Local Calcium Transients Triggered by Single L-Type Calcium Channel Currents in Cardiac Cells

José Ramón López-López, Philip S. Shacklock, C. William Balke, Withrow Gil Wier*

Excitation-contraction coupling was studied in mammalian cardiac cells in which the opening probability of L-type calcium (Ca^{2+}) channels was reduced. Confocal microscopy during voltage-clamp depolarization revealed distinct local transients in the concentration of intracellular calcium ions ($[Ca^{2+}]_i$). When voltage was varied, the latency to occurrence and the relative probability of occurrence of local $[Ca^{2+}]_i$ transients varied as predicted if Ca^{2+} release from the sarcoplasmic reticulum (SR) was linked tightly to Ca^{2+} flux through L-type Ca^{2+} channels but not to that through the Na-Ca exchanger or to average $[Ca^{2+}]_i$. Voltage had no effect on the amplitude of local $[Ca^{2+}]_i$ transients. Thus, the most efficacious "Ca^{2+} signal" for activating Ca^{2+} release from the SR may be a transient microdomain of high $[Ca^{2+}]_i$ beneath an individual, open L-type Ca^{2+} channel.

The central paradox of excitation-contraction (EC) coupling in heart (*1*) concerns how the relatively small amount of Ca^{2+} that enters the cell via L-type Ca^{2+} channels can both activate (*2*) and control the release of a much larger amount (*3*) of Ca^{2+} from the SR. Release of Ca^{2+} from the SR appears to be controlled by Ca^{2+} entering the cell through L-type Ca^{2+} channels because such release can be halted by stopping the Ca^{2+} current (*4, 5*). The solution to this paradox may lie in the concept of a microscopic coupling between individual L-type Ca^{2+} channels and SR release channels [or ryanodine receptors (RyRs)], perhaps arranged in clusters (*6, 7*). Briefly, if such clusters (*6*) are functionally independent and are recruited (*6, 8*) only by Ca^{2+} that entered through closely associated L-type Ca^{2+} channels, then the Ca^{2+} current could control SR release, because the Ca^{2+} released from one cluster would not activate or influence Ca^{2+} flux from another. This view of EC coupling had been termed "local control" (*6, 9, 10*). In addition to the open probability of L-type Ca^{2+} channels (P_o), the amplitude of the single L-type Ca^{2+} channel current (i) appears to be an important determinant of the peak rate of release of Ca^{2+} from the SR (*5*). Calcium currents consisting of relatively few large, single-channel currents (low P_o, large i) are much more efficacious in activating Ca^{2+} release from the SR than Ca^{2+} currents comprised of a relatively large number of small, single-channel currents (high P_o, small i).

Spontaneous, spatially localized $[Ca^{2+}]_i$ transients, termed "calcium sparks," have been recorded and attributed to the opening of one or a small group of SR Ca^{2+} release channels (*11*). These calcium sparks were thought to occur in the absence of L-type Ca^{2+} channel openings. We have recorded simultaneously L-type Ca^{2+} currents and local $[Ca^{2+}]_i$ transients, similar to the spontaneous calcium sparks, during small voltage-clamp depolarizations of rat cardiac cells (*9, 12*). The spatial inhomogeneity of $[Ca^{2+}]_i$ transients during action potentials (*10*) has also been attributed to the spatial and temporal summation of calcium sparks.

Despite these recent studies, several important predictions of the theory of local control of EC coupling by means of calcium sparks had yet to be tested experimentally. Such predictions include the following: (i) $[Ca^{2+}]_i$ transients at all pulse potentials consist of local $[Ca^{2+}]_i$ transients (*9*). (ii) Local $[Ca^{2+}]_i$ transients are evoked with a voltage dependence and temporal pattern that are consistent with activation by voltage-dependent, L-type Ca^{2+} channels that operate stochastically. (iii) Ca^{2+} entering the cell through L-type Ca^{2+} channels is more efficacious in inducing local $[Ca^{2+}]_i$ transients than a general increase in $[Ca^{2+}]_i$. (iv) Calcium sparks or evoked local $[Ca^{2+}]_i$ transients are uniform in size (that is, independent of voltage) (*9*).

To investigate these predictions, we used verapamil to reduce the probability, but not the amplitude, of the single L-type Ca^{2+} channel currents (*13, 14*). Thus, verapamil is expected to change the frequency of triggering events (single-channel currents), but not their effectiveness in activating Ca^{2+} release from the SR. Under these conditions, infrequent local $[Ca^{2+}]_i$ transients could be observed at all pulse potentials in the absence of contraction. In the presence of verapamil (10 μM), local $[Ca^{2+}]_i$ transients were evoked at most pulse voltages (Fig. 1). The local $[Ca^{2+}]_i$ transients were similar to those observed as a result of small depolarizations (*9*) in the absence of verapamil and to those observed during action potentials in the presence of the Ca^{2+} channel blocker Cd^{2+} (*10, 11*). The voltage-clamp pulse protocol (Fig. 1) was designed to ensure both uniform loading of the SR through reverse mode Na-Ca exchange (Ca^{2+} influx dependent on intracellular Na^+) at positive potentials (*15*) and a constant amount of use-dependent block of the Ca^{2+} current by verapamil. The local $[Ca^{2+}]_i$ transients under these conditions appeared to result from Ca^{2+} released from the SR, because local $[Ca^{2+}]_i$ transients were never observed in the presence of 50 μM ryanodine (*16*), which is similar to the results reported by Cheng *et al.* (*11*).

We devised an objective way to characterize the numbers (probability of occurrence), times of occurrence (latencies), and amplitudes of the local $[Ca^{2+}]_i$ transients so that these parameters could be compared with the corresponding parameters of L-type Ca^{2+} channel currents. Power spectral analysis (*9, 10*) or image processing of line-scan images of $[Ca^{2+}]_i$ during an action potential (*10*) can provide information on the heterogeneity of the Ca^{2+} images, but not on these specific parameters. In our experiments, the probability of occurrence of local $[Ca^{2+}]_i$ transients was so low that each could be distinguished unequivocally. To measure the size of local $[Ca^{2+}]_i$ transients (peak amplitude and image area) (Fig. 2), we developed a method that accounted for the variability in background $[Ca^{2+}]_i$ at the time that local $[Ca^{2+}]_i$ transients occurred (Fig. 2). We tested the effect of voltage on the size of local $[Ca^{2+}]_i$ transients by plotting the peak amplitudes and image areas of all the local $[Ca^{2+}]_i$ transients against the pulse potential (Fig. 2, B and C). No effect of potential on amplitude or image area could be detected. The substantial variation in amplitude at each pulse voltage may reflect the fact that some events originate outside the plane of focus and therefore appear smaller. Nevertheless, both spontaneous and evoked local $[Ca^{2+}]_i$ transients seem to constitute a single population with a peak of 201 ± 8.86 nM and an image area of 104.8 ± 4 pixels (mean ± SEM, n = 168). This amplitude is very similar to that of spontaneous calcium sparks in rat cells (*11*).

If local $[Ca^{2+}]_i$ transients are evoked by the opening of L-type Ca^{2+} channels, then the waiting time histogram of evoked local $[Ca^{2+}]_i$ transients under the low probability

J. R. López-López and W. G. Wier, Department of Physiology, University of Maryland, School of Medicine, Baltimore, MD 21201, USA.
P. S. Shacklock, Department of Medicine, University of Maryland, School of Medicine, Baltimore, MD 21201, USA.
C. W. Balke, Department of Physiology and Department of Medicine, University of Maryland, School of Medicine, Baltimore, MD 21201, USA.
*To whom correspondence should be addressed.

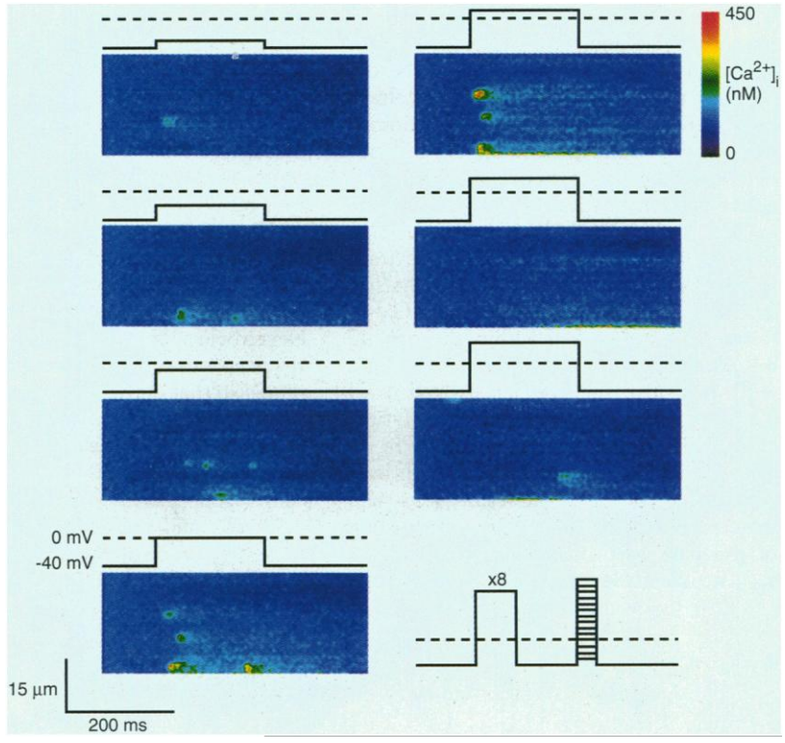

Fig. 1. Local $[Ca^{2+}]_i$ transients (LCTs) evoked by voltage-clamp depolarization of a guinea pig ventricular myocyte. Images are line-scan images obtained by confocal scanning microscopy in one dimension (*9*). Position along the cell is represented vertically, and successive scan lines in time are displayed horizontally. Isolated guinea pig ventricular cells were obtained by a standard enzymatic dispersion technique as described (*23*). Single cardiac myocytes were subjected to whole cell recordings in an external solution comprised of 140 mM NaCl, 10 mM dextrose, 10 mM Hepes, 10 mM CsCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$, with the pH adjusted to 7.3 to 7.4 with NaOH. Temperature was 21° to 23°C. When necessary, verapamil (10 μM) or ryanodine (50 μM) was added to this solution. The solution filling the electrode used for whole cell recording and loading with fluorescent dye was composed of 130 mM cesium glutamate, 10 mM NaCl, 10 mM Hepes, 20 mM tetraethylammonium chloride, 0.33 mM $MgCl_2$, 4 mM adenosine 5′-triphosphate (Mg salt), and 0.1 mM fluo 3 (pentapotassium salt), with the pH adjusted to 7.1 to 7.2 with CsOH. (**Inset**) Voltage-clamp protocol. The holding voltage was −40 mV. Depolarizing pulses were 200 ms in duration and varied between −30 and +100 mV, in increments of 10 mV. Each test pulse was preceded by eight conditioning pulses to +80 mV, at a frequency of 1 Hz and a duration of 400 ms. The whole cell Ca^{2+} currents were too small to be measured reliably against the background of other ionic currents. The dashed line represents 0 mV. $[Ca^{2+}]_i$ was calculated from the fluo 3 fluorescence, with a self-ratio method using an equation and calibration parameters given previously (*11*). Computations and image analysis were performed on an IBM Risc System/6000 workstation (IBM, Armonk, New York) with the software IDL (Research Systems, Boulder, Colorado).

conditions used here should be explicable in terms of L-type Ca^{2+} channel gating. Release units or clusters of RyRs are thought to be activated by the first opening of an L-type Ca^{2+} channel (8). However, L-type Ca^{2+} channels can continue to open and reopen throughout the duration of a depolarizing pulse and can exhibit an active-late pattern of gating (*17*). Because the mean open lifetime is so brief (*17*), the time course of the macroscopic current is essentially the time course of the probability that an L-type Ca^{2+} channel will open. If every such opening could evoke a local $[Ca^{2+}]_i$ transient, then the time course of the latencies to occurrence of local $[Ca^{2+}]_i$ transients should be exactly the same as that of the macroscopic current. It seems unlikely, however, that each opening of an L-type Ca^{2+} channel could evoke a local $[Ca^{2+}]_i$ transient, because once release occurs, its time course may be governed by the intrinsic properties of the RyR, which adapt to a maintained $[Ca^{2+}]_i$ (*18*).

Therefore, a reasonable expectation is that the time course of the occurrence of local $[Ca^{2+}]_i$ transients during a depolarizing pulse should peak rapidly, within the time during which the current is observed to activate fully, and then decline more rapidly than the macroscopic current (reflecting the failure of some openings to evoke local $[Ca^{2+}]_i$ transients). It is known that in rat heart cells, net cellular SR Ca^{2+} flux does decline more rapidly than the

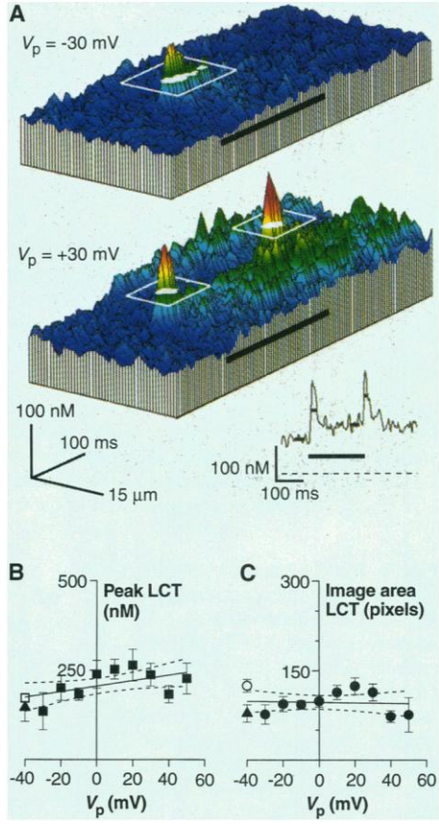

Fig. 2. Amplitude and image area of evoked LCTs are independent of pulse voltage. (**A**) Line-scan images of LCTs evoked by pulse depolarization to −30 mV (upper plot) or to +30 mV (first LCT, lower plot) or by repolarization to −40 mV (second LCT, lower plot) are represented as surface plots. Black bars indicate the period of pulse depolarization (200 ms). (**Inset**) Line plot of $[Ca^{2+}]_i$ through the peaks of the LCTs shown in the lower surface plot. The dashed line represents no $[Ca^{2+}]_i$. The white boxes on the surface plots show the selected areas of the image used to make the measurements of amplitudes and image areas for each LCT. Background $[Ca^{2+}]_i$ was calculated from the average local $[Ca^{2+}]_i$ just before the onset of the LCT (indicated by solid lines preceding LCTs on the line plot). The amplitude of the LCT was taken to be the difference between the peak and this background. The image area was defined as the number of pixels in which $[Ca^{2+}]_i$ exceeded the half-amplitude of the LCT (white regions on the surface plots). This image area is expected to reflect the distance over which the LCTs occurred and the time they persisted. The latency to occurrence (or waiting time) of LCTs was measured as the time elapsed from the onset of the pulse depolarization to the peak of the LCT minus 5.0 ms (average time to peak). Measured amplitudes (■) and image areas (●) of LCTs obtained from 12 different cells are plotted as a function of the voltage pulses (V_p) in (**B**) and (**C**), respectively. Open symbols represent the amplitude and image area of LCTs occurring after repolarization to −40 mV. Amplitudes and image areas of spontaneously occurring calcium sparks are represented by filled triangles. Values are means ± SEM. The total number of LCTs that were averaged at each value of V_p varied from 5 to 41. Data were fit by linear regression (solid lines). The dashed lines represent 90% confidence intervals of the fit.

whole cell Ca^{2+} current (*19*). Clearly, however, the time course of the probability of the local $[Ca^{2+}]_i$ transients should be dependent on voltage, reflecting the voltage dependence of the macroscopic current, which arises from the voltage dependence of channel gating patterns, including the active-late pattern (*17*).

We tested these predictions by measuring the latencies of evoked local $[Ca^{2+}]_i$ transients at different clamp-pulse potentials in 12 experiments (Fig. 3A). Most local $(Ca^{2+})_i$ transients were evoked within the first 40 ms. At pulse voltages between −20 mV and +10 mV, the numbers of evoked local $[Ca^{2+}]_i$ transients declined sharply with time and the latency histograms could be fit by exponentially declining functions with time constants (τ_{LCT}). At more negative and more positive potentials, exponential fitting of the data was somewhat uncertain, but τ_{LCT} was clearly less than at the intermediate potentials. The latency of local $[Ca^{2+}]_i$ transients evoked on repolarization (Fig. 3A, V_p = +70 mV) was short, falling almost entirely into the first bin (20 ms) after repolarization. Direct comparison of the time course of the L-type Ca^{2+} current with the time course of the latencies was difficult because the probability of the local $[Ca^{2+}]_i$ transient was low and because verapamil had reduced the macroscopic L-type Ca^{2+} current to such small amounts that the actual time course of the Ca^{2+} current could not be determined. Nevertheless, it is clear that the value of τ_{LCT} depends on pulse potential in a fashion similar to that of the slow component (τ_s) of the inactivation of the L-type Ca^{2+} current in guinea pig myocytes at 20° to 25°C (*20*). In such cells, τ_s varies with pulse potential, being about 550 ms at −10 mV, 100 ms at +10 mV, and ~250 ms at +30 mV. Although the plot of the dependence on potential of both τ_s and τ_{LCT} is U-shaped, it seems clear that the values for τ_{LCT} are generally smaller than those of τ_s.

We have suggested that the amplitude of the unitary L-type Ca^{2+} channel current, *i*, is an important determinant of the probability of evoking release (as opposed to the amplitude of the whole cell current) (*5*). In this case, a bell-shaped dependence of the number of evoked local $[Ca^{2+}]_i$ transients at times less than 40 ms on pulse potential would be expected, because P_o increases with voltage, but *i* decreases (*21*). We tested this prediction by plotting the peak number of events (peak n_{LCT}) in each of the latency histograms as a function of the clamp potential (Fig. 3B). At a particular voltage, we can define the relative probability of evoking a local $[Ca^{2+}]_i$ transient (P_{LCT}) as $n_{LCT}/n_{LCT(max)}$, where $n_{LCT(max)}$ is the maximum number of local $[Ca^{2+}]_i$ transients that occurred at a selected potential (+10 mV in Fig. 3A). Therefore, P_{LCT} will be given by the product of two other, independent probabilities according to

$$P_{LCT} = k \times P_{i,LCT} \times P_{o,L} \qquad (1)$$

where $P_{o,L}$ is the probability that the L-type Ca^{2+} channel will be open, $P_{i,LCT}$ is the probability that the current of Ca^{2+} (*i*) through it will trigger the release of Ca^{2+} from the cluster of RyRs, and *k* is a scaling factor to make $P_{i,LCT}$ equal to 1.0 at a pulse potential of −40 mV. Values for $P_{i,LCT}$ were computed according to Eq. 1 from the data and $P_{o,L}$ (*21*) (Fig. 3C). The dependence on pulse potential of $P_{i,LCT}$ follows approximately the expected dependence on voltage of *i*, which suggests that $P_{i,LCT}$ is a function of the single L-type Ca^{2+} channel current.

A controversial issue, important to the theory of local control of EC coupling, has been whether Ca^{2+} entering cells through Na-Ca exchange can also trigger SR Ca^{2+} release (*22*). To investigate this issue, we voltage-clamped cells for 200 ms to membrane potentials as high as +100 mV, where entry of Ca^{2+} through Na-Ca exchange occurs but not entry of Ca^{2+} via L-type Ca^{2+} channels (*23*) (Fig. 4). Local $[Ca^{2+}]_i$ transients were not observed during

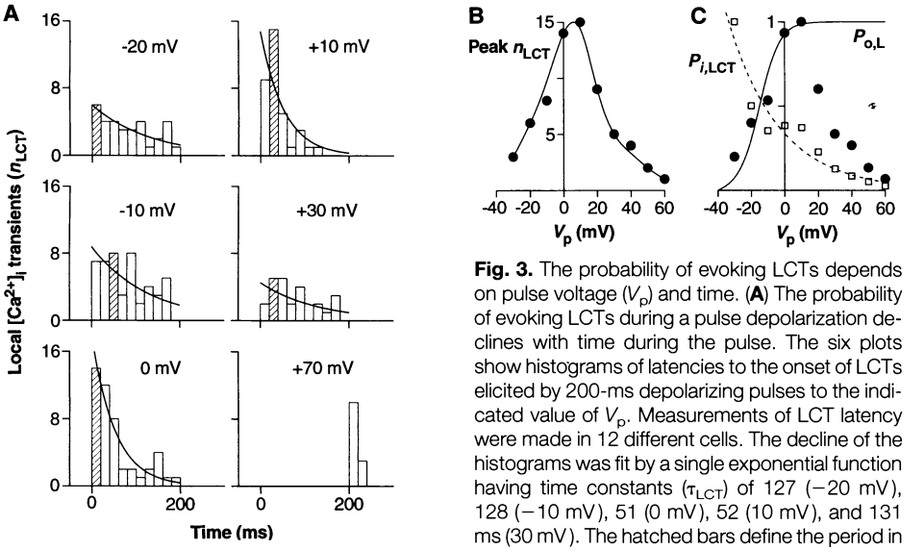

Fig. 3. The probability of evoking LCTs depends on pulse voltage (V_p) and time. (**A**) The probability of evoking LCTs during a pulse depolarization declines with time during the pulse. The six plots show histograms of latencies to the onset of LCTs elicited by 200-ms depolarizing pulses to the indicated value of V_p. Measurements of LCT latency were made in 12 different cells. The decline of the histograms was fit by a single exponential function having time constants (τ_{LCT}) of 127 (−20 mV), 128 (−10 mV), 51 (0 mV), 52 (10 mV), and 131 ms (30 mV). The hatched bars define the period in which the maximum numbers of LCTs (peak n_{LCT}) were evoked at each value of V_p. (**B**) The probability of evoking LCTs depends on voltage. Peak n_{LCT} was plotted as a function of V_p. The solid line represents a visual fit to the data. (**C**) Data from (B) (peak n_{LCT}) were normalized (P_{LCT}) and replotted (●). The solid line represents the open probability ($P_{o,L}$) of L-type Ca^{2+} channels (*21*). Values for P_{LCT} were divided by those for $P_{o,L}$ to obtain the probability of evoking an LCT ($P_{i,LCT}$). The dashed line is an exponential fit to $P_{i,LCT}$.

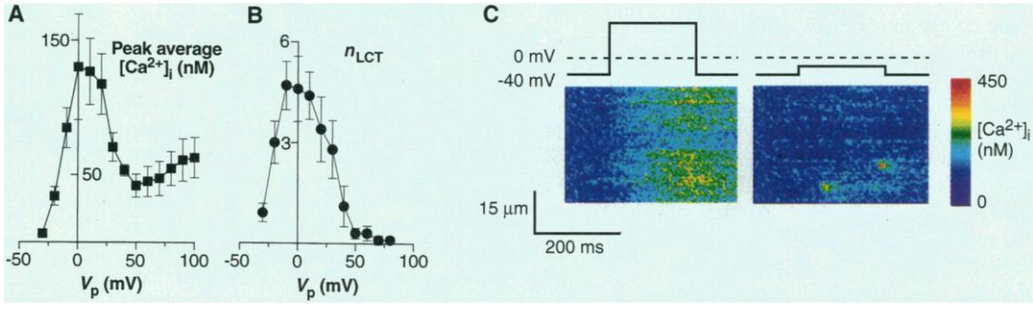

Fig. 4. The total number of LCTs evoked during clamp pulse depolarization is independent of peak spatially averaged $[Ca^{2+}]_i$. (**A**) Peak spatially averaged $[Ca^{2+}]_i$ (■) was plotted as a function of V_p after a resting $[Ca^{2+}]_i$ of 100 mM was subtracted. The increase in peak spatially averaged $[Ca^{2+}]_i$ at pulse voltages greater than +50 mV results from the entry of Ca^{2+} via the Na-Ca exchanger (*2*). (**B**) The number of LCTs (n_{LCT}; mean ± SEM) at each value of V_p in 12 cells is shown as a function of V_p. (**C**) Line-scan images of $[Ca^{2+}]_i$ are shown for pulse depolarizations to +80 mV (left) and to −20 mV (right) from a holding potential of −40 mV. LCTs were evoked during the pulse to −20 mV but not during that to +80 mV, despite the relatively homogeneous increase in $[Ca^{2+}]_i$ at +80 mV.

pulses to these potentials, although the spatially averaged $[Ca^{2+}]_i$ increased slowly to relatively high concentrations (Fig. 4, A and B). Calcium entry through L-type Ca^{2+} channels (V_p = −20 mV) was much more effective in triggering local $[Ca^{2+}]_i$ transients than an increase in the average $[Ca^{2+}]_i$ in the cell (V_p = +80 mV) (Fig. 4C). Local $[Ca^{2+}]_i$ transients at high positive clamp voltages were observed only in cells showing Ca^{2+} waves (*16*).

Our data support the concept that $[Ca^{2+}]_i$ transients under normal conditions (that is, without verapamil) can be explained by recruitment of more local $[Ca^{2+}]_i$ transients with the same characteristics as those observed here. First, the bell-shaped voltage dependence of the number of local $[Ca^{2+}]_i$ transients (Figs. 3B and 4B) is similar to that of the spatially averaged $[Ca^{2+}]_i$ transient (*24*), after accounting for the average $[Ca^{2+}]_i$ due to Na-Ca exchange. Second, the latency histograms are explicable in terms of the gating of L-type Ca^{2+} channels in guinea pig cardiac cells (*17*), although channel gating may be modified in the presence of verapamil (*14*). Latency histograms cannot yet be compared with measurements of whole cell SR Ca^{2+} release flux because these are not yet available for guinea pig cells. Accordingly, we determined how many local $[Ca^{2+}]_i$ transients must be evoked to produce the normal whole cell $[Ca^{2+}]_i$ transient. At 0 mV, we observed that three local $[Ca^{2+}]_i$ transients were elicited, on average, in the first 40 ms. This yields a peak frequency of ~2 s^{-1} μm^{-1} along the length of the scanned cell. As the peak average $[Ca^{2+}]_i$ is 120 nM, the contribution of each local $[Ca^{2+}]_i$ transient to the average $[Ca^{2+}]_i$ is 40 nM. Therefore, to produce a whole cell $[Ca^{2+}]_i$ transient of 1.5 μM under normal conditions, the peak frequency of local $[Ca^{2+}]_i$ transients would have to be at least 12 times greater than that observed here in the presence of verapamil. In summary, the $[Ca^{2+}]_i$ transient in the whole cell can be explained in terms of the recruitment of single, stereotyped unitary events (local $[Ca^{2+}]_i$ transients), controlled locally by single L-type Ca^{2+} channels in the plasma membrane.

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12. The resolution of the confocal microscope is presently not adequate to address the issue of whether calcium sparks originate from one single RyR or from a cluster of RyRs. The optical section is approximately 1 μm thick (*9*), and the point scanned is limited by diffraction at 1 μm. That means that the fluorescence represented at each pixel in the line scan is detected from a volume of approximately 1 μm^3.
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14. To understand the possible relation of the local $[Ca^{2+}]_i$ transients to single L-type Ca^{2+} channel currents during clamp-pulse depolarization, it is necessary to consider how phenylalkylamines such as verapamil (and its methoxy derivative D 600) block Ca^{2+} currents. In guinea pig myocytes, D 600 reduced the mean open channel lifetime and the frequency of opening without changing the length of time until the first opening or the single-channel current amplitude (*13*). Channels could still reopen in D 600, and therefore the time course of the ensemble average current (with Ba^{2+} as the charge carrier) was not affected. Therefore, we assume that L-type Ca^{2+} channels in the presence of verapamil opened with the same first latencies, closed somewhat more quickly (0.2 ms), still reopened occasionally thereafter, and conducted the same current as control channels, but did so much less frequently (that is, the L-type open probability, $P_{o,L}$, was reduced).
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25. Supported by U.S. Public Health Service awards HL29473 (W.G.W.), HL02466 (C.W.B.), and HL50435 (C.W.B.), an American Heart Association, Maryland Affiliate, grant-in-aid (C.W.B.), and the Division of Cardiology, Department of Medicine, University of Maryland at Baltimore, J.R.L.-L. is a Fogarty Scholar. We thank J. M. Miller for expert technical assistance in cell isolation.

7 December 1994; accepted 23 February 1995

The Control of Calcium Release in Heart Muscle

M. B. Cannell, H. Cheng, W. J. Lederer*

The control of calcium release from intracellular stores (the sarcoplasmic reticulum) in cardiac muscle was examined with the use of a confocal microscope and voltage clamp techniques. Depolarization evoked graded calcium release by altering the extent of spatial and temporal summation of elementary calcium release events called "calcium sparks." These evoked sparks were triggered by local L-type calcium channel currents in a stochastic manner, were similar at different potentials, and resembled spontaneous calcium sparks. Once triggered, the calcium release from the sarcoplasmic reticulum during a calcium spark was independent of the duration of the triggering calcium influx. These results were used to develop a unifying model for cardiac excitation-contraction coupling that explains the large (but paradoxically stable) amplification of the trigger calcium influx by a combination of digital and analog behavior.

In cardiac muscle, the archetypical ryanodine receptor (RyR) is gated by Ca^{2+} influx across the plasmalemma and serves to amplify the Ca^{2+} influx by releasing sufficient Ca^{2+} from the sarcoplasmic reticulum (SR) to activate contraction. This process is the cornerstone of excitation-contraction (EC) coupling and has been termed Ca^{2+}-induced Ca^{2+}release (CICR) (*1*). Because the Ca^{2+} efflux from the SR is between 10 and 65 times larger than that due to the Ca^{2+} current (I_{Ca}) through channels in the plasma membrane (*2*, *3*), the release of Ca^{2+} will tend to cause further release, leading to uncontrolled regenerative behavior (positive feedback). However, Ca^{2+} release from the SR seems to be tightly regulated by both the amplitude and duration of I_{Ca} (*2*, *4*, *5*). Ca^{2+}-dependent inactivation of Ca^{2+} release (*1*) could have provided a solution to this paradox; however, this mechanism has not been observed in intact cells (*4*, *6*). To

M. B. Cannell, Department of Pharmacology and Clinical Pharmacology, St. George's Hospital Medical School, Cranmer Terrace, London SW17 ORE, UK.
H. Cheng and W. J. Lederer, Department of Physiology and Medical Biotechnology Center, University of Maryland School of Medicine, 660 West Redwood Street, Baltimore, MD 21201, USA.

*To whom correspondence should be addressed.