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Short communication

Propagation of excitation-contraction coupling into ventricular myocytes

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Abstract. This paper examines the $[Ca^{2+}]_i$ transient in isolated rat heart cells using a laser scanning confocal microscope and the calcium indicator fluo-3. We find that the depolarization-evoked $[Ca^{2+}]_i$ transient is activated synchronously near the surface and in the middle of the heart cell with similar kinetics of activation. The time of rise of the transient did not depend on whether the sarcoplasmic reticulum (SR) Ca-release was abolished (by thapsigargin and ryanodine). The synchrony of activation and the similarity of levels of $[Ca^{2+}]_i$ at the peripheral and deeper myoplasm (regardless of the availability of SR Ca-release) shows that sarcolemmal Ca channels and SR Ca-release channels are distributed throughout the rat heart cell and that the propagation of the action potential into the interior of the cell is rapid. In addition, the activation of calcium release from the SR by CICR is rapid (<<2 ms) when compared to the time-course of calcium influx via the sarcolemmal Ca channel.

Key Words: Heart - Cardiac muscle - Contraction - E-C coupling - Calcium channels - Ryanodine receptors - Intracellular calcium.

Introduction

The heart muscle action potential causes contraction by activating intracellular calcium release. The release of calcium from intracellular stores depends on "trigger" calcium entering the cytosol from the extracellular space by activated sarcolemmal (SL) calcium channels [1,3,4] and by calcium entry on the Na/Ca exchanger [2]. The trigger calcium activates SR Ca-release channels by the process of "calcium-induced calcium-release" (CICR) which amplifies the modest increase in $[Ca^{2+}]_i$ [3,4] caused by the SL influx pathways across the sarcolemma to provide sufficient calcium to activate the contractile proteins.

Previous investigations into the time-course of the $[Ca^{2+}]_i$ transient have not specifically examined the uniformity of the rise in $[Ca^{2+}]_i$ across the diameter of the cell [5]. This lack of information about the time-course of the rise in $[Ca^{2+}]_i$ across the cell can be attributed to (1) inadequate temporal resolution of data obtained at video rates and (2) limited spatial resolution in wide-field fluorescence microscopy due to out-of-focus fluorescence.

In the experiments presented here we have specifically examined the synchrony and kinetics of excitation-contraction (E-C) coupling using a laser scanning confocal microscope and the fluorescent calcium indicator, fluo-3. We show that there is no significant difference in the delay associated with E-C coupling between the deep centre and outer edge of a single ventricular cardiac cell.

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Materials and Methods

Cells. Single rat cardiac myocytes were dissociated as previously described [7]. 50 μg of fluo-3 acetoxymethylester (AM) and 25 μg Pluronic (Molecular Probes, Eugene, OR, USA) were dissolved in 100 μl dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA). The stock fluo-3 AM solution was diluted to 5 μM with bathing solution and cells were exposed to this solution for 5 min to load them with fluo-3, followed by a 30 min. incubation in bathing solution to allow indicator de-esterification All experiments were performed at room temperature (25°C). Selected quiescent cells were free of blebs, had clear sharp striations and only contracted by 5 - 20% on electrical stimulation.

Solutions. The standard bathing solution contained (in mM): 137 NaCl, 5.4 KCl, 1.2 MgCl2, 1 CaCl2 20 HEPES (pH = 7.4 at 25°C). Thapsigargin (Sigma) was made up as a 10 mM stock solution in DMSO. Ryanodine (S.B. Pennick & Co, New York, NY, USA) was prepared as a 10 mM stock in water.

Confocal microscope. A Biorad MRC 600 confocal scanner and detector were attached to a Nikon Diaphot microscope. A Zeiss Neofluor 63 x 1.25 N.A. objective lens was used in all experiments. The detector aperture was set to 0.75 of maximum, providing a z resolution of 0.8 μm and X-Y resolution of 0.4 μm (measured as FWHM fluorescence of a 0.1 μm fluorescent bead . The illumination intensity from a Laser Ion Technology 20 mW Ar ion laser was set by neutral density filters (ND 0.3 - ND 3.0) while the excitation wavelength (488 nm Ar line) was selected using interference filters.

Cellular imaging. Electrical stimulation was delivered with 2 ms voltage pulses at 2.0 times threshold through parallel platinum wires in the experimental chamber. The timing of the stimulator was linked to the timing of the confocal microscope scan by custom electronics designed by the authors.

Image analysis. The confocal microscope interface was hosted by a 66 MHz 486 computer (Gateway, North Sioux City, SD, USA). Image processing was performed on an IBM RS/6000 workstation (IBM, Boca Raton, FL, USA)

running IDL software (Research Systems, Boulder, CO, USA). Final images were photographed from the computer monitor. Line-scan images were normalized to correct for dye distribution by dividing the experimental line-scan images by a control line-scan image so that an estimate of $[Ca^{2+}]_i$ can be obtained using a self-ratioing method [6]. Assuming that: (1) the apparent concentration of the fluorescent indicator is constant (so that a fixed level of fluorescence will be measured at some known $[Ca^{2+}]_i$) and (2) the fluorescence of the calcium free form of the indicator and cell autofluorescence are negligible (the measured fluorescence of the calcium free form of the indicator was only 1.4% of the fluorescence of the calcium-bound form and cell autofluorescence was similarly negligible) then at a single wavelength: $[Ca]_i = KR/\{(K/[Ca]_{rest}) - R + 1\}$; where K is the affinity of the indicator for calcium, R the normalized fluorescence signal (i.e. the fluorescence signal divided by the signal at rest) [6]. $[Ca^{2+}]_{rest}$ was assumed to be 100 nM. Athough no conclusions here depend on the accuracy of this calibration, estimates of $[Ca^{2+}]_i$ are given to allow direct comparison with other studies.

Results and discussion

The activation of a single rat ventricular myocyte (loaded with fluo-3) in response to electrical stimulation was recorded along a single confocal scan line (starting and ending outside the cell and crossing the center of the cell) at 2 ms intervals (the maximum rate of the MRC 600). Fig. 1A shows a control image while Fig. 1B was obtained 10 min after inhibiting SR calcium metabolism (with 1 μM ryanodine to block the SR Ca-release channel and 1 μM thapsigargin to inhibit SR calcium uptake) leaving only the calcium influx component of the $[Ca^{2+}]_i$ transient. In both pairs of images, there is an increase in the fluorescence of the cell after electrical stimulation. The line-scan images were constructed by placing the fluorescence signal from successive line-scans beneath each other. Thus time increases with vertical position (from top to bottom) in the image while distance along the scan line is plotted horizontally. The step-like increase in fluorescence shows that, in both conditions, the increase in $[Ca^{2+}]_i$ occurs at very similar times at all points along the scanned line. In addition, the increase in fluorescence was much smaller in the presence of thapsigargin and ryanodine.

A more quantitative analysis of the amplitude of the changes in fluorescence in these experiments is presented in Fig. 1C. Under control conditions, the normalized peak fluorescence was about 4.1 which corresponds to a $[Ca^{2+}]_i$ of about 1.82 μM (given the assumptions detailed in Methods). After exposure to ryanodine and thapsigargin the peak of the normalized fluorescence was reduced to 1.45, corresponding to a peak $[Ca^{2+}]_i$ of about 163 nM (see also Fig. 2). Thus the inhibition of SR calcium metabolism by ryanodine and thapsigargin resulted in a profound reduction in the amount of calcium released into the cytoplasm. In addition, the half-time of decline of $[Ca^{2+}]_i$ was increased from a control value of 85 ms to 1560 ms, demonstrating the importance of SR calcium metabolism to the decline of $[Ca^{2+}]_i$ [4,8]. At an expanded time scale (fig. 1D), there is a clear 4 ms delay after stimulation before the fluorescence begins to increase.

The fluorescence increased uniformly across the cell width after stimulation, in agreement with previous observations [9,10] and this point was further clarified by examining the signal from two regions (indicated by bars in fig. 1), one near

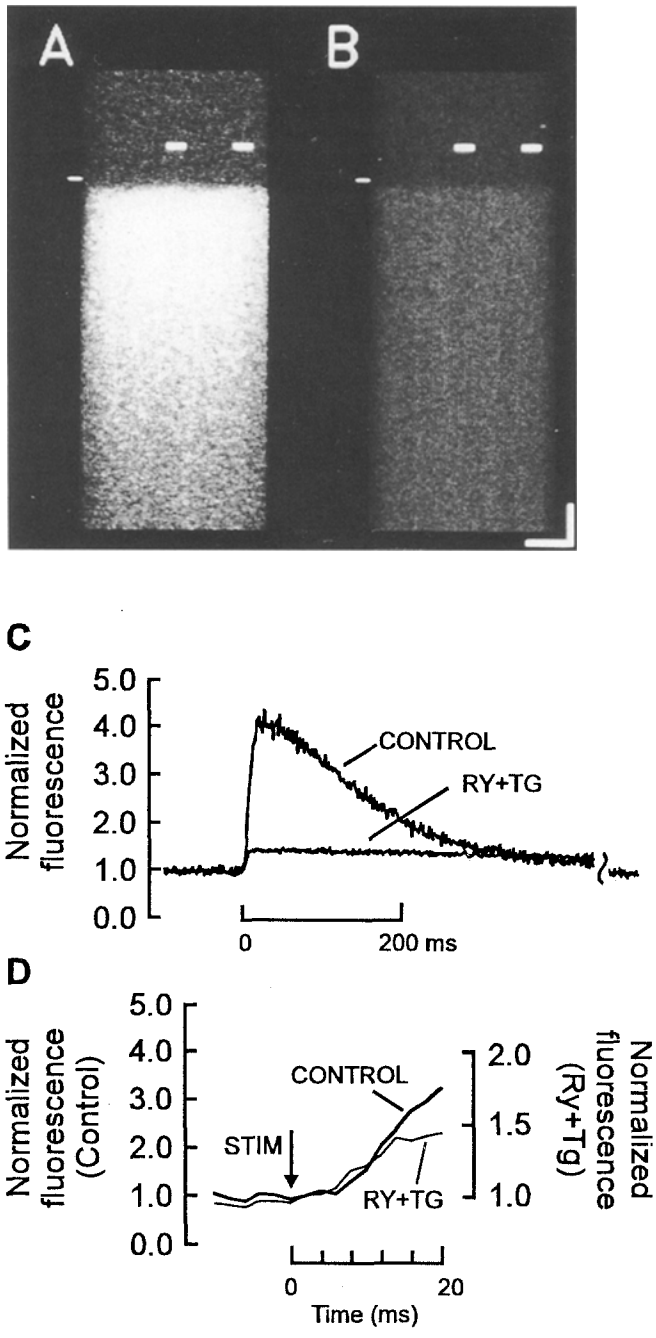

Fig. 1. Depolarization-activated calcium transients. A. Fluorescence line-scan image of a heart cell during electrical stimulation. The fluorescence intensity of a confocal scan line is repeatedly plotted (from the top of the image to the bottom). After 130 ms, the cell was field stimulated at the scan line indicated by the small horizontal bar. Note that the fluorescence increases as a step in the line-scan image shortly after the stimulus indicating a rapid and uniform increase in $[Ca^{2+}]_i$. B. Fluorescence line-scan image obtained from a the rat heart cell shown in panel A after exposure to 1 μM ryanodine and 1 μM thapsigargin (RY+TG) to block both SR Ca-release and SR Ca-uptake respectively. Note that the increase in fluorescence still occurs as a step but is much smaller. The scale bar for both line-scan images is 50 ms vertically and 5 μm horizontally. The time-course of spatial averaged fluorescence from the line-scan images shown in panels A & B is shown in panel C .(Normalized Fluorescence obtained 2.8 sec after the stimulus follows the record to show a steady state level.) Note the reduction in the amplitude of the transient and its slower decay in the presence of thapsigargin and ryanodine. D. The data in panel C shown at an expanded time scale to show the kinetic delay associated with cell activation. Similar results were observed in 6 other cells.

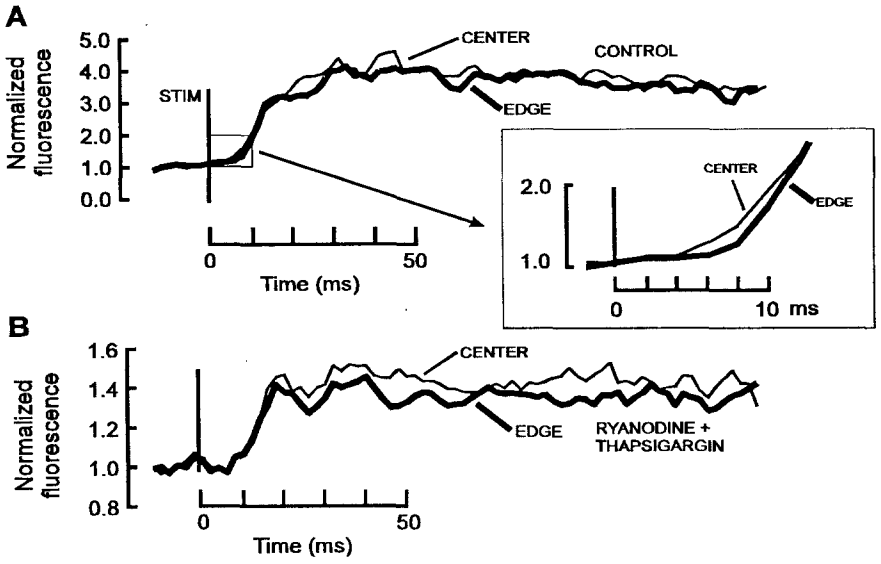

Fig. 2 A. Comparison of the time-course of fluorescence change at the center and edge of the cell in control conditions. These plots were obtained from the average intensity of the 2 μm wide regions indicated by bars in Fig. 1A&B. The inset panel shows an enlarged view of the data around the time of the stimulus in the top panel. Note that there is no detectable difference in the time-course of the $[Ca^{2+}]_i$ transient between the edge and the center of the cell. B. After treatment with thapsigargin & ryanodine (see text) the transient amplitude was reduced but had the same time-course in the center and at the edge of the cell. Note the slowing of the declining phase of the transient compared to panel A.

the cell center and the other near the cell surface (see fig. 2). In both control conditions (fig. 2A) and after treatment with ryanodine and thapsigargin (fig. 2B) there are no systematic differences in the time-course of the fluorescence signal recorded from either the center or the edge of the cell. The scanning delay between the two regions examined was 65 μs, so that the lack of detectable difference in the rise time of the signals recorded from the center and edge of the cell shows that excitation propagates rapidly into the cell center.

The rapid propagation of the stimulus into the center of the cell and the uniformity in the 4 ms delay before the increase in $[Ca^{2+}]_i$ occurred shows that E-C coupling throughout the cell does not depend on the diffusion of a second messenger from the surface of the cell to the center. Since it is generally assumed that calcium is the trigger for calcium release from the SR [1,2,3,4,5,6,8], the finding that, after SR inhibition, the trigger calcium increases synchronously throughout the cell can explain why calcium release also occurs synchronously throughout the cell. Thus, the calcium influx mechanisms which trigger CICR must be distributed throughout the t-tubular system.

Since the L-type calcium current is probably responsible for most of the rise in $[Ca^{2+}]_i$ observed in the presence of SR inhibitors [8], we conclude that *functional* L-type calcium channels must exist in the t-tubular membrane (since the rise in calcium due to the calcium current is synchronous across the cell). While most authors have assumed this function of the t-tubular system when discussing E-C coupling and CICR, the data presented here is a direct demonstration that calcium influx and calcium release occur throughout the cell nearly synchronously. These data also show that the delay between the stimulus and the appearance of calcium in the myoplasm is about 4 ms irrespective of whether the SR is able to release calcium. Since this delay appeared to be constant throughout the cell and inhibiting the SR had no effect on the onset of the rise in $[Ca^{2+}]_i$, we conclude that the majority of the delay in the rise in $[Ca^{2+}]_i$ after stimulation resides in the activation of calcium influx across the SL rather than the step in E-C coupling which links the trigger influx to SR calcium release (CICR). In other words, the release of calcium from the SR follows the activation of the calcium current with little delay (<<2 msec).

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References

1. Fabiato, A.(1985) Simulated calcium current can both cause calcium loading and trigger calcium release from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. Journal of General Physiology 85: 291-320
2. Leblanc, N.and Hume, J. R.(1990) Sodium current-induced release of calcium from cardiac sarcoplasmic reticulum. Science 248: 372-376
3. Fabiato, A. (1986) Release of calcium from the sarcoplasmic reticulum. *Cardiac Muscle: The Regulation of Excitation and Contraction*, pp. 283-295, Academic Press, Inc., New York, NY
4. Wier, W. G.(1990) Cytoplasmic $[Ca^{2+}]$ in mammalian ventricle: Dynamic control by cellular processes. Annual Review of Physiology 52: 467-485
5. Cannell, M. B., Berlin, J. R., and Lederer, W. J. (1987) Intracellular calcium in cardiac myocytes: Calcium transients measured using fluorescence imaging. *In Cell Calcium and the Control of Membrane Transport* (ed. Mandel, L. J. and Eaton, D. C.) pp. 202-214, Rockefeller University Press, New York
6. Cheng, H.; Lederer, W. J.and Cannell, M. B.(1993) Calcium sparks: Elementary events underlying excitation-contraction coupling in heart muscle. Science 262: 740-744
7. Niggli, E.and Lederer, W. J.(1990) Real-time confocal microscopy and calcium measurements in heart muscle cells: Towards the development of a fluorescence microscope with high temporal and spatial resolution. Cell Calcium 11:121-130
8. Sipido, K. R.and Wier, W. G.(1991) Flux of Ca^{2+} across the sarcoplasmic reticulum of guinea-pig cardiac cells during excitation-contraction coupling. Journal of Physiology 435: 605-630
9. Williams, D. A., Delbridge, L. M., Cody, S. H., Harris, P. J. and Morgan, T. O. (1992) Spontaneous and propagated calcium release in isolated cardiac myocytes viewed by confocal microscopy. American Journal of Physiology 262 (cell physiology 31): C731-C742
10. Lipp, P. and Niggli, E. (1993) Ratiometric confocal Ca^{2+}-measurments with visible wavelength indicators in isolated cardiac myocytes. Cell Calcium 14:359-372