multicellular preparations (26) studied at physiological temperatures, 25–80% of contraction amplitude was retained in the presence of ryanodine and/or thapsigargin. However, these earlier studies did not separate effects of ryanodine or thapsigargin on the two components of EC coupling examined in this study.

Contraction-voltage relationships determined from a \(v_{PC}\) of −70 mV include both VSRM and \(I_{Ca-L}\) Components of contraction. Both changes in conditioning-pulse voltage and ryanodine caused very large reductions in amplitudes of contractions initiated by steps from −70 mV. The component of contraction inhibited by both of these manipulations had a sigmoidal voltage dependence. The magnitude of the component that was inhibited was large even at very positive membrane potentials near the reversal potential of \(I_{Ca-L}\). In contrast, the component that was inhibited was very small at positive membrane potentials when the VSRM was inactivated by a \(v_{PC}\) of −40 mV. Thus, when both the VSRM and \(I_{Ca-L}\) are available, most of the contraction elicited at potentials corresponding to the action potential peak and plateau appears to attributable to the VSRM.

Our studies also demonstrated an inhibitory effect of ryanodine on \(I_{Ca-L}\) that was apparent in the I-V relationships. It is unlikely that ryanodine directly inhibits the L-type Ca\(^{2+}\) channel, because experiments by others in which intracellular Ca\(^{2+}\) levels were strongly buffered showed no effect of ryanodine on \(I_{Ca-L}\) (1). In our experiments, in which intracellular Ca\(^{2+}\) was not buffered, inhibition of \(I_{Ca-L}\) by ryanodine may reflect Ca\(^{2+}\)-mediated inhibition of L-type Ca\(^{2+}\) channels (21). Low concentrations of ryanodine lock the SR Ca\(^{2+}\) release channel in an open subconducting state; however, the SR can still take up Ca\(^{2+}\) (25). This Ca\(^{2+}\) can gradually leak out of the SR over a period of several hundred milliseconds (3, 26). In the protocols used to determine I-V relationships, test steps were preceded by conditioning pulses and a postconditioning period of 500 ms. A possible explanation for the effect of ryanodine on \(I_{Ca-L}\) is that the L-type Ca\(^{2+}\) channels may have been inhibited by Ca\(^{2+}\) leaking from the SR during the period between the last conditioning pulse and the test step. At present we have no direct evidence supporting or refuting this explanation. However, it is interesting to note that thapsigargin, which does not lock the release channel in an open subconducting state, had no effect on \(I_{Ca-L}\).

The effects of ryanodine and conditioning-pulse voltage on \(I_{Ca-L}\) and on \(I_{Ca-L}\) contractions were slightly different depending on the voltage-clamp protocol used. For example, the \(I_{Ca-L}\) contraction was not significantly inhibited by conditioning pulses to −40 mV when sequential activation steps to −40 and 0 mV were used, but was significantly inhibited when contraction-voltage relations were determined. This difference might be attributable to differences in the activation sequence between the two protocols. With the sequential steps, the VSRM is activated immediately before the \(I_{Ca-L}\) contraction. Because the VSRM contraction is not accompanied by \(I_{Ca-L}\) in this protocol, activation of the
VSRM might partially reduce SR Ca\(^{2+}\) and decrease the sensitivity of I\(_{\text{Ca-L}}\) contractions to changes in SR load.

The third goal of this study was to examine the role of the VSRM in recovery of contraction after a previous activation, a process termed restitution of contraction (5). In the present study we were able to demonstrate restitution of contraction in isolated myocytes under voltage-clamp conditions. Restitution was measured as the change in magnitude of test contractions initiated at different intervals after a previous activation. We found that the magnitude of the test contraction was negligible at short intervals but gradually increased with longer test intervals. Contractions initiated by depolarizing steps from −65 to 0 mV, to activate both VSRM and I\(_{\text{Ca-L}}\) components of contraction, showed restitution of contraction with a time constant near 60 ms. In contrast, the time constant of restitution of I\(_{\text{Ca-L}}\) was near 30 ms, which is similar to previous reports when one takes temperature into account (21). Thus the time course of restitution of contractions was slower than restitution of I\(_{\text{Ca-L}}\). Therefore, restitution of contraction is not limited by recovery of I\(_{\text{Ca-L}}\).

We found that contractions initiated by the VSRM, selectively activated by steps from −65 to −30 mV, also exhibited restitution. The magnitude of restitution of VSRM contractions was similar to that observed when both VSRM and I\(_{\text{Ca-L}}\) components of contraction were activated by steps from −65 to 0 mV. Thus recovery of the VSRM could account for much of restitution of contraction. This observation further demonstrates that restitution of contraction is not limited by recovery of I\(_{\text{Ca-L}}\), because the VSRM contractions were elicited by voltage steps that did not activate measurable I\(_{\text{Ca-L}}\). Indeed, I\(_{\text{Ca-L}}\) contractions initiated from a VPC of −35 mV showed only minimal restitution and only during the first 25–50 ms after the previous activation.

Manipulations to decrease SR Ca\(^{2+}\) load greatly attenuated restitution of total contractions initiated by voltage steps from −65 to 0 mV. The same manipulations, conditioning pulses to −40 mV or exposure to ryanodine, also reduced restitution of contractions initiated by the VSRM. These observations indicate that the VSRM plays an important role in restitution of cardiac contraction and that restitution of the VSRM is very sensitive to manipulations designed to reduce SR Ca\(^{2+}\) load.

The component of EC coupling that determines the time course of restitution of contraction has yet to be identified. It is very unlikely that the time course of restitution is limited by replenishment of SR Ca\(^{2+}\) stores because this is believed to be completed very quickly and in advance of recovery of SR Ca\(^{2+}\) release channels (3). Because of these considerations, restitution generally has been attributed to recovery of the SR release channels (3). However, our results suggest that the recovery process could be either at the level of the SR release channel or at the voltage sensor. Additional studies are needed to identify the precise component that determines restitution.

Our observations demonstrate that a component of contraction, which we have called a VSRM, plays an important part in EC coupling in heart. When the VSRM is inactivated, the maximum magnitude of contraction is reduced and contraction is weak at membrane potentials corresponding to the peak and plateau phases of the ventricular action potential. However, when the VSRM is available for activation, the magnitude of contraction is greater and becomes independent of voltage at potentials positive to approximately −20 mV. The VSRM then operates as a trigger mechanism that initiates maximal contractions over the entire range of potentials corresponding to the overshoot, initial repolarization, and plateau of the action potential. The VSRM also plays an important role in determining restitution of contraction following a previous activation and is very sensitive to manipulations that alter SR Ca\(^{2+}\) loading. I\(_{\text{Ca-L}}\) activates a component of contraction that is less sensitive than the VSRM to loading of SR stores and also plays an important role in loading SR Ca\(^{2+}\) stores. Our observations support the hypothesis that the VSRM is a major link that couples cardiac contraction to membrane potential, activation of contraction, and SR Ca\(^{2+}\) loading.

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