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Cardiac expression of ryanodine receptor subtype 3; a strategic component in the intracellular Ca^{2+} release system of Purkinje fibers in large mammalian heart

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ABSTRACT

Background: Three distinct Ca^{2+} release channels were identified in dog P-cells: the ryanodine receptor subtype 2 (***RyR2***) was detected throughout the cell, while the ryanodine receptor subtype 3 (***RyR3***) and inositol phosphate sensitive Ca^{2+} release channel (***InsP3R***) were found in the cell periphery. How each of these channels contributes to the Ca^{2+} cycling of P-cells is unclear. Recent modeling of Ca^{2+} mobilization in P-cells suggested that Ca^{2+} sensitivity of *Ca^{2+} induced Ca^{2+} release* (***CICR***) was larger at the P-cell periphery. Our study examined whether this numerically predicted region of Ca^{2+} release exists in live P-cells. We compared the regional Ca^{2+} dynamics with the arrangement of intracellular Ca^{2+} release (**CR**) channels.

Methods: Gene expression of CR channels was measured by qPCR in Purkinje fibers and myocardium of adult Yucatan pig hearts. We characterized the CR channels protein expression in isolated P-cells by immuno-fluorescence, laser scanning confocal microscopy, and 3D reconstruction. The spontaneous Ca^{2+} activity and electrically-evoked Ca^{2+} mobilization were imaged by 2D spinning disk confocal microscopy. Functional regions of P-cell were differentiated by the characteristics of local Ca^{2+} events. We used the Ca^{2+} propagation velocities as indicators of channel Ca^{2+} sensitivity.

Results: RyR2 gene expression was identical in Purkinje fibers and myocardium (6 hearts) while *RyR3* and *$InsP_3R$* gene expressions were, respectively, 100 and 16 times larger in the Purkinje fibers. Specific fluorescent immunostaining of Ca^{2+} release channels revealed an intermediate layer of *RyR3* expression between a near-membrane *InsP3R*-region and a central *RyR2*-region. We found that cell periphery produced two distinct forms of spontaneous Ca^{2+}-transients: (1) large asymmetrical Ca^{2+} sparks under the membrane, and (2) typical Ca^{2+}-wavelets propagating exclusively around the core of the cell. Larger cell-wide Ca^{2+} waves (**CWWs**) appeared occasionally traveling in the longitudinal direction through the core of Pcells. Large sparks arose in a micrometric space overlapping the *InsP3R* expression. The *InsP3R* antagonists 2-aminoethoxydiphenyl borate (2-APB; 3 μM) and xestospongin C (XeC; 50 μM) dramatically reduced their frequency. The Ca^{2+} wavelets propagated in a 5–10 μm thick layered space which matched the intermediate zone of *RyR3* expression. The wavelet incidence was unchanged by 2-APB or XeC, but was reduced by 60% in presence of the *RyR3* antagonist dantrolene (10 μM). The velocity of wavelets was two times larger (86 ± 16 μm/s; n = 14) compared to CWWs' (46 ± 10 μm/s; n = 11; $P < 0.05$). Electric stimulation triggered a uniform and large elevation of Ca^{2+} concentration under the membrane which preceded the propagation of Ca^{2+} into the interior of the cell. Elevated Ca_i propagated at 150 μm/s (147 ± 34 μm/s; n = 5) through the region equivalent to the zone of *RyR3* expression. This velocity dropped by 50% (75 ± 24 μm/s; n = 5) in the central region wherein predominant RyR2 expression was detected.

Conclusion: We identified two layers of distinct Ca^{2+} release channels in the periphery of Pcell: an outer layer of *$InsP_3Rs$* under the membrane and an inner layer of *RyR3s*. The propagation of Ca^{2+} events in these layers revealed that Ca^{2+} sensitivity of Ca^{2+} release was larger in the RyR3 layer compared to that of other sub-cellular regions. We propose that *RyR3* expression in P-cells plays a role in the stability of electric function of Purkinje fibers.

Abbreviations: Ca_i, intracellular Ca^{2+} concentration; **CR**, Ca^{2+} release; ***CICR***, Ca^{2+} induced-Ca^{2+} release; ***SOICR***, store overload–induced Ca^{2+} release; **ER/SR**, endoplasmic/sarcoplasmic reticulum; ***InsP3R***, inositol 1,4,5-trisphosphate receptor; **P-cell**, cardiac Purkinje cell; ***RyR***, ryanodine receptor; ***RyR1-3***, ryanodine receptor subtypes 1-3.

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1. Introduction

In cardiac cells, the elevation of intracellular Ca^{2+} concentration (**Ca_i**) activates the sarcomere contraction, but also controls the membrane repolarization during the action potential (**AP**) and can mediate after -depolarizations and APs through Ca^{2+} sensitive depolarizing currents (see [1] for review). Ca_i elevations are produced by the intracellular Ca^{2+} release (**CR**) from the endo/sarcoplasmic reticulum (**ER**). The release operates through opening of the ryanodine receptor (*RyR*) which belongs to a superfamily of Ca^{2+} channels encompassing the inositol 1,4,5-trisphosphate receptors (*InsP3Rs*) [2–4]. *RyR* gating function is controlled by both cytosolic and intra-reticular Ca^{2+} levels. Thus, CR can be triggered by a rise in Ca^{2+} concentration (1) on the cytosolic side (*Ca^{2+}-induced Ca^{2+} release*; CICR [5,6]) and/or (2) on the intra-reticular side of the channel (*Ca^{2+} Store Overload–Induced Ca^{2+} release*; SOICR [7]). Three *RyR* isoforms (*RyR1*, *RyR2*, and *RyR3*) have been identified in mammalian tissues. These isoforms are encoded by three distinct genes (*ryr1*, *ryr2*, and *ryr3*) and share a high degree of sequence homology [8–10]. *RyR1* was primarily detected in skeletal muscle [8,11] while *RyR2* is the most prevalent *RyR* isoform in the heart [9,12]. The third isoform, *RyR3*, was found in a wide range of other tissues including smooth and skeletal muscles [13–15]. *RyR1* and *RyR2* have clearly identified roles in cell functions and, notably, in the excitation-contraction coupling. In contrast, the fundamental role of *RyR3* expression is somewhat "enigmatic" [16]. *RyR3* complements other CR channels to implement specific functions [14,17–20,20–22], and fulfills particular tasks at given stages of tissue development (e.g. skeletal muscle [23,24]), or in specific regions of the same organ (e.g. the brain [10,25,26]). *RyR3* seems to have also different roles depending upon the nature of interaction(s) with the other co-existing forms of channel [16]. In the heart, the presence of *RyR3* has been detected in the Purkinje fibers [27], suggesting a specific role in cardiac conduction. In dog P-cells, *RyR3* co-exists with *InsP3R* in the cell periphery while *RyR2* is largely predominant in the core. Recent modeling of intracellular Ca^{2+} dynamics in pig P-cells suggested that *CICR* in the cell periphery was more sensitive to Ca^{2+} compared to other sub-cellular regions [28] and this feature participated in the Ca^{2+} mobilization process of those cells. The current study explored the hypothesis that this computationally predicted CR region exists in P-cells and directly relates to the peripheral expression of *RyR3*. We compared remarkable features of intracellular Ca^{2+} dynamics under spontaneous and electrically-evoked conditions with the nature and localization of ER-Ca^{2+} release channels in pig P-cells.

Spontaneous Ca_i elevations owing to abnormal CR in P-cells are thought to account for pro-arrhythmic activity in the His-Purkinje system [1,29]. Our study suggests that RyR3 expression plays an important role in Ca^{2+} cycling of P-cells, thus suggesting a new potential actor in the arrhythmogenicity of cardiac Purkinje system.

2. Materials and methods

2.1. Cell preparation

Free running Purkinje bundles (Fig. 1A) were dissected from the left and right ventricular chambers of adult (25–35 kg) Yucatan miniature swine. Single cells and 2–4 cells aggregates were enzymatically dispersed using method modified from [27,30–32]. Ventricular myocytes were collected during the procedure from the same hearts for comparisons. All cells were isolated from same age male Yucatan swine. Veterinary examination confirmed the absence of pathology in these animals. Freshly isolated cells exhibited a typical elongated shape and clear striation visible under phase contrast. The selected cells were free of blebs or vesicles, responded to field stimulation and showed a steady basal Ca^{2+} activity with standard 2 mM external Ca^{2+} concentration at 35 °C and pH 7.3. The typical finger-like shape of cell ends was an additional criterion of P-cell identification [30].

2.2. Gene expression

Gene expression was measured for *RyR1* (ryr1), *RyR2* (ryr2), *RyR3* (ryr3), and *InsP3R1* (itpr1) in Purkinje fibers, myocardium and skeletal muscle of the same animal. Relative transcription level was evaluated by quantitative real-time PCR methods as described in [33]. For analyses of real-time PCR data, the comparative threshold cycle (C_T) method was used to calculate the relative quantities of gene expression for samples. Statistical testing was made by one-way ANOVA with PCR data combined from two-independent experimental runs (6 animals per run). Data are reported as the mean ± sem for the relative expression ratio of target genes to reference samples. Difference with $P < 0.05$ was considered significant.

2.3. Immunofluorescence

Freshly isolated P-cells were fixed with 2% formaldehyde, permeabilized with 0.3% triton X-100 and incubated with primary antibodies overnight at 4 °C. The preparations were then exposed for 3 h to fluorescent secondary antibodies. The details of the procedure and list of antibodies used in the study are given in expanded methods in Supplement section. Fluorescence was imaged by laser scanning confocal microscopy at 60× magnification. Antibody distribution was assessed in the cell volume by optical sectioning (z step: 0.25 or 0.5 μm) and 3D reconstruction.

2.4. Intracellular Ca^{2+} imaging

Single cells and cell-aggregates were loaded with the fluorescent Ca^{2+} dye Fluo-4 and placed in a temperature-controlled chamber on the stage of a motorized inverted microscope. Fluorescence of Fluo-4 was imaged by 2D spinning disk confocal microscopy at 33 frames per second (fps). Ca^{2+} concentration was expressed by the F/Fo ratio where F was the instantaneous fluorescence intensity and Fo, the reference fluorescence intensity (measured prior to the event of interest). F/Fo variation (ΔF/Fo) of 1 was considered as equivalent to a 0.1 μmol·L^{-1} variation of Ca^{2+} concentration (ΔCa_i) [27,34,35]. AP-evoked Ca^{2+} transients were imaged from electrically paced cells (stimulation frequency: 0.3 Hz). Experiments were conducted at 35 °C, pH 7.3 and, unless otherwise specified in the text, 2 mM external Ca^{2+} concentration. Under standard conditions, cytosolic Ca^{2+} dynamics was imaged at mid-thickness of the cell (see expanded methods in appendix). Ca^{2+} imaging experimental conditions were identical to those set prior fixation of cells for immunofluorescence.

2.5. Image processing and data analysis

Images were processed and analyzed by using the NIH open-source program image-J, Olympus Metamorph software and custom IDL routines as indicated in expanded methods.

2.6. Statistics

Qualitative observations reported in this study were obtained from cells prepared from different hearts ($N > 5$) and representative examples are shown in the figures. Quantitative data were expressed as mean ± SEM or mean ± SD depending on number of samples. Comparisons of groups of normally distributed data were made by paired *t*-tests. Wilcoxon signed-ranks test was used for non-Gaussian distributions. Significant differences were considered for $P < 0.05$.

The utilization of animals as described in the protocols was approved by the Institutional Animal Care Committee of Memorial University of Newfoundland, and complied with the guidelines of the Canadian Council of Animal Care.