Sustained Subthreshold-for-Twitch Depolarization in Rat Single Ventricular Myocytes Causes Sustained Calcium Channel Activation and Sarcoplasmic Reticulum Calcium Release

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ABSTRACT Single rat ventricular myocytes, voltage-clamped at -50 to -40 mV, were depolarized in small steps in order to define the mechanisms that govern the increase in cytosolic [Ca^{2+}] (Ca_i) and contraction, measured as a reduction in myocyte length. Small (3-5 mV), sustained (seconds) depolarizations that caused a small inward or no detectable change in current were followed after a delay by small (<2% of the resting length), steady reductions in cell length measured via a photodiode array, and small, steady increases in Ca_i measured by changes in Indo-1 fluorescence. Larger (greater than -30 and less than -20 mV), sustained depolarizations produced phasic Ca^{2+} currents, Ca_i transients, and twitch contractions, followed by a steady current and a steady increase in Ca_i and contraction. Nitrendipine (or Cd, verapamil, or Ni) abolished the steady contraction and always produced an outward shift in steady current. The steady, nitrendipine-sensitive current and sustained increase in Ca_i and contraction exhibited a similar voltage dependence over the voltage range between -40 and -20 mV. 2 μM ryanodine in the presence of intact Ca^{2+} channel activity also abolished the steady increase in Ca_i and contraction over this voltage range. We conclude that when a sustained depolarization does not exceed about -20 mV, the resultant steady, graded contraction is due to SR Ca^{2+} release graded by a steady ("window") Ca^{2+} current. The existence of appreciable, sustained, graded Ca^{2+} release in response to Ca^{2+} current generated by arbitrarily small depolarizations is not compatible with any model of Ca^{2+}-induced Ca^{2+} release in which the releasing effect of the Ca^{2+} current is due to real SR Ca^{2+} release graded by a steady ("window") Ca^{2+} current.
channel current is mediated solely by Ca\(^{2+}\) entry into a common cytosolic pool. Our results therefore imply a distinction between the triggering and released Ca\(^{2+}\) pools.

**INTRODUCTION**

Studies of excitation-contraction coupling in bulk mammalian cardiac muscle using voltage-clamp techniques have identified the presence of a Ca\(^{2+}\) current, \(I_{Ca}\), and its relationship to contraction (Beeler and Reuter, 1970a, b; New and Trautwein, 1971; Gibbons and Fozzard, 1975). In individual cardiac cells that have had their sarcolemma mechanically removed (Fabiato and Fabiato, 1975; Fabiato, 1981), the rapid application of a Ca\(^{2+}\) "trigger" can cause a release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR). This has led to a hypothesis that \(I_{Ca}\) may provide this trigger function in cells with intact sarcolemma (Fabiato, 1985b). Recent studies in voltage-clamped single adult cardiac myocytes with intact sarcolemmal function have more precisely defined the characteristics of \(I_{Ca}\) (Isenberg and Klockner, 1982; Mitchell et al., 1983; Josephson et al., 1984b) and the voltage dependence of current, and of the cytosolic Ca\(^{2+}\) (\(C_{a}\)) transient or contraction (Isenberg, 1982; London and Krueger, 1986; Barcenas-Ruiz et al., 1987; Cannell et al., 1987; Mitchell et al., 1987a, b; Beuckelmann and Wier, 1988; Callewaert et al., 1988; Houser et al., 1988; Isenberg et al., 1988; Talo et al., 1988). While these studies have provided evidence consistent with the notion that \(I_{Ca}\) per se, is sufficient to trigger Ca\(^{2+}\) release, a role for depolarization in addition to Ca\(^{2+}\) influx via \(I_{Ca}\) has been suggested (Cannell et al., 1987). Thus, additional information is needed to determine how \(I_{Ca}\) might be coupled to SR Ca\(^{2+}\) release and thus to contraction.

The careful observation and definition of near-threshold events for contraction and Ca\(^{2+}\) release in skeletal muscle has provided critical information regarding excitation-contraction coupling mechanisms in that tissue (for review, see Schneider, 1986). This suggested to us that studies of near-threshold events in cardiac myocytes could possibly provide novel information regarding Ca\(^{2+}\)-induced Ca\(^{2+}\) release and the role of \(I_{Ca}\) in coupling this release to membrane depolarization. In this study we measured current, contraction, and changes in \(C_{a}\) using the fluorescent probe, Indo-1 AM, in rested-state, single rat cardiac myocytes, with particular emphasis on steady, near-threshold-for-twitch events. Our major findings are that sustained depolarizations of only a few millivolts from holding potentials of \(-40\) to \(-50\) mV, which are subthreshold for a twitch, can lead to a sustained small increase in \(C_{a}\) and a small steady contraction; these events require functional Ca\(^{2+}\) channels as they are abolished by Ca\(^{2+}\) channel blockers. Thus, a small depolarization itself cannot increase \(C_{a}\) and cause a contraction. Further, \(I_{Ca}\) does not directly cause the increase of \(C_{a}\) under these conditions; rather it appears to result from Ca\(^{2+}\) release from the SR, as it is abolished by ryanodine. That continued "low grade" Ca\(^{2+}\) release from SR can occur without becoming regenerative and producing a rapid phasic increase and twitch may define a boundary condition for models that attempt to explain Ca\(^{2+}\)-induced Ca\(^{2+}\) release from SR in cardiac myocytes.
METHODS

Myocyte Isolation

Left ventricular myocytes were obtained from 2–6-mo-old rats by retrograde perfusion of the aorta with a low Ca\(^{2+}\) collagenase bicarbonate buffer as previously described (Capogrossi et al., 1986). After the dissociation procedure cells were resuspended in HEPES buffer of the following composition (in mM): 137 NaCl, 1.2 MgSO\(_4\), 5 KCl, 20 HEPES, and 16 d-glucose. CaCl\(_2\), unless otherwise indicated, was 1 mM; pH was 7.4.

Current Measurements

Petri dishes containing cells were placed in a chamber on the stage of an inverted microscope (Leitz Diavert; Wetzlar, FRG). The chamber was slowly perfused with the solution described above and kept at +26°C. In experiments in which changes in Ca\(^{2+}\) were simultaneously measured with contraction and current an IM-35 microscope (Zeiss, Oberkochen, FRG) was used and temperature was 23°C (see below). After the application of the patch electrode to the center of the myocyte, formation of the gigaseal, and lifting-up of the cell the pipette voltage was adjusted to −70 mV and the cell membrane inside the electrode was broken by suction. Cells chosen for the study were those in which there were no or minimal excitation results from this procedure. Whole-cell currents were measured by the patch-clamp technique using an amplifier (either a model 8800 [DAGAN Corp., Minneapolis, MN] or an AXOPATCH-1A [Axon Instruments, Burlington, CA]).

The electrodes were drawn of borosilicate glass with a fiber, heat polished, and filled with a solution containing 150 mM KCl, 10 mM HEPES, and 5 μM EGTA, pH 7.2, adjusted by KOH. Adding 5 μM EGTA buffered the [Ca] of the fluid to <10^{-7} M so that the electrode was not a Ca\(^{2+}\) source for the cell. However, while the small amount of EGTA did not affect electrically evoked contractions, it usually prevented the occurrence of spontaneous SR Ca\(^{2+}\) release that is often observed in rat myocytes in 1 mM bathing Ca\(^{2+}\) (Capogrossi et al., 1986). For comparisons, some experiments were conducted by adding 50 μM EGTA into the pipette. This resulted in a gradual slow reduction of velocity and amplitude of electrically stimulated contractions. A further increase in EGTA concentration to 500 μM or 1 mM in other cells accelerated this reduction of the contraction amplitude.

We were concerned with two methodological problems that could affect current measurement in these cells. First, the resting cell length of single adult cardiac myocytes (i.e., those used in the present study) can be as great as 150 μm and can produce I\(_{\text{Ca}}\) exceeding 1 nA. This may limit the fidelity of the whole-cell voltage-clamp unless sufficiently large tip diameter pipettes are used (Hamill et al., 1981). Our preliminary experiments with two simultaneous gigaseal electrodes showed that adequate voltage control in these cells may not always be obtained when the electrode resistance is >5 MΩ. Thus, the resistance of all electrodes used in the present study ranged from 1 to 3 MΩ with the majority being <2 MΩ. Second, with time during the experiment the electrode tip occasionally becomes partially resealed by the membrane and thus causes the access resistance not to remain stable with time after the break into the whole-cell mode. In the present study this was monitored by periodically comparing the current signal resulting from the voltage step to that in the beginning of the recording after capacitive compensation was made. If a decrease in the current signal at the same holding potential was observed, the pipette was reopened by applying additional suction or gentle (back) pressure.

Positive voltage steps of varying amplitude lasting up to 5 s were made with 1 min rest between the steps. To inactivate Na\(^+\) and transient outward K\(^+\) currents (Josephson et al.,
1984a, b), clamp steps were made from the holding potential of $-50$ to $-40$ mV. The steady $\text{Ca}^{2+}$ current, measured at the end of the clamp step, was taken as a difference between the control current and that measured after nitrendipine (1 $\mu$M). Voltage steps of long duration to membrane potentials positive to 10 mV were not used routinely to avoid excessive $\text{Ca}^{2+}$ loading of the myocytes, indicated by appearance of spontaneous contraction waves that gradually disappeared after stepping back to the holding potential.

Contractive Measurements

The cell contraction was measured optically by projecting the cell's image on a self-scanning photodiode array (model RC100B; Reticon, Sunnyvale, CA) consisting of 1,040 diodes. The array was scanned every 5 ms. Since the cell was fixed only to the patch electrode positioned in its center it was free to contract. Measurements of the cell contraction were made while the cell was lifted from the bottom of the chamber by the patch electrode.

In experiments in which contraction was measured simultaneously with measurements of the intracellular free $\text{Ca}^{2+}$ the cell length was measured by a video edge-tracking technique as previously described (Capogrossi et al., 1986), or by a diode system as described above that used a Starlight (Reticon) diode array. In these studies the cells were lightly attached to the bottom of the perfusion chamber (Capogrossi et al., 1986).

Measurements of Changes in $\text{Ca}_i$

In some experiments, in addition to current and contraction, the change in $\text{Ca}_i$ in response to depolarization was assessed by loading cells with the fluorescence $\text{Ca}^{2+}$ probe, Indo-1 AM (Poenie et al., 1986). The apparatus and procedure that we used to monitor changes in $\text{Ca}_i$ in single cardiac myocytes has recently been reported (Spurgeon et al., 1988a). Briefly, single cardiac myocytes were loaded with Indo-1 AM at 23°C in a chamber on the stage of the microscope. The method utilizes Indo-1 AM dissolved in dimethylsulfoxide and mixed with fetal calf serum and a dispersing agent, Pluronic F-127 (Poenie et al., 1986). Indo-1 fluorescence was excited by epi-illumination with 3.8-μs flashes of 350-nm light at repetition rates of up to 250 Hz. Indo-1 emission was collected by paired photomultipliers to simultaneously measure spectral windows centered on 410 and 490 nm, selected by bandpass interference filters. The fluorescence emission from each flash was collected by a pair of fast integrator sample-and-hold circuits of custom design under the control of a VAX 11/730 (Digital Equipment Corp., Marlboro, MA) computer which, for each flash, calculated the ratio of Indo-1 emission at the two wavelengths as a measure of $\text{Ca}_i$. The degree of Indo-1 loading achieved in the present study was sufficient to permit measurements of $\text{Ca}_i$ changes in response to individual voltage-clamp steps and thus avoided averaging the results of multiple clamps. By using red light (700–750 nm) for a bright-field image and a dichroic mirror to transmit the fluorescent light (380–550 nm) and reflect the red light, changes in cell length and $\text{Ca}_i$ were obtained simultaneously without cross talk. In these experiments cell length was measured from the image of the cell via the video edge tracking method described above.

Drugs

Nitrendipine was dissolved in DMSO to achieve a 1-mM stock solution which was kept refrigerated. This was added directly into the bath to result in a 1-μM concentration. During this procedure the flow through the bath was stopped. Ryanodine (Pennick, Lyndhurst, NJ) was dissolved in distilled water (1 mM stock solution) and added to the superfusate to achieve desired concentrations.
RESULTS

Voltage Dependence of Ca\textsuperscript{2+} and Contraction in Rested Rat Myocytes

Fig. 1 shows the effect of the magnitude of depolarization on membrane current and contraction in a representative myocyte, rested for 2 min, that had not been loaded with the Ca\textsuperscript{2+} indicator. Depolarization of sufficient magnitude from a holding potential of $-45\text{ mV}$ produces a phasic inward current and a phasic shortening of the cell, i.e., a “twitch” contraction. When depolarization is sustained, as in this case, the cell relengthens, but not to the fully relaxed resting length; thus, a steady or “tonic” contraction persists. Both the amplitude of the phasic twitch shortening and phasic current are graded with the magnitude of the depolarization. The figure also shows that small depolarizations that are subthreshold for producing a phasic current or twitch nonetheless cause a small (<50 pA), rapid step change in current. A main point of the figure is that following a delay (up to 100 ms) after the onset of this small but sustained inward current, a slowly developing, small sustained contraction occurs; this contraction does not have a phasic component. A continuity among the “tonic” contraction in response to a subthreshold-for-twitch depolarization, the “phasic” twitch and the post-twitch “tonic” contraction can be appreciated from
stacked plots of the contraction tracings measured across a wide voltage range (Fig. 2). In the midst of this continuum, the twitch emerges as a phasic event that interrupts what would otherwise be a steady slowly developing contraction that increases in amplitude as the magnitude of the depolarization increases and that persists at a "tonic" level until repolarization occurs.

Fig. 2 also shows that the delay between depolarization and the first detectable contraction, whether it be the monophasic steady contraction resulting from a subthreshold-for-twitch depolarization, or a twitch, is dependent on the magnitude of the voltage step. The voltage dependence of this delay time to the onset of contraction for all non-Indo-loaded cells studied is shown in Fig. 3. The steep part of the voltage dependence occurs from \(-40\) to \(-20\) mV and the delay intervals range from 400 to 50 ms. Depolarization to levels within this range encompasses both the subthreshold-for-twitch and slowly developing phasic twitches; the amplitudes of both are graded with the magnitude of the depolarization. That the delay time for first detectable contraction after depolarization varies as a continuous function of voltage, regardless of whether or not the resulting contraction eventually becomes a "twitch" or just a sustained, low grade, steady contraction without ever evolving into a "twitch" suggests a link between the source of the subthreshold-for-twitch and the twitch events. Depolarizations to >\(-20\) mV elicit twitches after a nearly constant delay of \(~50\) ms.

Fig. 4 shows membrane current, cell length, and Indo-1 fluorescence measured simultaneously in a myocyte that had been loaded with the \(\text{Ca}^{2+}\) indicator. \(A\) and \(B\)
show that a voltage step from \(-45\) mV to \(-37.5\) or \(-35\) mV gives rise to slow increases in Indo-1 fluorescence and shortening of the cell. Removal of the small depolarization step permits a reduction in fluorescence and cell relaxation. A step to \(0\) mV (D) elicits a phasic inward current followed by rapid phasic increase in \(\text{Ca}_i\) and a phasic contraction, which are then followed by tonic components during the sustained depolarization. Subsequent repolarization to the holding potential results

**FIGURE 3.** Delay from the depolarizing voltage step to the beginning of the contraction as a function of the membrane voltage. Each circle is an individual measurement.

**FIGURE 4.** Simultaneous recordings of membrane current (bottom trace), changes in \(\text{Ca}_i\) (top trace), and cell length (middle trace) in a representative voltage-clamped rat ventricular myocyte in response to step increases in membrane potential of varying magnitude. Calcium was measured as the ratio of Indo-1 fluorescence emitted at 410 nM to that at 490 nM. Increase of \(\text{Ca}_i\) is upwards, and cell shortening is downwards. See text for further details.
in a decay of Ca$_i$ to the resting level and full relengthening of the cell. C illustrates the response to a voltage step that is intermediate between those in B and D. Fig. 5 shows, in another cell, that a threefold increase in bathing [Ca$^{2+}$] produces only minor changes in the steady increase in Ca$_i$ during sustained depolarization.

**Ca$^{2+}$ Currents Are Required Not Only for Twitches but Also for Subthreshold-for-Twitch, and Post-Twitch "Tonic" Contractions**

Fig. 6, A and B, shows that the mechanism that couples depolarization to the phasic increase in Ca$_i$ and contraction in response to a large depolarization (to 0 mV) is nitrendipine sensitive. That nitrendipine concomitantly abolishes the Ca$^{2+}$ current is compatible with the hypothesis that activation of L-type Ca$^{2+}$ channels is involved in coupling the depolarization to the rapid increase in Ca$^{2+}$ (London and Krueger, 1986; Barcenas-Ruiz and Wier, 1987; Callewaert et al., 1988; Isenberg et al., 1988; Talo et al., 1988). However, it could be argued that this nitrendipine effect to block the Ca$_i$ transient and contraction after depolarization is due to a depletion of the SR Ca$^{2+}$ load. That this is not the case is shown in C, as caffeine spritzed onto the same cell 80 ms after the onset of a depolarization in the presence of nitrendipine (C) still elicits a large increase in Ca$_i$ and contraction. The caffeine-induced increase in Ca$_i$ and contraction represents Ca$^{2+}$ release from the SR as it is abolished by ryanodine (D).

Fig. 7 shows the effect of nitrendipine on the steady current and steady contraction in response to a sustained depolarization. In A nitrendipine abolishes the steady contraction, and this is accompanied by an outward small sustained change in current. Not all small depolarizations that led to "subthreshold-for-twitch" contractions produced an inward current. Rather, in some cells the small, steady contraction in response to a subthreshold-for-twitch depolarization occurred in the absence of a
detectable change in current, and in some others a small outward current was recorded, as exemplified in the cell depicted in Fig. 7B. Nonetheless, even when no change or an outward shift of the current occurred in response to the subthreshold-for-twitch depolarization, in every instance (n = 12) blockade of Ca\(^{2+}\) channels by nitrendipine (or by other L-type channel antagonists (1 mM Mn, 0.1 mM Cd, or 1 \(\mu\)M Verapamil) produced a net outward current shift during the depolarization and abolished contraction.

Fig. 7C shows that in response to larger depolarizations that elicit phasic contractions, nitrendipine abolishes not only the phasic current and resultant contractions but also the time-dependent steady component of current and contraction that follows the twitch during long depolarizations. The abolition of "tonic" contraction after a twitch was accompanied by an outward shift in the steady current.

![Figure 6](image)

**Figure 6.** A, Depolarization to 0 mV elicits a rapid increase in Ca\(_{\text{in}}\), measured as an increase in the Indo-1 fluorescence ratio, and contraction. B, Both events in the same cell as A are blocked by nitrendipine (Nitr, 2 \(\mu\)M). C, In the same cell as in A and B, in the continued presence of nitrendipine, caffeine (Caf) can still elicit a large increase in Ca\(_{\text{in}}\) (Caffeine was spritzed onto the cell via a second micropipette [caffeine concentration = 10 mM] at 80 ms after the initiation of the 300-ms voltage step to 0 mV). Before that time the depolarization itself, as in B, did not elicit a rapid increase in Ca\(_{\text{in}}\). D, In the continued presence of nitrendipine plus 5 \(\mu\)M ryanodine in the same cells as A–C, caffeine fails to elicit a large increase in Ca\(_{\text{in}}\).

The average nitrendipine sensitive steady contraction and steady nitrendipine sensitive current, i.e., that just before repolarization, measured across a wide range of clamp potentials that encompasses the sub-twitch steady contractions, and twitches followed by steady contractions are illustrated in Fig. 8. The current depicted is the difference current between that measured prior to and in the presence of nitrendipine in each cell. It closely resembles that measured recently in another study of rat myocytes (Cohen and Lederer, 1987) from activation–inactivation parameters of the Ca\(^{2+}\) current. The main point of the figure is that over the range of depolarization steps (i.e., from -40 to about -20 mV) the magnitude of the small, steady contraction varies directly with the magnitude of the steady
Figure 7. A, The effect of nitrendipine (Nitr; 1 μM) on the maintained shortening (top trace) and on the holding current (middle trace) during a 10-mV voltage step from a holding potential of -50 mV. In the presence of nitrendipine the current during the step shifts outward and the contraction disappears. B, Nitrendipine produces a net outward shift during sustained subthreshold-for-twitch depolarization, even when that depolarization caused a small outward current before drug addition. C, In response to a larger depolarization (-45 to -15 mV) nitrendipine abolishes the twitch and post-twitch tonic contraction, abolishes the phasic inward current, and causes an outward shift in the steady current.

nitrendipine-sensitive current. However, with larger voltage steps (i.e., positive to -20 mV) magnitude of the steady (post-twitch) nitrendipine-sensitive current becomes reduced while the mean steady contraction that follows the twitch does not. This indicates that a second, steady, voltage-dependent, nitrendipine-insensitive
process contributes to the steady contraction that persists after a twitch at these more positive lower membrane potentials.

The results of Figs. 7 and 8 clearly show that depolarization per se, to voltages negative to about −20 mV, cannot be the cause of steady contractile activation because this contraction is abolished during the depolarization in the presence of nitrendipine. Fig. 6 shows that a greater depolarization (i.e., to zero mV) is not sufficient to cause a rapid phasic increase in Ca, and twitch in the presence of full SR Ca\(^{2+}\) loading when Ca\(^{2+}\) current is blocked. Thus, a requirement for Ca\(^{2+}\) current appears to be a common mechanism required for the continuum of the steady contraction resulting from small subthreshold-for-twitch steady depolarizations and post-twitch “tonic” contraction during sustained depolarization to −20 mV or less as well as for the phasic rapid twitch elicited by depolarization of any magnitude.

**FIGURE 8.** Voltage dependence of the average nitrendipine-sensitive current (shaded area) and sustained contraction scaled to a common maximum value at −20 mV. Bars indicate SEM; \(n = 8\) for current and \(12\) for length. Note that the maximal average sustained shortening is small (i.e., only 2\% of the cell resting length).

*Intact Sarcoplasmic Reticulum Function Is Required for the Steady Subthreshold-for-Twitch Contraction and Post-Twitch Tonic Contractions, as Well as for Rapid Phasic Twitches*

Ryanodine inhibits SR Ca\(^{2+}\) release in cardiac preparations (Sutko, 1985); in single cardiac myocytes this effect can be attributed to a depletion of SR Ca\(^{2+}\) content (Hansford and Lakatta, 1987; Silverman et al., 1988; Spurgeon et al., 1988b). We used ryanodine to determine the role of intact SR Ca\(^{2+}\) function not only in producing a phasic twitch, but also for its possible role in the sustained contractile activation due to the subthreshold-for-twitch depolarization, and in the sustained contraction after a twitch during sustained depolarization. If the role of Ca\(^{2+}\) current demonstrated above is to provide Ca\(^{2+}\) which directly activates myofilaments,
then ryanodine should be without an effect, or might even lead to an enhancement of these steady contractions, due to its effect of promoting a Ca$^{2+}$ leak into the cytosol (Hansford and Lakatta, 1987).

Fig. 9 shows that ryanodine, while not reducing the associated inward current, abolishes the steady contraction in response to a subthreshold-for-twitch depolarization. This suggests that the sustained myofilament activation that results from small depolarizations involves Ca$^{2+}$ release from the SR and cannot be the result of direct activation of the myofilaments via transsarcolemmal Ca$^{2+}$ influx due to the depolarization.

The combined results of Figs. 6-9 suggest that the steady contraction resulting from subthreshold-for-twitch depolarization, the phasic twitch, and the "tonic" contraction that follows the twitch during sustained depolarization all require both intact SR function and functional Ca$^{2+}$ channels, since all three are abolished by either nitrendipine in the presence of intact SR function or by ryanodine in the presence of intact Ca$^{2+}$ channels.

![DISCUSSION](image)

**FIGURE 9.** Ryanodine (lower traces) abolishes the increase in Ca$_i$ and contraction in response to small voltages steps.

**DISCUSSION**

*Increase in Ca$_i$ and Contraction Induced by Depolarization That Is Subthreshold for a Phasic I$_{Ca}$ and Twitch*

A novel and most important result of this study is that the small subthreshold-for-twitch depolarization can cause a sustained net inward current that is blocked by maneuvers (nitrrendipine, Cd, Ni$^{2+}$) that inhibit I$_{Ca}$. The resultant small, steady increase in Ca$_i$ and contraction require the presence of this current, because in its absence they do not occur. The small increase in Ca$^{2+}$ and small contraction caused by a subthreshold-for-twitch depolarization are inhibited by ryanodine. We interpret this to indicate that the immediate source of steady Ca$^{2+}$ increase in Ca$_i$ that sustains the small, steady contraction is SR Ca$^{2+}$ release.
In prior studies of the bulk of cardiac ventricular muscle events of such a small magnitude as these, subthreshold-for-twitch events could not be clearly defined. Among the possible reasons for this are a lack of sensitivity and difficulty in clamping membrane voltage, the relative insensitivity of the photoprotein aequorin to small changes of $\mathrm{Ca}_i$ from the resting level, and an inability to measure small changes in myofilament contraction due to muscle end stray compliance, necessitated by mounting constraints.

While L-type Ca$^{2+}$ channel current, $\mathrm{Ca}_n$, and contraction have been measured in single cardiac cells, relatively little attention has been given to those events that are subthreshold for triggering a phasic twitch. Lee and Tsien (1982) had noticed a small noninactivating Ca$^{2+}$ current at this voltage range. However, it has been recently reported (Cannell et al., 1987) that depolarization from about $-64$ to about $-50$ mV in rat myocytes increased $\mathrm{Ca}_n$ but did not produce a detectable Ca$^{2+}$ current. In this study the thresholds for Ca$^{2+}$ release and contraction are not dissociated or shifted leftward of the activation of $I_{\mathrm{Ca}}$ (Cannell et al., 1987) because small depolarizations in the presence of nitrendipine fail to elicit an increase in $\mathrm{Ca}_n$ or contraction. In guinea pig cells small depolarizations that elicited Ca$^{2+}$ release were also accompanied by a verapamil-sensitive current (Beuckelmann and Wier, 1988). The subthreshold-for-twitch events reported here for single cells can easily escape detection if just current measurements rather than simultaneous measurement of current and contraction or $\mathrm{Ca}_i$ are made. In other words, a small, steady change in current induced by a small (5 mV) depolarization in the absence of a simultaneous measurement of contraction and $\mathrm{Ca}_i$ are made. Additionally, as the near-threshold events develop slowly and after a delay of up to 400 ms, their presence would not have been detected during short depolarizing clamps. Small depolarizations of shorter duration can fail to detect this; thus, for short duration clamps (e.g., 200 ms or less) from the holding potential range used in the present study the threshold for Ca$^{2+}$ release would have been shifted to more positive membrane potentials than for longer clamps. Thus, when the delay to onset of Ca$^{2+}$ release in response to small clamp steps (Fig. 5) exceeds the clamp duration, a gradually increasing monotonic event is observed and the near-threshold Ca$^{2+}$ release appears as a delayed phasic event with a slow rate of rise (i.e., a small, slowly developing phasic twitch).

**Post-Twitch “Tonic” Increase in $\mathrm{Ca}_i$ and Tension during Sustained Depolarization**

Larger depolarizations that are sufficient to trigger a phasic increase in $\mathrm{Ca}_k$ and a twitch contraction, if sustained, lead to a steady "tonic" increase in $\mathrm{Ca}_i$ and contraction. Post-twitch "tonic" levels of $\mathrm{Ca}_i$ and contraction are less in magnitude than those of the twitch. This indicates that under conditions of this study cell membrane (i.e., SR and sarcolemmal), mechanisms that modulate Ca$^{2+}$ fluxes can reduce $\mathrm{Ca}_k$ even in the presence of sustained depolarization. Thses mechanisms include a time-dependent reduction in the number of open Ca$^{2+}$ channels (inactivation) during the sustained depolarization; a purported $\mathrm{Ca}_k$ inhibition of SR Ca$^{2+}$ release; and a $\mathrm{Ca}_k$-induced stimulation of the SR Ca$^{2+}$ pump or Na$^+$/Ca$^{2+}$ exchanger. The present results indicate, however, that these post-twitch "tonic" events due to depolarization to $-15$ or 20 mV or less, like the steady increase in $\mathrm{Ca}_k$ and
contraction in response to small, subthreshold-for-twitch depolarization, require both sustained Ca\(^{2+}\) current and SR Ca\(^{2+}\) release; thus, the resultant sustained contraction is not due to direct myofilament Ca\(^{2+}\) activation via transsarcolemmal Ca\(^{2+}\) influx. These data support a hypothesis raised in earlier studies, i.e., that transsarcolemmal Ca\(^{2+}\) influx does not necessarily lead to direct myofilament Ca\(^{2+}\) activation; rather, it is initially sequestered by intracellular Ca\(^{2+}\) sinks, the most prominent of which is the SR (Reiter et al., 1984; Fabiato, 1985a).

The L-type Ca\(^{2+}\) current dependence of this post-twitch sustained increase in Ca\(_i\) is compatible with studies in myocyte suspensions which show that application of KCl, which leads to sustained depolarization to about −25 mV, causes a sustained increase in systolic Ca\(^{2+}\) that can in many instances be totally blocked by Ca\(^{2+}\) channel blockers (Sheu et al., 1986). That ryanodine, as well as Ca\(^{2+}\) channel blockers, eventually completely inhibit the sustained increase in Ca\(_i\) and contraction in response to depolarization over this range (i.e., −40 to about −20 mV, but up to 5 mV in some cells), suggests that in the cells used in this study, the Na\(^+/Ca^{2+}\) exchanger does not have a major role in the sustained contraction (over the noted clamp potential range). However, it is well recognized that larger depolarizations can produce Ca\(^{2+}\) influx via Na\(^+/Ca^{2+}\) exchange and can contribute to a steady increase in Ca\(_i\). The magnitude of this contribution depends on the extent of cell Na\(^+\) loading as well as on the duration and magnitude of depolarization (Kimura et al., 1986; Barcenas-Ruiz et al., 1987; Isenberg et al., 1988). In this regard, in the present study we did observe that in some cells depolarization to −20 mV or above produced a low amplitude contraction that developed slowly and after a delay of several seconds and was not abolished by Ca\(^{2+}\) channel inhibitors. This contraction was probably mediated via Na\(^+/Ca^{2+}\) exchange. The contribution of Na\(^+/Ca^{2+}\) exchange to cell Ca\(^{2+}\) homeostasis in this study may have been underestimated by dilution of cell [Na\(^+\)], by the Na\(^+\)-free electrolyte pipette solution. This, our results do not specifically address the role of Na\(^+/Ca^{2+}\) exchanger in cell Ca\(^{2+}\) homeostasis. However, the conditions used, perhaps by blunting the role of Na\(^+/Ca^{2+}\) exchange by diluting the cell Na\(^+\) content at constant extracellular [Na\(^+\)], in essence, may have resulted in a specific Na\(^+/Ca^{2+}\) inhibition, thus permitting a clear definition of a role of Ca\(^{2+}\) currents and intact SR function.

Depolarization-induced Phasic I\(_{Ca}\) Cytosolic Ca\(^{2+}\) Transients, and Twitch Contractions

The present observations using Ca\(^{2+}\) channel blockers or ryanodine show that large depolarization (to >−30 mV) in rat cells are similar to those of recent studies in single adult mammalian cardiac cells that have been interpreted to indicate a necessary role for I\(_{Ca}\) in the rapid phasic increase in Ca\(_i\) or twitch contraction that is elicited by depolarization (Isenberg, 1982; Barcenas-Ruiz and Wier, 1987; Cannell et al., 1987; Beuckelmann and Wier, 1988; Callewaert et al., 1988; Houser et al., 1988; Isenberg et al., 1988). This large body of data is consistent with the hypothesis that I\(_{Ca}\) mediates the release of Ca\(^{2+}\) from the SR to cause a twitch (Fabiato, 1985a). However, recent studies in skeletal muscle have been interpreted to indicate that nitrendipine not only blocks Ca\(^{2+}\) current but also blocks voltage-dependent, asymmetric intramembranous charge movement that is thought to couple the
depolarization to SR Ca\(^{2+}\) release (Rios and Brum, 1987). It might be argued that in the present study this type of charge movement and not Ca\(^{2+}\) current couple a large depolarization to the rapid phasic increase in Ca\(_i\) which leads to a twitch. That this is not the case is suggested by other experiments that show that depolarization exceeding the reversal potential for Ca\(^{2+}\) channels (i.e., to \(-100\) mV), which abolishes Ca\(^{2+}\) current but ought not to decrease intramembranous charge movement, abolishes Ca\(^{2+}\) release or contraction in rat and guinea pig cardiac cells (London and Krueger, 1986; Cannell et al., 1987; Beuckelmann and Wier, 1988; Isenberg et al., 1988; Nabauer et al., 1989). Failure of depolarization to this level to elicit a rapid and large increase in Ca\(_i\) also indicates that the Na-Ca exchanger is not the physiologic trigger for SR Ca\(^{2+}\) release. Thus, the nitrendipine effect to abolish a rapid phasic Ca\(^{2+}\) release and twitch contraction in the present study (Figs. 6 and 7 C) is interpreted to result from its effect to block L-type Ca\(^{2+}\) current, and is not specifically due to a possible concomitant inhibition of intramembranous charge movement. Our observation that cadmium, which supports depolarization-induced intramembranous charge movement but fails to elicit a contract in response to small depolarizations, also supports this interpretation. That the intracellular Ca\(^{2+}\) storage site from which Ca\(^{2+}\) is released by \(I_{Ca}\) is the SR is strongly suggested by the previous (Barcenas-Ruiz and Wier, 1987) and present observations that ryanodine, which specifically abolishes SR Ca\(^{2+}\) release (Sutko, 1985), probably by depleting the SR of Ca\(^{2+}\) (Bers, 1985, 1987; Hansford and Lakatta, 1987; Silverman et al., 1988), abolishes the depolarization-induced rapid phasic increase in Ca\(_i\) and twitch while the Ca\(^{2+}\) current is preserved. A novel finding of the present study is that the abolition of the Ca\(_i\) transient by nitrendipine is not due to a depletion of SR Ca\(^{2+}\) loading, as a large Ca\(_i\) transient can be elicited after nitrendipine by rapid application of caffeine (Fig. 6 C).

**Implications of Graded SR Ca\(^{2+}\) Release on Models of Ca\(^{2+}\)-induced Ca\(^{2+}\) Release**

The present results can be interpreted to indicate that the amount of Ca\(^{2+}\) current produced by a depolarization is a determinant of the extent of Ca\(^{2+}\) release caused by that depolarization. When Ca\(^{2+}\) current activation produced by depolarization is of sufficient magnitude to produce a large phasic current, it causes, with a minimal delay, a rapid and large SR Ca\(^{2+}\) release, which leads to a phasic increase of Ca\(_i\) and contraction that has been traditionally referred to as a “twitch.” After a phasic current, if depolarization persists, a noninactivating Ca\(^{2+}\) current persists which leads to sustained SR Ca\(^{2+}\) release. In contrast, Ca\(^{2+}\) channel activity elicited by a small depolarization step is insufficient to mobilize a phasic Ca\(^{2+}\) current as measured in the whole-cell patch clamp. Rather, an abrupt, small, inward, steady shift in current occurs, which, following a delay, leads only to a small, steady increase in Ca\(_i\). Over a range of depolarization steps (40 to 20 mV) the steady ryanodine-dependent contraction that results from the steady inward current is graded in magnitude with the magnitude of the steady nitrendipine-sensitive current (Fig. 8). This suggests that gradations in steady rates of SR Ca\(^{2+}\) release can be achieved by graded Ca\(^{2+}\) current triggers.

That graded, SR-dependent Ca\(^{2+}\) release can occur in response to small depolarization, places important constraints on possible models of excitation-contraction
coupling via "Ca\(^{2+}\)-induced Ca\(^{2+}\) release." In particular, it excludes models in which excitation-contraction coupling is mediated entirely by communication through a single cytosolic pool of Ca\(^{2+}\). If SR Ca\(^{2+}\) release is triggered by the rise in Ca\(_i\), the system possesses positive feedback, which will tend to make it unstable. In order for the cell to be stable in the resting state, it is necessary that the "loop gain" of the positive feedback be sufficiently small to prevent regenerative release. But if the loop gain is small, the amplification provided by SR release cannot be large (i.e., the contribution of SR Ca\(^{2+}\) release to the increase in Ca\(_i\) cannot be dominant), as we have shown it to be in these studies.

Fabiato (1985b) has pointed out that it is fallacious to assume that a system with positive feedback must generate an all-or-none rather than a graded response. He has argued that the presence of negative feedback mechanisms, such as Ca\(^{2+}\) inhibition of Ca\(^{2+}\) release, can control the regenerative twitch and make its amplitude graded as a function of the trigger. While it is true that such models can be constructed which demonstrate a limited degree of gradation of the twitch as a function of the trigger, they are complex and not robust, and depend on the extreme nonlinearities involved in the full-blown regenerative twitch (Stern, M. D., unpublished observations).

These arguments do not apply to the small, subthreshold graded release described in this article. If we assume that our subthreshold stimuli to Ca\(^{2+}\) release are indeed small, in the sense that they remain within the range in which the cell behaves as a linear system, then it may be shown quite generally that the ratio of the SR Ca\(^{2+}\) release to the Ca\(^{2+}\) influx via the inward current can only be large when the static loop gain of the positive feedback is near unity. On the other hand, for the cell to be stable at rest (i.e., not spontaneously oscillate), the loop gain cannot exceed unity. Therefore, for the entire class of excitation-contraction coupling models in which the stimulus to Ca\(^{2+}\) release is a variation in the size of a common ("cytosolic") Ca\(^{2+}\) pool, a large contribution of SR Ca\(^{2+}\) release can be present (in the linear regime) only when the cell is precariously perched at the edge of resting instability. As none of the cells used in this study exhibited features of spontaneous SR Ca\(^{2+}\) release and, as shown in Fig. 5, substantial graded subthreshold Ca\(^{2+}\) release occurs over a wide range of Ca\(^{2+}\) loading, it is unlikely that it depends on the cell being critically poised near the threshold of oscillation. The entire range of common-pool Ca\(^{2+}\)-induced Ca\(^{2+}\) release models is thereby excluded by our results, provided that one accepts that a linear regime of Ca\(^{2+}\) regulation is being examined, and that all cells in the study were not marginally unstable, despite the wide range of conditions in which they were studied. This argument may be made mathematically rigorous using standard methods of feedback control theory (Stern, M. D., unpublished results).

Thus, it seems necessary to seek a more complex model of excitation-Ca\(^{2+}\) release coupling. Such a model might include selective access of the Ca\(^{2+}\) current to the SR Ca\(^{2+}\) channel, with Ca\(^{2+}\) release occurring at a site slightly removed from Ca\(^{2+}\) sensing, so that feedback is reduced. An ultrastructural relationship between the sarcolemmal and SR Ca\(^{2+}\) channels via junctional "foot processes" (Inui et al., 1987; Lai et al., 1987, 1988; Block et al., 1988) occurs in some but not all types of cardiac cells as contact between junctional SR and transverse tubules or the sarcolemma varies greatly among species and within species during development (Anderson et al.,
1976). However, evidence in support of a “privileged access” of Ca\(^{2+}\) channels to SR Ca\(^{2+}\) release channel comes from recent studies in rat cardiac myocytes during neonatal development (Cohen and Lederer, 1988) and recent efforts to model Ca\(^{2+}\)-dependent Ca\(^{2+}\) release have used such separation (Wong et al., 1987). 

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