A New Approach to Study the Relationship between Calcium Waves and Diastolic [Ca\textsuperscript{2+}]\textsubscript{i} in Heart Muscle Cells

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In the present study, changes in calcium waves with alternation of diastolic [Ca\textsuperscript{2+}]\textsubscript{i} were investigated in a single common rat neonatal cultured heart muscle cell without changes in extracellular conditions. Heart muscle cells were loaded with fluo-3 by the "whole cell clamp" technique. Rapid changes in diastolic [Ca\textsuperscript{2+}]\textsubscript{i} by variations in the membrane potential clarified the influences on calcium waves. When the membrane potential was held at 0 mV, inter wave basal [Ca\textsuperscript{2+}]\textsubscript{i} were maintained at a high level. The membrane-holding potential was then momentarily altered to -80 mV and the basal [Ca\textsuperscript{2+}]\textsubscript{i} registered a lower level within 1 sec. During these events, spontaneous calcium waves were analyzed by confocal laser scanning microscopy (X-t scanning mode). The frequency and velocity of calcium waves at 0 mV significantly increased compared with those at -80 mV. Moreover, these changes were more pronounced with increasing extracellular [Ca\textsuperscript{2+}]. These results suggest that diastolic [Ca\textsuperscript{2+}]\textsubscript{i} directly influences calcium waves.

Key Words: Diastolic [Ca\textsuperscript{2+}]\textsubscript{i}, Calcium wave, Whole cell clamp, Confocal laser scanning microscope

I. Introduction

The calcium wave in heart muscle cells is an abnormal Ca\textsuperscript{2+}-transient [9, 10, 16, 17, 22], and is usually encountered when cells are Ca\textsuperscript{2+}-overloaded. Ischemia-reperfusion [7] and injury of papillary muscles [20] are typical examples of such a phenomenon. Therefore, they are thought to be related with certain arrhythmiae [7, 16, 17, 19, 20]. It is known that extracellular perfusates containing high Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]) induce high-frequency calcium waves [9, 12]. However, the direct relationship between the level of diastolic intracellular free Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]\textsubscript{i}) and calcium waves remains unclear. High extracellular [Ca\textsuperscript{2+}] not only produce elevation of diastolic [Ca\textsuperscript{2+}]\textsubscript{i}, but also influence cellular Ca\textsuperscript{2+}-handling. All such events contribute to a compensation mechanism regulating the calcium waves.

The purpose of this study was to clarify the direct relationship between diastolic [Ca\textsuperscript{2+}]\textsubscript{i} and calcium waves under constant extracellular conditions. Confocal laser scanning microscopy (CLSM) [18, 19] and rapid alternation of diastolic [Ca\textsuperscript{2+}], with the "whole cell clamp" technique [6] enabled us to analyze the direct effects of variations in diastolic [Ca\textsuperscript{2+}]\textsubscript{i} on calcium waves in a single common heart muscle cell.

II. Materials and Methods

Cultured heart muscle cell preparation

Heart muscle cells were obtained by enzymatic digestion of ventricular tissues from neonatal (2-4 days of age) Wistar rats as described previously [12]. The minced ventricular cubes were suspended and disaggregated in phosphate-buffered solution (pH 7.3) containing 0.2% collagenase (type I, Sigma Chem. Co.) for 60 min at 37°C. The cells were incubated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum for 3-5 days.
Perfusion
Cells were perfused with a modified Tyrode's solution containing 145 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 5 mM or 10 mM CaCl₂, 10 mM D-glucose and 10 mM N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid (HEPES) (pH 7.3; adjusted with NaOH). All experiments were conducted at room temperature (20-24°C).

Whole cell clamp
Cells were loaded with fluo-3 through a micropipette electrode by rupturing a patch in the cell membrane. Electrodes were filled with a medium containing 100 μM fluo-3, 2 mM ATP (dipotassium salt), 140 mM KCl, 12 mM NaCl, 2 mM MgCl₂ and 10 mM HEPES (pH 7.2; adjusted with KOH). Cells were voltage-clamped using the "whole-cell recording" technique [6]. The membrane-holding potential was initially adjusted to 0 mV. After [Ca²⁺] increased homogeneously with excitation-contraction coupling (E-C coupling), calcium waves followed with a high frequency. The membrane-holding potential was then changed to -80 mV momentarily. The changes in [Ca²⁺] and calcium waves via this sequence of events were analyzed by CLSM (X-t scanning mode; 4 sec/frame, successive 2 frames, 8 msec/line) [6, 19].

Confocal microscopy and image analysis
The system for CLSM consisted of the following subsystems; a confocal laser scanning apparatus, an inverted microscope (Olympus IMT-2, Japan), an image processor (Imaging Technology Inc. Series 151, USA) and electrophysiological equipment. These subsystems were controlled by a 32-bit personal computer (Sanyo MBC-18TJ, Japan) using the "C" language, library of subroutines available for GP-IB and analog-digital interfaces (National Instruments, USA).

The argon laser beam (λ=488 nm, 10 mV) was projected via a beam expander and a dichroic mirror (λ=500 nm) to establish a fluorescent light, which was then directed to a photomultiplier through the dichroic mirror and a pinhole aperture (100 μm) to create a confocal image. A viewing image was generated from the fluorescence intensity data, which were stored in a 512 × 512 × 8-bit frame memory. In the present study, we employed a line scan method (maximum speed: 2 msec/line), where

![Image of calcium waves generation](image-url)
scanning on a line corresponding to the long axis of a cultured heart muscle cell along the X axis was done (X-t scanning) to facilitate quantitative study of rapid changes of [Ca\(^{2+}\)].

III. Results

Membrane potential and [Ca\(^{2+}\)]

While the membrane-holding potential was maintained at 0 mV, inter calcium wave basal [Ca\(^{2+}\)] remained higher than at -80 mV (Fig. 1C). After the membrane-holding potential was rapidly restored to -80 mV, basal [Ca\(^{2+}\)] recovered to the resting [Ca\(^{2+}\)], level within 1 sec. Because basal [Ca\(^{2+}\)], levels at 0 mV and -80 mV were consistent, the influence by changes in the loading conditions or photo-bleaching of the Ca\(^{2+}\) indicator could be ignored in this study.

Frequency and velocity of calcium waves

When basal [Ca\(^{2+}\)] was maintained at a high level with membrane-holding potential at 0 mV, the frequency of calcium waves significantly increased compared with that at -80 mV (Fig. 1B). In addition, the propagating speed of calcium waves was significantly increased with elevation of the basal [Ca\(^{2+}\)].

Effect of extracellular [Ca\(^{2+}\)]

When the perfusate [Ca\(^{2+}\)] was adjusted at 10 mM, changes due to variations in the basal [Ca\(^{2+}\)], were enhanced. In addition, calcium sparks [5] occurred more frequently and distinct calcium waves were occasionally unable to be detected for significantly elevated basal [Ca\(^{2+}\)], (Fig. 2).

IV. Discussion

In our present study, the direct effects of diastolic [Ca\(^{2+}\)], on calcium waves in a single common heart muscle cell were tested. This novel approach enabled us to acquire various basal [Ca\(^{2+}\)], without producing structural changes in the organelles and generating differences in loading conditions of Ca\(^{2+}\) indicators and extracellular conditions including the perfusate [Ca\(^{2+}\)]. This is an important stipulation. In changing the perfusate [Ca\(^{2+}\)], diastolic [Ca\(^{2+}\)], is certain to increase, altering subsequently the many other factors controlling cellular Ca\(^{2+}\)-handling, such as the Ca\(^{2+}\) channels, Ca\(^{2+}\) pumps and Na\(^+\)-Ca\(^{2+}\) exchanger. Therefore, changes in calcium waves

![Fig. 2. Generation of calcium waves at 10 mM perfusate [Ca\(^{2+}\)]. Changes in the calcium waves with variations in basal [Ca\(^{2+}\)], were more pronounced and were accompanied by frequent calcium sparks. In addition, calcium waves at 0 mV were occasionally unclear for remarkably elevated basal [Ca\(^{2+}\)].](image-url)
may be dependent on not only elevated diastolic [Ca^{2+}]_{i} but also these factors. In this study, we managed to monitor calcium waves at various diastolic [Ca^{2+}]_{i} levels without changing the extracellular factors.

On holding the membrane potential at 0 mV for more than 10 sec, Ca^{2+} channels at the cell membrane are inactivated [4]. Although the membrane-potential-dependent [11] Na^{+}/Ca^{2+} exchanger makes a difference in the basal [Ca^{2+}]_{i}, the membrane potential per se does not directly influence Ca^{2+}-induced Ca^{2+} release (CICR) [3, 13, 14], which is regarded as a propagating mechanism of the calcium wave [9, 10, 16, 17, 22]. This is because CICR in heart muscle cells is mainly dependent on the Ca^{2+}-releasing channel and Ca^{2+}-restoring pump in sarcoplasmic reticulum (SR), which are thought to be independent of the membrane potential itself [1, 14]. The present study clarified that alternations in calcium wave propagation are controlled by basal [Ca^{2+}]_{i}. Therefore, it is suggested that diastolic [Ca^{2+}]_{i} influences the Ca^{2+}-releasing channel and Ca^{2+}-restoring pump in SR directly. However, it should be elucidated hereafter whether the difference in membrane potential would involve the function of the Ca^{2+} channel and Ca^{2+} pump in SR completely.

Our findings revealed that frequency and velocity of calcium waves increased at a high basal [Ca^{2+}]_{i}. The propagating speed was dependent on the intervals between each Ca^{2+} release from the neighboring SR. There are two possibilities accounting for these changes: firstly, Ca^{2+} pooling in SR is so great that released Ca^{2+} rapidly reaches the threshold of CICR under elevated diastolic [Ca^{2+}]_{i} conditions [8, 15, 21]; secondly, the relative threshold decreases with elevation in basal [Ca^{2+}]_{i}. Because the frequency is dependent on the basal [Ca^{2+}]_{i}, the latter appears to be more rational, whereas the former warrants further studies.

It is thought that the calcium wave is an abnormal Ca^{2+} transient, and this phenomenon is closely related to mechanism of various arrhythmiae. Our findings suggest that spontaneous calcium waves can be easily induced and propelled at high basal [Ca^{2+}]_{i}. These events may expound the frequent occurrence of arrhythmiae at high diastolic [Ca^{2+}]_{i}, as in the case of a heart failure [2].

V. References