Increases in diastolic [Ca\(^{2+}\)] can contribute to positive inotropy in guinea pig ventricular myocytes in the absence of changes in amplitudes of Ca\(^{2+}\) transients

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Coupling of electrical excitation to cardiac contraction (EC-coupling) is accomplished through a rapid increase in cytosolic [Ca\(^{2+}\)] that initiates contraction in mammalian heart (9, 13–15, 32). CICR is not all or none; rather, the amplitudes of Ca\(^{2+}\) transients are graded by the amplitude of I\(_{Ca-L}\). Gradation of CICR is believed to occur by way of local control of Ca\(^{2+}\) release by L-type Ca\(^{2+}\) channels (3, 32). SR Ca\(^{2+}\) is released in units called Ca\(^{2+}\) sparks (8). Ca\(^{2+}\) sparks represent the coordinated opening of a number of RyRs, which function as a Ca\(^{2+}\) release unit (8). A Ca\(^{2+}\) release unit can be activated by Ca\(^{2+}\) entry via an adjacent L-type Ca\(^{2+}\) channel. However, these Ca\(^{2+}\) release units are not normally activated by Ca\(^{2+}\) released by neighboring groups of RyRs (8, 3, 32). Therefore, the number of L-type Ca\(^{2+}\) channels that open determines the number of local Ca\(^{2+}\) release units activated (3, 32). The cardiac action potential is believed to activate a large number of L-type Ca\(^{2+}\) channels simultaneously and therefore activate a large number of Ca\(^{2+}\) release units to produce a large Ca\(^{2+}\) transient. In contrast, if the depolarization is weak and few L-type Ca\(^{2+}\) channels are activated, then fewer release units will open and the transient will be smaller (5).

Studies have shown that increases in SR Ca\(^{2+}\) load also can increase the amplitude of Ca\(^{2+}\) transients (22). Increased SR Ca\(^{2+}\) load is thought to increase the amount of Ca\(^{2+}\) released in each Ca\(^{2+}\) spark by increasing the driving force for SR Ca\(^{2+}\) release (3, 41). In addition, increased SR Ca\(^{2+}\) content also can increase RyR sensitivity to single L-type channel currents, thereby increasing the probability of activation of a Ca\(^{2+}\) release unit by any given Ca\(^{2+}\) current (3, 8). Thus increased SR Ca\(^{2+}\) load can increase the amplitude of Ca\(^{2+}\) transients through an increase in the amount of SR Ca\(^{2+}\) released through each cluster of RyRs, as well as by increasing the number of Ca\(^{2+}\) release units activated (3, 8, 41).

Although I\(_{Ca-L}\) and SR Ca\(^{2+}\) load are important mediators of inotropic effects in the heart, several lines of evidence suggest that diastolic Ca\(^{2+}\) levels also may play a role in regulation of the amplitude of cardiac contraction (3). Frampton et al. (19) reported that, when extracellular [Ca\(^{2+}\)] was increased, or when stimulation rate was increased, diastolic [Ca\(^{2+}\)] increased. The authors suggested that this increase in diastolic Ca\(^{2+}\) contributed to positive inotropic effects by increasing SR stores (19).

Interestingly, Hattori et al. (20) showed that increases in diastolic Ca\(^{2+}\) might play a direct role in mediating positive staircases, which were generated by stimulating cardiac myocytes following a period of rest. In this study, positive staircases were directly related to progressive increases in...
diastolic [Ca\(^{2+}\)] and were preserved when SR Ca\(^{2+}\) release was blocked by ryanodine (20). Ryanodine should remove the contribution of SR Ca\(^{2+}\) release to staircases, and therefore this observation suggests a role for changes in diastolic Ca\(^{2+}\) in generation of staircases (20).

Despite evidence demonstrating a role for diastolic Ca\(^{2+}\) in inotropy, the contribution of diastolic [Ca\(^{2+}\)] to regulation of contraction amplitude is disputed. duBell and Houser (11) showed that Ca\(^{2+}\) transient amplitude and SR Ca\(^{2+}\) content are the primary contributors to positive staircases of contraction. Also, Suda and Kubunken (34) did not detect significant changes in diastolic Ca\(^{2+}\) when they elevated extracellular [Ca\(^{2+}\)] to produce a positive inotrop effect. Although some studies show that positive staircases and inotropic responses to elevation of extracellular [Ca\(^{2+}\)] occur in the absence of changes in diastolic Ca\(^{2+}\), they do not eliminate the possibility that diastolic Ca\(^{2+}\) could contribute to positive inotropy. Thus the role of diastolic Ca\(^{2+}\) in positive inotropy in the heart remains controversial.

In the present study, we examined inotropic effects of diastolic Ca\(^{2+}\) during positive staircases initiated by electrical stimulation following rest and in response to elevated extracellular [Ca\(^{2+}\)] in isolated guinea pig ventricular myocytes at physiological temperatures. As elevation of extracellular [Ca\(^{2+}\)] increases the amplitudes of contractions in intact cardiac muscle (7) and amplitudes of \(I_{\text{Ca-L}}\) and contraction in isolated myocyte models (19, 22, 4), we used elevation of extracellular [Ca\(^{2+}\)] as an experimental manipulation to produce positive inotropy. Positive inotropy was measured as an increase in the degree of unloaded cell shortening. Although cell shortening does not definitively describe whole heart changes in force of contraction, inotropic responses recorded from unloaded cells parallel responses measured from loaded cells and intact tissue (6, 7). Increased extracellular [Ca\(^{2+}\)] can modulate EC coupling through several factors that affect CICR, including peak amplitude of \(I_{\text{Ca-L}}\), SR Ca\(^{2+}\) load, and/or diastolic [Ca\(^{2+}\)]. The specific objectives of this study are 1) to determine whether diastolic Ca\(^{2+}\) plays a role in positive staircases and 2) to determine whether diastolic Ca\(^{2+}\) contributes to the inotropic effects caused by experimental increases in extracellular [Ca\(^{2+}\)] in myocytes at physiological temperatures.

**METHODS**

**Myocyte isolation.** Experiments were approved by the Dalhousie University Committee on Laboratory Animal Care, in accordance with Canadian Council on Animal Care guidelines. Guinea pig ventricular myocytes were prepared as described previously (38). Briefly, guinea pigs were anesthetized with pentobarbital sodium (Somnotol; 120 mg/kg ip), coinjected with heparin (3,300 IU/kg). Following induction of anesthesia, the chest was opened, and the heart was cannulated in situ. The heart was perfused with a nominally Ca\(^{2+}\)-free buffer. Cells were dissociated from the tissue by gentle agitation and were filtered through a 225-μm filter (Spectra/Mesh). Isolated cells, suspended in 1.0–1.5 ml of high-K\(^{+}\) buffer, were incubated for 15–20 min, at room temperature in the dark, with 5 μM fura-2 AM (fura-2 AM stock solution in anhydrous DMSO). Myocytes were then transferred to an experimental chamber installed on the stage of an inverted microscope (Nikon TE200) and were allowed to settle on the glass coverslip bottom of the chamber. Myocytes were superfused with a buffer of the following composition (mM): 45 NaCl, 100 choline chloride, 10 HEPES, 10 glucose, 1 MgCl\(_2\), 2 CaCl\(_2\), 0.3 lidocaine (37°C). Superfusion of myocytes and isolated cardiac muscle with buffers at hypothermic temperature (22°C) has been shown to produce dynamic changes in EC coupling. Hypothermia inhibits Na\(^{+}\)/pump function (12), elevates SR Ca\(^{2+}\) load, alters myofilament Ca\(^{2+}\) sensitivity, and changes \(I_{\text{Ca-L}}\) amplitude and inactivation (4). The superfusion buffer, therefore, was warmed to 37°C to approximate guinea pig core temperature (39°C; Ref. 30).

**Voltage clamp.** Discontinuous single-electrode voltage clamp at a sampling rate of 7–10 kHz was conducted with an Axoclamp 2B current and voltage-clamp amplifier (Axon Instruments, Foster City, CA) and ClampEx software (version 8.1, Axon Instruments). In most experiments, cells were impaled with high-resistance glass micropipettes (15–25 MΩ) filled with 2.7 M KCl. In some experiments, myocytes were voltage clamped with patch pipettes (1–2 MΩ). Patch pipettes were filled with a nominally Na\(^{+}\)-free intracellular solution of the following composition (in mM): 70 KCl, 70 potassium aspartate, 1 MgCl\(_2\), 10 HEPES, 0.5 EGTA, 4 Mg-ATP, 2.5 KH\(_2\)PO\(_4\), 0.12 CaCl\(_2\), and 0.05 8-bromo cAMP, pH adjusted to 7.2 (16). All test steps were preceded by trains of ten 200-ms-long conditioning pulses from −80 to 0 mV, delivered at a rate of 2 Hz. Na\(^{+}\) current was inhibited with 300 μM lidocaine. Cells were voltage clamped at a holding potential of −80 mV, except following conditioning pulse trains when a holding potential of either −60 or −50 mV preceded the test step to facilitate inhibition of Na\(^{+}\) current by lidocaine. At concentrations <0.5 mM, lidocaine has been reported to have little effect on \(I_{\text{Ca-L}}\) (26) and no effect on the arrhythmogenic transient inward current (26) or on the T-type Ca\(^{2+}\) current (36).

[Ca\(^{2+}\)] in the control solution was 2.0 mM, and this solution was used to superfuse the cells throughout the preceding trains of 10 conditioning pulses. Extracellular [Ca\(^{2+}\)] was rapidly changed from 2.0 mM to 0.1, 0.5, or 5.0 mM by bathing the cell in buffers of different [Ca\(^{2+}\)]. A computer-controlled rapid solution changer was used to rapidly expose the cell to solutions with different [Ca\(^{2+}\)] for 3 s following the conditioning pulse train and throughout the test step (17). In some experiments, the extracellular Mg\(^{2+}\) concentration also was elevated from 1 to 4 mM. The elevated Mg\(^{2+}\) was applied with the rapid solution changer. The rapid solution changer also was used in other experiments where 0.1 mM Cd\(^{2+}\) was added to a 5.0 mM extracellular [Ca\(^{2+}\)] test solution. Finally, the rapid solution changer was used to rapidly expose the cell to a buffer containing 10 mM caffeine (1.0 s application). Caffeine was used to release SR Ca\(^{2+}\) and thereby estimate SR Ca\(^{2+}\) load. To inhibit extrusion of Ca\(^{2+}\) from the cytosol by the Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX), caffeine was applied in a nominally Ca\(^{2+}\)- and Na\(^{+}\)-free solution (23) of the following composition (in mM): 140 LiCl, 4 KCl, 10 glucose, 5 HEPES, 4 MgCl\(_2\), and 10 caffeine. In a few experiments, caffeine was applied in a buffer of the following composition (in mM): 145 NaCl, 10 HEPES, 10 glucose, 4 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 0.3 lidocaine, and 10 caffeine.

**Measurement and analysis.** Fura-2 fluorescence was recorded and measured as previously described (18). Briefly, an aperture in front of the photomultiplier tube was used to isolate the fura-2-loaded cell in a sampling window. Ca\(^{2+}\) bound and unbound fura-2 were excited by UV light at 340 and 380 nm, respectively, by a DeltaRam fluorescence system (Photon Technologies International). Fluorescence emission at 510 nm was measured, at 100 samples/s for each of the 340- and 380-nm excitation wavelengths. Following fluorescence recordings from cells, background fluorescence was determined. To determine intracellular [Ca\(^{2+}\)], the background values for each excitation wavelength were subtracted from the recordings made during the experi-
mental protocol. The ratio of fluorescence emission recorded during excitation at 340 and 380 nm was determined. Emission ratios were converted to [Ca\textsuperscript{2+}] with an in vitro calibration curve determined from known concentrations of Ca\textsuperscript{2+}. Simultaneous recordings of contractions and fluorescence were made by splitting the light from the microscope with a dichroic cube. Red light was sent to the closed-circuit camera and video edge detection system, while the remaining light was delivered to the photomultiplier tube for fluorescence measurement. Contraction amplitudes were recorded by tracking one edge of the cell with a Zenetics Electronics (Sandy, UT) video edge detector at 120 Hz. In some experiments, resting cell length was determined by tracking both ends of the cell. To determine resting cell length and resting intracellular [Ca\textsuperscript{2+}], cells were activated with a short activation protocol to show that they responded to electrical stimuli with contractions and Ca\textsuperscript{2+} transients. Cells were then voltage clamped at a resting potential of −80 mV and exposed to different extracellular [Ca\textsuperscript{2+}]. Cells that generated spontaneous Ca\textsuperscript{2+} waves were excluded.

Contractions amplitudes, cell length, and whole cell currents were digitized by a Digidata 1322A analog-to-digital interface (Axon Instruments). Digitization rates varied from 0.7 to 4.7 kHz, depending on the duration of the specific voltage clamp protocol. Contraction amplitudes, cell length, and currents were recorded with ClampEx 8.1 (Axon Instruments) software and analyzed with ClampFit 8.1 (Axon Instruments) software. SigmaPlot 2001 (Jandel Scientific, SPSS) was used to construct graphs. Statistical analyses were performed with either SigmaPlot 2001 or Sigmaplot, version 2.03 (Jandel Scientific, SPSS). Mean data were expressed ± SE. * denotes P < 0.05.

Following steps from −50 to 0 mV, peak amplitudes of $I_{\text{Ca-L}}$ were measured as the peak inward current with respect to net current at the end of the 250-ms test step. Following steps from −60 to 0 mV, peak amplitudes of $I_{\text{Ca-L}}$ were measured as the peak current with respect to net current at the time point where $I_{\text{Ca-L}}$ was 90% inactivated on a step to 0 mV. In some experiments, identity of $I_{\text{Ca-L}}$ was confirmed, and measurement techniques validated, with application of Cd\textsuperscript{2+} and determination of Cd\textsuperscript{2+}-sensitive current amplitude. These experiments established that measurement of peak inward current, as described above, resulted in minimal underestimation of $I_{\text{Ca-L}}$. Contraction amplitudes were measured as the difference between peak contraction and the diastolic value immediately preceding the onset of contraction. Following conversion of the fura-2 fluorescence ratio to [Ca\textsuperscript{2+}], diastolic [Ca\textsuperscript{2+}] (the concentration immediately preceding the test step), peak systolic [Ca\textsuperscript{2+}], and Ca\textsuperscript{2+} transient amplitude (differ-ence between systolic and diastolic) were measured.

Sources of chemicals. Lidocaine, choline chloride, HEPES buffer, MgCl\textsubscript{2}, anhydrous DMSO, and caffeine were purchased from Sigma Aldrich Canada (Oakville, ON). Invitrogen (Burlington, ON) was the supplier for furan-2 AM. All other chemicals were purchased from BDH (Toronto, ON).

RESULTS

Role of diastolic [Ca\textsuperscript{2+}] in increasing contraction amplitude during positive staircases. In our first set of experiments, we simultaneously recorded contractions and Ca\textsuperscript{2+} transients from voltage-clamped guinea pig ventricular myocytes during positive staircases initiated by regular depolarization following a rest period. Myocytes were activated with trains of ten 200-ms rectangular pulses from −80 to 0 mV, following a 4-s rest period. Pulses were delivered at a rate of 2 Hz. Figure 1 shows a schematic of the activation train (A), as well as representative recordings of contractions and intracellular Ca\textsuperscript{2+} transients (B and C, respectively). The recording of contractions in Fig. 1B shows that the first contraction initiated after a period of rest was small. However, the amplitude of contractions increased stepwise with each sequential activation during the first part of the train. Therefore, these contractions exhibit positive staircases. In contrast, Ca\textsuperscript{2+} transient amplitudes appeared to remain constant throughout the train (Fig. 1C). Mean amplitudes of contraction and intracellular [Ca\textsuperscript{2+}] during positive staircases are shown in Fig. 1, D–F. Only data from responses to activation pulses 2–10 were fitted, as the first response represents a rest response which followed a long period of quiescence. All responses were normalized to the mean magnitude of the 9th and 10th responses to compensate for cell-to-cell variability. Contraction amplitudes increased significantly during the pulse train (Fig. 1D). The solid regression line shows that amplitude of contraction increased over the first half of the train and then remained constant. Figure 1E illustrates the relationship between Ca\textsuperscript{2+} transient amplitudes and beat number for the 10 pulses. Amplitudes of Ca\textsuperscript{2+} transients only increased slightly at the end of the train, but this was not significant. There was no clear relationship between Ca\textsuperscript{2+} transient amplitude and contraction. However, as shown in Fig. 1F, diastolic intracellular [Ca\textsuperscript{2+}] increased significantly over the series of pulses. The regression line for diastolic [Ca\textsuperscript{2+}] appeared to increase in parallel with the regression for mean amplitudes of contractions. Both peak systolic Ca\textsuperscript{2+} and diastolic Ca\textsuperscript{2+} increased over the 10-pulse train.

These data suggest that changes in contraction amplitudes are more closely related to changes in diastolic [Ca\textsuperscript{2+}] than amplitudes of Ca\textsuperscript{2+} transients. To examine this, data were replotted as shown in Fig. 2. Figure 2A shows the relationship of amplitude of contraction to that of Ca\textsuperscript{2+} transient, and Fig. 2B shows the relationship of amplitude of contraction to diastolic [Ca\textsuperscript{2+}]. Amplitudes of contractions show no relationship to amplitudes of Ca\textsuperscript{2+} transients (Fig. 2A). In contrast, amplitude of contraction shows a strong direct linear relationship to diastolic [Ca\textsuperscript{2+}], as illustrated by the regression line to the data ($r^2 = 0.92$) in Fig. 2B. These results indicate that amplitude of contraction during positive staircases increased in parallel with increasing diastolic [Ca\textsuperscript{2+}], but not with amplitude of Ca\textsuperscript{2+} transients.

Effect of increased extracellular [Ca\textsuperscript{2+}] on resting cell length and diastolic [Ca\textsuperscript{2+}]. In additional experiments, we determined whether extracellular [Ca\textsuperscript{2+}] could alter diastolic intracellular [Ca\textsuperscript{2+}] in resting myocytes in the absence of electrical stimulation and whether this would affect diastolic cell length. Initially, cells were superfused with 2.0 mM [Ca\textsuperscript{2+}], then extracellular [Ca\textsuperscript{2+}] was increased to 5.0 mM, and simultaneous recordings of cell length and intracellular [Ca\textsuperscript{2+}] were made for a minimum of 5 s at each extracellular [Ca\textsuperscript{2+}]. Figure 3A shows representative recordings of intracellular [Ca\textsuperscript{2+}] (top trace) and resting cell length (bottom trace) during a transition in extracellular [Ca\textsuperscript{2+}]. When extracellular [Ca\textsuperscript{2+}] was elevated, intracellular [Ca\textsuperscript{2+}] increased slowly without initiating a rapid Ca\textsuperscript{2+} transient. During the transition in extracellular [Ca\textsuperscript{2+}] from 2.0 to 5.0 mM, cell length decreased, and this decrease in resting cell length mirrored the increase in intracellular [Ca\textsuperscript{2+}]. Figure 3B compares mean intracellular [Ca\textsuperscript{2+}] and resting cell length recorded in 2.0 and 5.0 mM extracellular [Ca\textsuperscript{2+}] after resting cell length and intracellular Ca\textsuperscript{2+} had reached a steady state. Mean intracellular [Ca\textsuperscript{2+}] increased significantly, and mean resting cell length decreased significantly in 5.0 mM extracellular [Ca\textsuperscript{2+}] However, when extracellular [Ca\textsuperscript{2+}] was elevated from 2.0 to 5.0 mM, diastolic [Ca\textsuperscript{2+}] increased significantly, and this increase
in diastolic [Ca\(^{2+}\)] was sufficient to decrease cell length, presumably by activating the contractile myofilaments.

Effect of increasing extracellular [Ca\(^{2+}\)] on amplitudes of I\(_{\text{Ca-L}}\) contractions, and Ca\(^{2+}\) transients. In the next set of experiments, we investigated the role of diastolic Ca\(^{2+}\) in determining amplitudes of contraction when myocytes were electrically stimulated while being superfused with different concentrations of extracellular Ca\(^{2+}\). Manipulation of extracellular Ca\(^{2+}\) was used as an experimental treatment, to produce changes in the amplitude of I\(_{\text{Ca-L}}\), and to thereby produce positive inotropy. Figure 4 shows a schematic of activation train, test step, and test solution application. Amplitudes of contractions, I\(_{\text{Ca-L}}\), and Ca\(^{2+}\) transients were recorded simultaneously. Mean data are expressed in panels D, E, and F. Mean data were normalized to the means of the 9th and 10th responses. The first response was omitted.

**Fig. 1.** Positive staircases of contraction occur in parallel with increasing diastolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)], not increasing Ca\(^{2+}\) transient amplitude. A: schematic representation of voltage-clamp protocol used to activate the cells. Contractions and Ca\(^{2+}\) transients were elicited by a series of ten 200-ms square pulses from \(-80\) to \(0\) mV at 2 Hz. Representative examples of contractions (B) and Ca\(^{2+}\) transients (C) were recorded simultaneously. Mean data are expressed in panels D, E, and F. Mean data were normalized to the means of the 9th and 10th responses. The first response was omitted. D: mean contraction amplitudes increased significantly during the train of pulses. E: mean Ca\(^{2+}\) transient amplitudes do not differ significantly between the 9th and 10th responses. F: mean diastolic Ca\(^{2+}\) significantly over the train of 10 pulses. n = 9 Cells. All data are paired. *P < 0.05 as tested by one-way repeated-measures ANOVA.

**Fig. 2.** During positive staircases, increasing contraction amplitudes show a direct relationship to the increase in diastolic [Ca\(^{2+}\)]. A: contraction-Ca\(^{2+}\) transient amplitude relationship from the data shown in Fig. 2. Ca\(^{2+}\) transient amplitude shows no relationship to increasing contraction amplitude. B: contraction-diastolic Ca\(^{2+}\) relationship determined from the data presented in Fig. 2. Contraction amplitude is directly related to diastolic Ca\(^{2+}\). n = 9 Cells. All data are paired.
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Thus, although Ca\textsuperscript{2+} transient amplitude appeared to saturate (Fig. 4F), the amplitudes of contraction and \(I_{\text{Ca-L}}\) (E and G) continued to increase exponentially with increasing extracellular [Ca\textsuperscript{2+}].

As we were interested in the roles of diastolic Ca\textsuperscript{2+}, \(I_{\text{Ca-L}}\), and Ca\textsuperscript{2+} transient amplitude in regulation of the amplitude of contraction, we compared mean amplitudes of contractions, \(I_{\text{Ca-L}}\), and Ca\textsuperscript{2+} transients when extracellular [Ca\textsuperscript{2+}] was elevated from 2.0 to 5.0 mM in Fig. 5. Figure 5A shows that contraction amplitude increased significantly in 5.0 mM extracellular [Ca\textsuperscript{2+}] compared with 2.0 mM [Ca\textsuperscript{2+}].

Elevation of extracellular [Ca\textsuperscript{2+}] from 2.0 to 5.0 mM also increased the amplitude of \(I_{\text{Ca-L}}\) significantly (Fig. 5B). As shown in Fig. 5C, diastolic and peak systolic intracellular [Ca\textsuperscript{2+}] also both increased with increasing extracellular [Ca\textsuperscript{2+}]. In contrast, elevation of the extracellular [Ca\textsuperscript{2+}] from 2.0 to 5.0 mM did not significantly affect the amplitudes of Ca\textsuperscript{2+} transients. Ca\textsuperscript{2+} transient amplitude appeared to have saturated in 5.0 mM extracellular Ca\textsuperscript{2+}. Interestingly, increasing extracellular [Ca\textsuperscript{2+}] from 2.0 to 5.0 mM led to positive inotropy under our experimental conditions. Although both the trigger for EC coupling and the final output, contraction, were increased, the positive inotropic effect occurred, despite saturation of Ca\textsuperscript{2+} transient amplitude.

We investigated the possibility that EGTA in the high-K\textsuperscript{+} buffer was responsible for the saturation of the Ca\textsuperscript{2+} transient. Ca\textsuperscript{2+} transients also were measured in myocytes, which were isolated in the absence of EGTA (0 EGTA in the high-K\textsuperscript{+} buffer). The amplitudes of Ca\textsuperscript{2+} transients recorded on a step from −50 to 0 mV in 5.0 mM extracellular Ca\textsuperscript{2+} did not differ significantly between myocytes isolated in the presence or absence of EGTA (75 ± 13 vs. 119 ± 35 mM, \(n = 7\) cells in each group).

Voltage dependence of amplitudes of \(I_{\text{Ca-L}}\), contractions, and Ca\textsuperscript{2+} transients in elevated extracellular [Ca\textsuperscript{2+}]. As amplitudes of contractions, \(I_{\text{Ca-L}}\), and Ca\textsuperscript{2+} transients vary with the voltage of test steps to different membrane potentials, it was important to examine responses to a wide range of test step potentials. It was our goal to determine whether the relationship between these variables remained similar to that seen with test steps to only one potential. To that end, test steps were made from a holding potential of −60 mV, following conditioning pulse trains. This activation protocol, shown as a schematic in Fig. 6A, was repeated eight times. The test step potential was increased by 20 mV following each repetition of the protocol to initiate responses by test steps from −60 to +80 mV. This whole sequence was then repeated with the extracellular [Ca\textsuperscript{2+}] increased to 5.0 mM for 3 s before and throughout the test step. Contraction, current, and Ca\textsuperscript{2+} transient amplitudes were plotted as functions of test step amplitudes to generate contraction-voltage, current-voltage, and Ca\textsuperscript{2+} transient-voltage relationships as shown in Fig. 6, B–D. Figure 6B shows that elevated extracellular [Ca\textsuperscript{2+}] caused a significant increase in the amplitude of contraction and that this increase was observed over a wide range of membrane potentials.

Figure 6C shows that, when extracellular [Ca\textsuperscript{2+}] was elevated from 2.0 to 5.0 mM, peak \(I_{\text{Ca-L}}\) amplitude was significantly increased at potentials from −20 to +60 mV. These contraction-voltage and current-voltage relationships show that the positive inotropy and increase in peak \(I_{\text{Ca-L}}\) amplitude
associated with elevated extracellular [Ca\textsuperscript{2+}] were not voltage dependent but rather occurred at a wide range of membrane potentials. The transient-voltage relationship shown in Fig. 6D shows that elevation of extracellular [Ca\textsuperscript{2+}] did not increase Ca\textsuperscript{2+} transient amplitude at any of the voltages tested. Thus Ca\textsuperscript{2+} transient amplitude appeared to saturate and did not increase in 5.0 mM extracellular [Ca\textsuperscript{2+}]. This saturation was independent of both voltage and contraction amplitude. This

Fig. 4. Contraction amplitudes, inward L-type Ca\textsuperscript{2+} current (I\textsubscript{Ca-L}), and Ca\textsuperscript{2+} transient amplitudes increase with increasing extracellular [Ca\textsuperscript{2+}]. A: different extracellular [Ca\textsuperscript{2+}] (0.1, 0.5, 2.0, and 5.0 mM) were applied with a computer-controlled rapid solution changer for 3 s before and throughout a step from −50 to 0 mV. The solution application and test step were preceded by 10 pulses at a rate of 2 Hz. Representative transmembrane currents (B), Ca\textsuperscript{2+} transients (C), and contractions (D) recorded in 0.1 (top), 0.5, 2.0, and 5.0 mM (bottom) extracellular [Ca\textsuperscript{2+}] are shown. Concentration-response curves for I\textsubscript{Ca-L} (E), Ca\textsuperscript{2+} transient (F), and contraction (G) amplitudes are shown. Dotted lines represent regressions to the mean data. I\textsubscript{Ca-L} (E) and contractions (G) increased in parallel with increasing extracellular [Ca\textsuperscript{2+}]. Ca\textsuperscript{2+} transient amplitude (F) increased with increasing extracellular [Ca\textsuperscript{2+}]; however, it appeared to saturate and did not increase in 5.0 mM extracellular [Ca\textsuperscript{2+}]. n = 6–14 Cells. All data are paired.
tion protocol is as shown in Fig. 4. Extracellular \([\text{Ca}^{2+}]\) was elevated. \(\text{Ca}^{2+}\) transient amplitude in 5.0 mM extracellular \([\text{Ca}^{2+}]\) was increased significantly in 5.0 mM extracellular \([\text{Ca}^{2+}]\) shown in Fig. 6, were measured directly preceding each of the eight test steps. Diastolic \([\text{Ca}^{2+}]\) increased significantly in 5.0 mM extracellular \([\text{Ca}^{2+}]\) for each of the voltages, and compared with intracellular \([\text{Ca}^{2+}]\). Activa-
tion protocol is as shown in Fig. 4A. Extracellular \([\text{Ca}^{2+}]\) was increased from 2.0 to 5.0 mM for 3 s following conditioning pulse train and throughout a test step from -50 to 0 mV. A and B: mean amplitudes of \(I_{\text{Ca-L}}\) and contractions increased significantly in 5.0 mM extracellular \([\text{Ca}^{2+}]\). C: diastolic and peak systolic intracellular \([\text{Ca}^{2+}]\) increased significantly when extracellular \([\text{Ca}^{2+}]\) was elevated. \(\text{Ca}^{2+}\) transient amplitude did not increase when extracellular \([\text{Ca}^{2+}]\) was elevated from 2.0 to 5.0 mM. n = 14 Cells. All data are paired.

indicates that the saturation of \(\text{Ca}^{2+}\) transient amplitude shown in Fig. 5 was not caused by an alteration in the voltage dependence of \(\text{Ca}^{2+}\) release in response to elevated extracellular \([\text{Ca}^{2+}]\). Mean diastolic \([\text{Ca}^{2+}]\) for each of the voltages, shown in Fig. 6E, were measured directly preceding each of the eight test steps. Diastolic \([\text{Ca}^{2+}]\) increased significantly when the extracellular \([\text{Ca}^{2+}]\) was elevated from 2.0 to 5.0 mM. The absence of changes in diastolic \(\text{Ca}^{2+}\) transient amplitude suggests that diastolic \([\text{Ca}^{2+}]\) is an important contributor to increased trans-
tract amplitude in 5.0 mM extracellular \([\text{Ca}^{2+}]\).

Surface charge screening caused by high extracellular divalent (21) concentrations might have influenced \(\text{Ca}^{2+}\) transient amplitude in 5.0 mM extracellular \(\text{Ca}^{2+}\). To further investigate this possibility, intracellular \(\text{Ca}^{2+}\) also was measured in myocytes exposed to 5.0 mM extracellular \(\text{Ca}^{2+}\) and 1.0 mM extracellular \(\text{Mg}^{2+}\) and compared with intracellular \(\text{Ca}^{2+}\) measurements from myocytes exposed to 2.0 mM extracellular \(\text{Ca}^{2+}\) and 4.0 mM \(\text{Mg}^{2+}\). Responses were elicited by the voltage protocol shown in Fig. 4A. Extracellular solution con-
taining 2.0 mM \(\text{Ca}^{2+}\) and 4.0 mM \(\text{Mg}^{2+}\) has the same divalent concentration as 5.0 mM \(\text{Ca}^{2+}\) and 1.0 mM \(\text{Mg}^{2+}\). When the mean responses of myocytes to 2.0 and 5.0 mM extracellular \(\text{Ca}^{2+}\) were compared at the same extracellular divalent concentration, diastolic and peak systolic \(\text{Ca}^{2+}\) increased significantly with increased extracellular \(\text{Ca}^{2+}\) (diastolic 167 ± 24 vs. 201 ± 28 mM and systolic 229 ± 29 vs. 276 ± 33 mM, n = 7, P < 0.05). \(\text{Ca}^{2+}\) transient amplitude, however, did not increase in 5.0 mM extracellular \(\text{Ca}^{2+}\) (62 ± 8 vs. 75 ± 13 mM, n = 7). Therefore, surface charge effects of elevated extracellular \([\text{Ca}^{2+}]\) did not alter \(\text{Ca}^{2+}\) transient amplitude.

**Effect of changing extracellular \([\text{Ca}^{2+}]\) on SR load.** In the next series of experiments, we determined whether elevation of the extracellular \([\text{Ca}^{2+}]\) and the subsequent elevation in diastolic \(\text{Ca}^{2+}\) levels had an effect on SR \(\text{Ca}^{2+}\) content. To that end, SR \(\text{Ca}^{2+}\) content was estimated by rapid application of caffeine. As in previous protocols, extracellular \([\text{Ca}^{2+}]\) was increased for 3 s following the conditioning pulse train. However, rather than a test step, a 1-s rapid application of 10 mM caffeine solution was used to induce a \(\text{Ca}^{2+}\) transient. A schematic representation of this protocol is shown in Fig. 7A. Figure 7B shows representative caffeine-induced \(\text{Ca}^{2+}\) trans-
sients. The amplitude of the caffeine-induced \(\text{Ca}^{2+}\) transient was not elevated when extracellular \([\text{Ca}^{2+}]\) was increased in this example. The mean data shown in Fig. 7C indicate that SR \(\text{Ca}^{2+}\) load was not significantly increased by elevation of extracellular \([\text{Ca}^{2+}]\) from 2.0 to 5.0 mM.

To further investigate SR \(\text{Ca}^{2+}\) load following a brief (3 s) application of 5.0 mM extracellular \(\text{Ca}^{2+}\), we also applied 10 mM caffeine in a solution containing 2.0 mM \(\text{Ca}^{2+}\) and 145 mM \(\text{Na}^{+}\) using the protocol shown in Fig. 7A. We measured the caffeine-induced \(\text{Ca}^{2+}\) transients and the corresponding inward, forward-mode NCX current induced by the caffeine application. Caffeine-induced \(\text{Ca}^{2+}\) transient amplitude and the integral of the inward current elicited in 145 mM \(\text{Na}^{+}\) following application of 5.0 mM \(\text{Ca}^{2+}\) (45 ± 6 mM and -367 ± 92 pC) did not differ significantly from those elicited in 2.0 mM extracellular \(\text{Ca}^{2+}\) (38 ± 9 mM and -271 ± 62 pC, n = 5; data are paired). We also investigated the possibility that EGTA in the high-\(\text{K}^{+}\) buffer influenced the peak caffeine-induced \(\text{Ca}^{2+}\) transient amplitude. To examine this possibility, caffeine-induced \(\text{Ca}^{2+}\) transient amplitudes were measured in myocytes, which were isolated in the absence of EGTA (0 EGTA in the high \(\text{K}^{+}\) buffer) and compared with data from cells isolated in the presence of EGTA. In myocytes isolated in the presence or absence of EGTA caffeine-induced \(\text{Ca}^{2+}\) transient amplitudes elicited in either 2.0 mM (144 ± 51 vs. 135 ± 32 nM) or 5.0 mM (190 ± 75 vs. 157 ± 38 nM; n = 3 and 4), extracellular \(\text{Ca}^{2+}\) did not differ significantly. Thus the positive inotropy seen under our experimental conditions occurred in the absence of changes in SR load. Also, the saturation of mean transient amplitude seen in Figs. 5 and 6 occurred in the absence of increased SR \(\text{Ca}^{2+}\) load.

**Effect of 100 \(\mu\text{M} \text{Cd}^{2+}\) on the increase in diastolic \(\text{Ca}^{2+}\) in myocytes at \(\text{Ca}^{2+}\) at \(\text{Ca}^{2+}\). Myocytes were exposed to buffer containing 100 \(\mu\text{M} \text{CdCl}_2\) to inhibit \(I_{\text{Ca-L}}\), window current, which could be activated when myocytes are held at \(-50 \text{ mV}\) for 3 s before the test step, as shown in the protocol in Fig. 8A. Figure 8B shows the mean intracellular \(\text{Ca}^{2+}\) responses of myocytes exposed to 2.0, 5.0, or 5.0 mM extracellular \(\text{Ca}^{2+}\) plus 100 \(\mu\text{M} \text{Cd}^{2+}\). \(\text{Cd}^{2+}\) (100 \(\mu\text{M})\) did not abolish the increases in diastolic.
and peak systolic intracellular Ca²⁺ associated with the application of 5.0 mM extracellular Ca²⁺. Therefore, in 5.0 mM extracellular Ca²⁺, diastolic Ca²⁺ does not increase due to prolonged activation of I_{Ca-L} window currents during the 3 s before the test step.

**Effect of inhibition of reverse mode NCX on diastolic Ca²⁺.**

To further investigate the mechanism responsible for the elevation in diastolic Ca²⁺ associated with the application of elevated extracellular [Ca²⁺], myocytes were voltage clamped with patch pipettes containing a nominally Na⁺-free intracellular solution. Dialysis of intracellular Na⁺ from the cytosol with patch pipettes should inhibit reverse-mode NCX (16). A schematic representation of the activation protocol is shown in Fig. 9A. Representative examples of fura-2 fluorescence recordings of the responses elicited on a step from −50 to 0 mV in 2.0 and 5.0 mM extracellular Ca²⁺ are shown in Fig. 9B. Diastolic and peak systolic Ca²⁺ appeared to be slightly elevated in 5.0 mM extracellular Ca²⁺. The mean data (Fig. 9C) indicate that only diastolic and peak systolic Ca²⁺ were significantly elevated, but Ca²⁺ transient amplitude was not. Thus diastolic Ca²⁺ was significantly elevated by a 3-s application of 5.0 mM extracellular Ca²⁺, even when myocytes were voltage clamped with patch pipettes containing a nominally Na⁺-free intracellular solution to inhibit reverse-mode NCX.

**DISCUSSION**

One of the objectives of this study was to determine whether diastolic Ca²⁺ plays a role in positive staircases. Our results showed that contraction amplitude depended on diastolic [Ca²⁺] in positive inotropic staircases of contraction generated in guinea pig ventricular myocytes. Ca²⁺ transient amplitudes remained constant, while diastolic [Ca²⁺] and contraction amplitudes increased in parallel over a series of stimulated pulses from rest. These findings are in agreement with results from some previous studies, which had shown positive inotropy in response to increasing diastolic [Ca²⁺] during positive staircases in guinea pig ventricular myocytes (20). In contrast, duBell and Houser (11) reported that progressive increases in Ca²⁺ transient amplitudes throughout positive staircases were the primary mediator of increasing contraction amplitude in ferret ventricular myocytes. However, duBell and Houser did not directly examine diastolic [Ca²⁺]. In our
saturation of the amplitudes of Ca\textsuperscript{2+} transients, was unanticipated. One possible explanation for our observation is that the amplitude of contractions increased because diastolic [Ca\textsuperscript{2+}] increased when extracellular [Ca\textsuperscript{2+}] was elevated. Bers (3, 4) has hypothesized that elevated diastolic [Ca\textsuperscript{2+}] should be inherently positively inotropic, because it necessarily increases the peak systolic [Ca\textsuperscript{2+}] and decreases the intracellular Ca\textsuperscript{2+} buffering capacity. The data presented in the present study support this hypothesis and demonstrate that positive inotropy can arise from elevation of diastolic [Ca\textsuperscript{2+}]. The elevation in diastolic [Ca\textsuperscript{2+}], which occurred in response to the increase in extracellular [Ca\textsuperscript{2+}], led to a corresponding increase in peak systolic [Ca\textsuperscript{2+}]. Thus positive inotropy occurred in the absence of increasing Ca\textsuperscript{2+} transient amplitude.

Figure 10A shows the hypothetical cell shortening-intracellular [Ca\textsuperscript{2+}] relationship (3, 4). When a Ca\textsuperscript{2+} transient of a given amplitude occurs, the intracellular [Ca\textsuperscript{2+}] increases from the diastolic [Ca\textsuperscript{2+}] to a peak systolic [Ca\textsuperscript{2+}], and cell shortening occurs. When the diastolic [Ca\textsuperscript{2+}] is elevated, as in Fig. 10B, a rightward shift in the starting point on the cell shortening-intracellular [Ca\textsuperscript{2+}] relationship occurs. This rightward shift leads to a larger contraction for a transient of the same amplitude as in Fig. 10A. This conclusion is supported by our contraction amplitude data, which show that contraction amplitude continues to increase for a constant Ca\textsuperscript{2+} transient amplitude where diastolic [Ca\textsuperscript{2+}] is increasing. Furthermore,
we have shown that increases in diastolic [Ca\(^{2+}\)] caused by increasing extracellular [Ca\(^{2+}\)] in resting myocytes cause cell shortening. This indicates that we are working in a range of diastolic [Ca\(^{2+}\)] where active resting tension can be produced.

We have shown that the contribution of diastolic Ca\(^{2+}\) to peak systolic Ca\(^{2+}\) was an important determinant of contraction amplitude when extracellular [Ca\(^{2+}\)] was increased and Ca\(^{2+}\) transient amplitude appeared to saturate. Also, a similar dependence of increasing contraction amplitude on diastolic [Ca\(^{2+}\)] exists for positive staircases of contraction. Other researchers also have shown positive inotropy in response to increasing diastolic [Ca\(^{2+}\)]. For example, Periyasamy et al. (29) showed that, when rat ventricular myocytes were exposed to uremic serum, diastolic [Ca\(^{2+}\)], systolic [Ca\(^{2+}\)], and percent shortening were significantly increased. However, although the authors did not measure it directly, there was no apparent effect of uremic serum on Ca\(^{2+}\) transient amplitude. Similar effects to those seen with uremic serum were seen with the Na\(^+\)-K\(^+\) pump inhibitor ouabain (29). Inhibition of the Na\(^+\)-K\(^+\) pump leads to accumulation of intracellular Na\(^+\) and promotes elevation of cytosolic Ca\(^{2+}\) through Na\(^+\)/Ca\(^{2+}\) exchange. Interestingly, Bers (4) hypothesized that blockade of the Na\(^+\)-K\(^+\) pump could lead to positive inotropy through elevation of diastolic [Ca\(^{2+}\)].

The mechanism responsible for the elevation in diastolic Ca\(^{2+}\) following an increase in extracellular Ca\(^{2+}\) was not clear. It is possible that Ca\(^{2+}\) influx through I\(_{\text{Ca-L}}\) window currents is responsible for the increase in diastolic Ca\(^{2+}\). Previous studies have shown that 100 µM Cd\(^{2+}\) blocks I\(_{\text{Ca-L}}\) window current (35). Interestingly, when we blocked I\(_{\text{Ca-L}}\) window currents with 100 µM Cd\(^{2+}\), we found that diastolic Ca\(^{2+}\) levels still increased in 5.0 mM extracellular [Ca\(^{2+}\)]. Thus the increase in diastolic Ca\(^{2+}\) associated with elevated extracellular Ca\(^{2+}\) cannot be attributed to I\(_{\text{Ca-L}}\) window currents.

Another mechanism that could account for the increase in diastolic Ca\(^{2+}\) is reverse mode NCX. However, when we dialyzed myocytes with patch pipettes containing a nominally Na\(^+\)-free intracellular solution to exclude the contribution of reverse mode NCX (16), diastolic Ca\(^{2+}\) increased significantly in 5.0 mM extracellular [Ca\(^{2+}\)]. Thus intracellular Ca\(^{2+}\) increased in response to increased extracellular [Ca\(^{2+}\)] when reverse-mode NCX was inhibited by the absence of intracellular Na\(^+\). Therefore, although NCX-induced Ca\(^{2+}\) entry can contribute to changes in intracellular Ca\(^{2+}\) in some situations (4, 24, 37), NCX-induced Ca\(^{2+}\) entry is not responsible for increases in diastolic Ca\(^{2+}\) in voltage-clamped myocytes held at −50 mV.

Therefore, the increased diastolic Ca\(^{2+}\), which occurs in response to elevated extracellular [Ca\(^{2+}\)], must arise through a mechanism other than I\(_{\text{Ca-L}}\) window current or reverse-mode NCX. Other possible mechanisms that might lead to the elevation of diastolic Ca\(^{2+}\) must, therefore, be responsible for the
increase in diastolic Ca\textsuperscript{2+} associated with elevated extracellular [Ca\textsuperscript{2+}]. Enhanced SR Ca\textsuperscript{2+} leak or a decrease in the extrusion of the Ca\textsuperscript{2+} that has leaked from the SR could contribute to the increase in diastolic Ca\textsuperscript{2+} in myocytes voltage clamped at \(-50\) mV.

Our results showed that Ca\textsuperscript{2+}-transient amplitudes did not increase in 5.0 mM extracellular [Ca\textsuperscript{2+}], despite increased \(I_{\text{Ca-L}}\) amplitude. This remained true when extracellular [Ca\textsuperscript{2+}] was elevated in the presence of 0.1 mM Ca\textsuperscript{2+}, when myocytes were voltage clamped with patch pipettes containing a nominally Na\textsuperscript{+}-free intracellular solution, throughout the entire transient-voltage relationship, and when extracellular divalent was balanced in 2.0 mM Ca\textsuperscript{2+} by increasing extracellular [Mg\textsuperscript{2+}]. Thus, despite many manipulations, we could not produce a statistically significantly larger Ca\textsuperscript{2+} transient, despite increased \(I_{\text{Ca-L}}\) amplitude, in 5.0 mM extracellular Ca\textsuperscript{2+}.

There are several possible explanations that could account for our finding that Ca\textsuperscript{2+} transient amplitudes did not increase when \(I_{\text{Ca-L}}\) amplitude continued to increase. First, it is possible that saturation of Ca\textsuperscript{2+} transient amplitude occurs in response to inhibition of CICR by elevated diastolic [Ca\textsuperscript{2+}]. Xu et al. (39) have shown that the RyR can be inhibited by high diastolic [Ca\textsuperscript{2+}], although the [Ca\textsuperscript{2+}] in their experiments were substantially higher than those seen in our experiments. Second, elevated intracellular [Ca\textsuperscript{2+}] may increase spark frequency (18), which would lead to an increase in the number of refractory spark units at any one time (33), and Ca\textsuperscript{2+} transient amplitude could saturate due to inactivation of that subset of refractory RyRs (33). Also, Ca\textsuperscript{2+} transient amplitude may saturate due to saturation of local control of CICR at the level of the RyR. A finite number of Ca\textsuperscript{2+} release units are coupled to L-type Ca\textsuperscript{2+} channels (32, 33); therefore, as the amplitude of \(I_{\text{Ca-L}}\) increases, the number of Ca\textsuperscript{2+} release units available for activation must approach a maximum. Once this maximum is reached, no further increase in Ca\textsuperscript{2+} transient amplitude could occur. Finally, it is possible that the saturation of Ca\textsuperscript{2+} transient amplitudes arises from a saturation of the amount of SR Ca\textsuperscript{2+} available for release in response to the trigger Ca\textsuperscript{2+} current. In support of this view, Janczewski et al. (22) have shown that, when extracellular Ca\textsuperscript{2+} is elevated, Ca\textsuperscript{2+} transient amplitude does not increase, despite increases in the amplitude of \(I_{\text{Ca-L}}\), unless SR Ca\textsuperscript{2+} load also is elevated. When the findings of Janczewski et al. are considered in conjunction with the findings of the present study, the data suggest strongly that saturation of the amount of Ca\textsuperscript{2+} available for release from the SR is responsible for the saturation of the Ca\textsuperscript{2+} transient, which we report in this study.

The results of this study may be relevant to disease states where diastolic Ca\textsuperscript{2+} is elevated. Following myocardial ischemia, in late reperfusion, Ca\textsuperscript{2+} transient amplitudes are depressed; however, diastolic [Ca\textsuperscript{2+}] is elevated (25). Elevated diastolic Ca\textsuperscript{2+} in this situation could contribute to larger contraction amplitudes, abrogating the contractile depression associated with myocardial stunning. However, in general, prolonged periods of elevated intracellular [Ca\textsuperscript{2+}] are damaging to the heart. Studies have shown that intracellular Ca\textsuperscript{2+} is increased in heart disease (40). In most disease states, such as heart failure, the increased intracellular [Ca\textsuperscript{2+}] levels do not improve contractile function (40). Rather, prolonged exposure to increased intracellular [Ca\textsuperscript{2+}] can activate pathways in which Ca\textsuperscript{2+} acts as a second messenger to produce deleterious genomic effects (1, 2, 10). Ca\textsuperscript{2+}-activated second-messenger signaling cascades, such as the Ca\textsuperscript{2+}-calcineurin/NFAT cascade, lead to gene expression changes seen in cardiac myocytes undergoing pathological hypertrophy (27, 28). Thus the increased diastolic [Ca\textsuperscript{2+}] level seen in heart failure is associated with deficits in contractile force generation, rather than improvements.

In summary, the results of this study demonstrate that diastolic [Ca\textsuperscript{2+}] can contribute to the positive inotropic effects that occur following rest or when extracellular [Ca\textsuperscript{2+}] is elevated. Therefore, although amplitudes of Ca\textsuperscript{2+} transients can be an important determinant of amplitudes of contractions, the amplitude of contraction can be further modulated by the contribution of diastolic [Ca\textsuperscript{2+}], which elevates the peak systolic [Ca\textsuperscript{2+}]. By causing a rightward shift in the cell shortening-intracellular [Ca\textsuperscript{2+}] relationship start point,
increases in diastolic \([Ca^{2+}]\) contribute significantly to the positive inotropy associated with the experimental manipulation of extracellular \([Ca^{2+}]\) and during positive staircases. In the situations examined throughout the course of this study, diastolic \([Ca^{2+}]\) was the primary contributor to the positive inotropic effects we observed.

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