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Original article

A modified local control model for Ca^{2+} transients in cardiomyocytes: Junctional flux is accompanied by release from adjacent non-junctional RyRs

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ABSTRACT

Excitation–contraction coupling in cardiomyocytes requires Ca^{2+} influx through dihydropyridine receptors in the sarcolemma, which gates Ca^{2+} release through sarcoplasmic ryanodine receptors (RyRs). Ca^{2+} influx, release and diffusion produce a cytosolic Ca^{2+} transient. Here, we investigated the relationship between Ca^{2+} transients and the spatial arrangement of the sarcolemma including the transverse tubular system (t-system). To accomplish this, we studied isolated ventricular myocytes of rabbit, which exhibit a heterogeneously distributed t-system. We developed protocols for fluorescent labeling and triggered two-dimensional confocal microscopic imaging with high spatiotemporal resolution. From sequences of microscopic images, we measured maximal upstroke velocities and onset times of local Ca^{2+} transients together with their distance from the sarcolemma. Analyses indicate that not only sarcolemmal release sites, but also those that are within 1 μm of the sarcolemma actively release Ca^{2+}. Our data also suggest that release does not occur at sites further than 2.5 μm from the sarcolemma. The experimental data are in agreement with results from a mathematical model of Ca^{2+} release and diffusion. Our findings can be explained by a modified local control model, which constrains the region of regenerative activation of non-junctional RyR clusters. We believe that this model will be useful for describing excitation–contraction coupling in cardiac myocytes with a sparse t-system, which includes those from diseased heart tissue as well as atrial myocytes of some species.

1. Introduction

It is well established that the central mechanism underlying excitation–contraction coupling in heart is Ca^{2+} induced Ca^{2+} release (CICR). This was largely established by Fabiato and Fabiato (e.g. [1]), who inferred that an abrupt elevation of Ca^{2+} in the solution surrounding skinned cardiac cells produced a release of Ca^{2+} from the sarcoplasmic reticulum (SR) sufficient to activate contraction. Direct evidence that the Ca^{2+} current triggered contractions and presumably SR Ca^{2+} release was subsequently obtained by London and Krueger [2]. This was important since it suggested that a small transmembrane Ca^{2+} flux could trigger a much larger SR release flux, i.e. an amplification of release as Fabiato and Fabiato first suggested [1]. Fabiato and Fabiato's work also suggested that, since Ca^{2+} itself triggers Ca^{2+} release one expects regenerative activation of all Ca^{2+} release sites in the cell.

However, Cannell et al. and Barcenas-Ruiz and Wier were the first to demonstrate in isolated ventricular myocytes from rat and guinea pig, respectively, that the size of this release could be graded with the size of the Ca^{2+} current under voltage clamp [3,4]. This created a difficulty. How could an inherently regenerative process (CICR) produce graded release? Stern proposed that one could explain graded release if it was controlled locally [5]. He proposed a "Ca^{2+} synapse" model consisting of various arrangements of dihydropyridine receptors (DHPRs) and ryanodine receptors (RyRs). With these proposals it was possible to construct local control models that produced graded release.

Based on structural studies, both Stern et al. [6] and Franzini-Armstrong et al. [7] subsequently proposed the existence of discrete Ca^{2+} release units or couplons in skeletal and cardiac myocytes, respectively. In cardiac myocytes couplons comprise more than one DHPR [8]

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in the sarcolemma, mainly in the transverse tubules (t-tubules). DHPRs are apposed to clusters of RyRs [9,10] on the terminal cisternae of the SR. DHPRs and RyR clusters are separated by a junctional region of ~12 nm across. Sparks or local release events are evoked in the couplons [11,12]. These are controlled locally [13] and sum to produce a macroscopic Ca^{2+} transient [14]. Moreover the recruitment of sparks and hence couplons was found to be graded with membrane voltage [13].

The existence of release events that could be controlled locally and recruited as a function of Ca^{2+} current magnitude supported Stern's original explanation of the way that graded release could occur (4). However, during an action potential it seems that couplon recruitment is at maximum [8], which is consistent with the finding of Janczewski et al. that Ca^{2+} transients saturate under voltage clamp [15]. If this is the case it seems likely that the transient magnitude is controlled by modulating SR Ca^{2+} content and/or size of DHPR current. Other factors that could modulate the transient magnitude include the degree to which SR Ca^{2+} release is synchronized [16].

This fairly simple picture of EC coupling has been clouded by a number of studies suggesting the existence of RyR clusters that are not associated with DHPRs (e.g. [9,17,18]). Jorgensen and Campbell originally proposed that these non-junctional RyRs are activated by a diffusional agent [19]. Such clusters are known to exist in atrial myocytes and are presumably activated by a mechanism of the type proposed by Jorgensen and Campbell [20]. Spontaneous sparks produced by couplons do not necessarily activate other couplons or non-junctional RyRs regeneratively; neither do spontaneous sparks produced by non-junctional RyRs evoke global Ca^{2+} release. This leads to a number of unanswered questions: for example, to what extent does the activation of non-junctional RyRs contribute to Ca^{2+} transients? What is the mechanism by which they are activated and can this activation be graded? These issues are of particular relevance to heart disease where non-junctional RyRs are produced by t-tubule loss [21].

To gain insight into these issues we acquired and analyzed two-dimensional images of the sarcolemma and Ca^{2+} transients at a high spatial and temporal resolution. The study was based on our previous work, in which we applied three-dimensional confocal microscopy to living left ventricular cardiac myocytes of adult New Zealand white rabbits to reconstruct and analyze their transverse tubular system (t-system) [22]. The image data revealed remarkable heterogeneity of t-system density in these cells. Regions with dense t-system exhibited a regular arrangement of tubules with spacing of ~2 μm in the longitudinal and ~1 μm in the transversal direction. However, in some intracellular regions t-system was absent and the distance to the closest sarcolemma was larger than in regions with a regular arrangement of the t-system. We took advantage of this spatial heterogeneity in isolated rabbit ventricular cells to investigate the relationship between Ca^{2+} release and the distance to sarcolemma. We tested the hypotheses that (1) Ca^{2+} release can occur at sites distant to the sarcolemma and (2) regions of ventricular myocytes lacking t-system do not exhibit Ca^{2+} release.

2. Materials and methods

2.1. Isolation of rabbit ventricular myocytes

Ventricular myocytes were isolated from adult New Zealand White rabbits (2–2.5 kg) as previously described [22]. The animals received an intravenous injection of sodium pentobarbital (50 mg/ml) and heparin (10,000 USP/ml). The heart was quickly excised and the aorta cannulated. We used Collagenase P (Roche, Indianapolis, IN) and protease XIV (Sigma-Aldrich, St. Louis, MO) for enzymatic isolation of the cells using retrograde perfusion of the heart as described previously [23]. We stored the cells in a 1 mM Ca^{2+}–HEPES-buffered saline solution at room temperature until usage. All procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and animal protection guidelines of the University of Utah.

2.2. Loading of cells with fluo-4 and di-8-ANEPPS

Isolated cardiomyocytes were loaded with di-8-ANEPPS and fluo-4-AM (Invitrogen, Carlsbad, CA) as a marker for sarcolemma and indicator for Ca^{2+}, respectively. We loaded the cells with 12.5 μM of fluo-4-AM (Invitrogen, Carlsbad, CA, USA) for 15 min at room temperature. After 10 min we added 6.25 μM of di-8-ANEPPS (Invitrogen, Carlsbad, CA, USA). After 15 min we placed the cells in an imaging chamber and let them set for 10 min. The glass slide at the bottom of the imaging chamber was coated with mouse laminin (BD Biosciences, San Jose, CA). Subsequently, the cells were constantly superfused at room temperature with a modified Tyrode solution containing (in mM) 4.4 KCl, 138 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 11 dextrose, 24 HEPES, and 0.5 probenecid (pH 7.4 adjusted with NaOH).

2.3. High speed scanning of living cardiomyocytes

Cell segments were imaged within 5 h after isolation. Imaging was performed with a confocal microscope (Zeiss Live 5 Duo, Carl Zeiss, Jena, Germany) using a 63× oil immersion lens having a numerical aperture of 1.4. A schematic representation of imaging setup is presented in Fig. S1. The measured point spread function (PSF) for this imaging system is shown in Fig. S2. The confocal aperture was set to an Airy number of 1. Images covered an area of 1024 × 96 pixels with a spatial sampling at 0.1 μm and a temporal resolution of 3.64 ms. The dyes were excited with a 489 nm laser diode. The laser intensity was set to a small level allowing for analysis of the signals to minimize photobleaching. The fluorescent emission was separated using a dichroic beam splitter (NFT 535 nm). The emitted light was filtered using band-pass filters of 505–610 nm and 560–675 nm. Image acquisition was triggered 36.4 ms before electrical stimulation of the imaged cell using an adjacent electrode. Typically 50 images of the fluo-4 and di-8-ANEPPS associated fluorescence were acquired. Only image scans before significant contraction of the myocytes were used for subsequent analysis.

2.4. Image segmentation from di-8-ANEPPS scans and generation of distance maps

The outer sarcolemma of myocyte segments was manually outlined in the initial di-8-ANEPPS image. Gaussian filtering (SD: $\sigma_x = \sigma_y =$ 0.05 μm, $\sigma_t =$ 3.64 ms) was applied to the image sequence. Thresholding of the filtered initial image was applied to identify the sarcolemma including t-system. The threshold t was calculated as:

$$t = m + 2\sigma$$

with the mode m and SD σ of the intensity distribution. We calculated distance maps, which specify the distance between each pixel in the cell and its closest pixel identified as sarcolemma. All methods of image processing and analyses were implemented in Matlab (R2012b, Mathworks, Natick, MA). For a more detailed description of the image processing methods we refer to, for instance, Gonzalez and Woods [24].

2.5. Analysis of fluo-4 images

The fluo-4 images provide local information on the initial phase of the Ca^{2+} transient configured by several processes including diffusion of Ca^{2+} from neighboring sites, sarcolemmal fluxes and release from the SR. Analysis of fluo-4 images was limited to regions adjacent to sarcolemma and in the cell interior. Mean background signal intensity was detected in the cell exterior and removed. Gaussian filtering (SD: $\sigma_x = \sigma_y =$ 0.1 μm, $\sigma_t =$ 3.64 ms) was applied to the image sequence. The images were self-ratioed using the mean fluo-4 intensity (I_0) in the cell interior before stimulation. Approximately 10 images were used to calculate I_0. A 4th-order polynomial was fit to the filtered self-ratioed transient of each pixel. Analysis of this polynomial