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Spontaneous Ca^{2+} transients in rat pulmonary vein cardiomyocytes are increased in frequency and become more synchronous following electrical stimulation

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ABSTRACT

The pulmonary veins have an external sleeve of cardiomyocytes that are a widely recognised source of ectopic electrical activity that can lead to atrial fibrillation. Although the mechanisms behind this activity are currently unknown, changes in intracellular calcium (Ca^{2+}) signalling are purported to play a role. Therefore, the intracellular Ca^{2+} concentration was monitored in the pulmonary vein using fluo-4 and epifluorescence microscopy. Electrical field stimulation evoked a synchronous rise in Ca^{2+} in neighbouring cardiomyocytes; asynchronous spontaneous Ca^{2+} transients between electrical stimuli were also present. Immediately following termination of electrical field stimulation at 3 Hz or greater, the frequency of the spontaneous Ca^{2+} transients was increased from 0.45 ± 0.06 Hz under basal conditions to between 0.59 ± 0.05 and 0.65 ± 0.06 Hz ($P < 0.001$). Increasing the extracellular Ca^{2+} concentration enhanced this effect, with the frequency of spontaneous Ca^{2+} transients increasing from 0.45 ± 0.05 Hz to between 0.75 ± 0.06 and 0.94 ± 0.09 Hz after electrical stimulation at 3 to 9 Hz ($P < 0.001$), and this was accompanied by a significant increase in the velocity of Ca^{2+} transients that manifested as waves. Moreover, in the presence of high extracellular Ca^{2+}, the spontaneous Ca^{2+} transients occurred more synchronously in the initial few seconds following electrical stimulation. The ryanodine receptors, which are the source of spontaneous Ca^{2+} transients in pulmonary vein cardiomyocytes, were found to be arranged in a striated pattern in the cell interior, as well as along the periphery of cell. Furthermore, labelling the sarcolemma with di-4-ANEPPS showed that over 90% of pulmonary vein cardiomyocytes possessed T-tubules. These findings demonstrate that the frequency of spontaneous Ca^{2+} transients in the rat pulmonary vein are increased following higher rates of electrical stimulation and increasing the extracellular Ca^{2+} concentration.

1. Introduction

The pulmonary veins are widely accepted to play a significant role in the development of atrial fibrillation [1,2]; however, the underlying pathophysiology is still incompletely understood. A potential mechanism that may contribute to this arrhythmia is ectopic electrical activity originating in the cardiomyocytes that form a sleeve surrounding the blood vessel, which then propagates to the atria and disrupts normal sinus rhythm [3]. Independent electrical activity within the pulmonary vein was first demonstrated by Cheung in 1981, when spontaneous action potentials were recorded from cardiomyocytes in the pulmonary vein [4]. Nevertheless, subsequent studies have shown that there is significant variability in the incidence of spontaneous electrical activity, both within [5–8] and between [9] different species.

There is increasing evidence that calcium (Ca^{2+}) release from the sarcoplasmic reticulum (SR) *via* the ryanodine receptor (RyR) plays an important role in the generation of ectopic electrical activity originating in the pulmonary vein. Low concentrations of ryanodine (0.5–2 μM), which would increase the opening probability of the RyRs, were shown to cause depolarisation of the cardiomyocyte resting membrane potential, and subsequent short trains of high frequency electrical stimuli triggered bursts of spontaneous action potentials that eventually self-terminated [6]. In contrast, spontaneous action potentials could not be induced by pacing the atrium in the presence of ryanodine,

Abbreviations: EFS, electrical field stimulation; FOV, field of view; LTCC, L-type Ca^{2+} channel; NCX, Na^+/Ca^{2+} exchanger; PSS, physiological salt solution; SR, sarcoplasmic reticulum; ROI, region of interest; RyR, ryanodine receptor

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indicating that there are fundamental differences between cardiomyocytes in the pulmonary vein and atrium [6]. High concentrations of ryanodine (10 μM), sufficient to inhibit the RyR, have been shown to completely suppress triggered firing in the pulmonary vein, which was initiated by autonomic nerve stimulation [10], and further support for the involvement of the RyR was provided by the finding that the RyR stabilizer, K201 reduced the frequency of spontaneous action potentials occurring in cardiomyocytes that were isolated from the pulmonary vein [11]. The link between intracellular Ca^{2+} and a change in the cardiomyocyte membrane potential is thought to be through the activation of the Na^+/Ca^{2+} exchanger (NCX), since inhibition of the exchanger has been found to suppress spontaneous electrical activity in pulmonary vein cardiomyocytes [5,12–14].

We have previously shown that the cardiomyocytes in the rat pulmonary vein display spontaneous Ca^{2+} transients that often manifest as waves occurring asynchronously in neighbouring cells [15,16], and a similar observation has recently been made regarding the mouse pulmonary vein [17]. A high concentration of ryanodine (20 μM) abolished the spontaneous Ca^{2+} transients in the majority of cardiomyocytes in the rat pulmonary vein [15], which is consistent with the notion that Ca^{2+} waves occur due to summation of the elementary Ca^{2+} sparks that result when Ca^{2+} is released from the SR [18]. Since spontaneous SR Ca^{2+} release typically occurs as a wave in the pulmonary vein cardiomyocytes, this will lead to a relatively slow increase in the activation of Ca^{2+}-activated currents as well as the NCX [19]. Indeed, it has been shown in isolated pulmonary vein cardiomyocytes that there is a noradrenaline induced inward current, which correlated with the size of the cytosolic Ca^{2+} transient during diastole [20]. Within the tissue, neighbouring cells will act as a current sink, diminishing any change in membrane potential that would occur through activation of the NCX [21]. Thus, these factors are likely to limit the ability of such asynchronous Ca^{2+} events to initiate spontaneous action potentials.

A direct mechanistic link between spontaneous Ca^{2+} transients and arrhythmogenic activity has previously been demonstrated in ventricular tissue. Electrical pacing (2 Hz) in the presence of isoproterenol and low extracellular K^+ induced an increase in the frequency of spontaneous Ca^{2+} transients after termination of stimulation, and this was accompanied by delayed after depolarisations (DADs) [22]. In another study on the ventricle, mathematical modelling showed that spontaneous Ca^{2+} transients occurred more synchronously after a period of high-frequency electrical stimulation (5 Hz), and that the spontaneous Ca^{2+} transient synchronicity correlated with the size of the resultant DADs [23]. Thus, it is apparent that for spontaneous Ca^{2+} transients to generate arrhythmogenic activity they must be entrained to occur more synchronously.

An important component of ectopic activity originating in the pulmonary vein is SR Ca^{2+} release *via* the RyR. Therefore, the aim of the present study was to investigate whether increasing cellular Ca^{2+} loading by raising the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$), in combination with brief periods of electrical stimulation, could synchronise the spontaneous Ca^{2+} transients and increase their potential arrhythmogenicity. The localisation of the RyRs and voltage-gated L-type Ca^{2+} channels (LTCCs) was also studied to provide further insight into how excitation-contraction coupling is controlled in pulmonary vein cardiomyocytes. Elucidating how intracellular Ca^{2+} signalling is regulated in the pulmonary vein is key towards understanding the causes of ectopic activity that can lead to atrial arrhythmias.

2. Materials and methods

2.1. Animals and pulmonary vein isolation

Adult male Sprague-Dawley rats, weighing 250–430 g, were euthanised by cervical dislocation according to Schedule 1 of the Animals (Scientific Procedures) Act, 1986. After opening the thoracic cavity, the heart and lungs were quickly removed *en bloc* and placed in ice cold physiological salt solution (PSS) of the following composition (in mM); 119 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.17 $MgSO_4$, 1.18 KH_2PO_3, 2.5 $CaCl_2$ and 5.5 glucose (pH 7.4 with 95% O_2 and 5% CO_2). The main pulmonary vein branches to the left and posterior right lung lobes were micro-dissected and cleaned of any surrounding parenchyma under a Nikon SMZ645 stereomicroscope, which resulted in two separate pulmonary vein branches, measuring approximately 10–15 mm in length and 2–3 mm in outside diameter.

2.2. Imaging intracellular Ca^{2+}

The pulmonary vein was incubated for 60 min in the dark at room temperature, in PSS containing 10 μM fluo-4 AM and cremophor EL (0.03% v/v). The tissue was then washed and pinned onto a Sylgard® coated tissue chamber containing 4 ml PSS. The preparation was allowed 15 min for equilibration before any recording was performed. All experiments were carried out at room temperature (21–24 °C).

The pulmonary vein was imaged by wide-field epifluorescence microscopy, using an upright Zeiss Axioscop 50 epifluorescence microscope (Carl Zeiss, Germany) and a 40× objective lens (Achroplan, Carl Zeiss, Germany). Fluo-4 was excited by light from a 50 W mercury short ARC lamp (Osram, Germany), passed through a 450–490 nm band pass excitation filter. The emitted fluorescence was passed through a 515 nm long pass emission filter and images were captured using a Hamamatsu multiformat CCD camera (C4880-80, Hamamatsu Photonics K. K., Japan). Images were acquired with an exposure time of 109 ms (~9 frames per second) using WinFluor V3.2.19 (Dr John Dempster, University of Strathclyde).

2.3. Electrical stimulation of the pulmonary vein

The pulmonary vein was stimulated by electrical field stimulation (EFS) using two platinum electrodes, positioned either side of the vein, approximately 1 cm apart. Rectangular pulses (2 ms duration) were applied at supramaximal voltage (80–100 V) using a Grass SD9 stimulator (Grass Instrument Co., USA).

2.4. Analysis of Ca^{2+} transients

Spontaneous Ca^{2+} transients were analysed for their peak amplitude and frequency using a custom-built plugin written for ImageJ. A region of interest (ROI) (20 × 20 pixels; equivalent to 5 × 5 μm) was selected in a cardiomyocyte and an initial record of pixel intensity over time was obtained. The plugin then fitted a polynomial 4th order curved baseline, and divided the raw data by the calculated baseline value at each time-point to correct for photobleaching. The plugin contained an algorithm that distinguished peaks and nadirs in the data where there was a general increase or decrease in the fluorescence signal, and the amplitude ($\Delta F/F_{min}$) was automatically calculated for each Ca^{2+} transient. For the spontaneous Ca^{2+} transients, the mean amplitude during the recording period was calculated for each ROI, and the number of peaks per second was also obtained for the frequency (Hz). The velocity of the spontaneous Ca^{2+} waves was measured as distance/time using the "Kymograph" plugin for ImageJ.

The synchronisation of spontaneous Ca^{2+} transients was analysed using a script written in the Python language. A mask was applied from a maximum projection image to isolate a region of tissue displaying spontaneous Ca^{2+} transients. The code then determined the minimum and maximum intensity of each pixel throughout an image series and then calculated the half maximal pixel intensity for every pixel. A threshold of 50% was applied in order to create a binary image series that distinguished pixels with a higher or lower intensity than the threshold, and the fraction of pixels with a higher intensity than the threshold was calculated for each frame providing a synchronisation index between 0 and 1 for the whole image series. For example, during an electrically evoked response all the pixels comprising the tissue

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