Digital Imaging System for Recording Rapid Changes in Intracellular Ca\(^{2+}\) Concentrations Triggered by Electrical Stimulation of Cardiac Myocytes

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Abstract. To examine the origin and spreading of the Ca\(^{2+}\) transient following electrical stimulation of isolated myocyte, a system capable of recording intracellular Ca\(^{2+}\) distribution with sufficient temporal and spatial resolution was constructed. The system consists of a fluorescence microscope with computer-controlled pulse illumination and a digital image analyzer. The results with this new equipment show that the Ca\(^{2+}\) transient originates from one or a few points within a myocyte, and spreads throughout the cell. During the initial 60-msec period, the distribution of Ca\(^{2+}\) within a myocyte was not uniform. The system may be used for better understanding of the excitation-contraction coupling mechanism occurring within a cardiac myocyte or of changes in intracellular Ca\(^{2+}\) concentrations in other cells in which Ca\(^{2+}\) plays a crucial role in signal transduction. (Keio J Med 39 (3): 168–172, September 1990)

Key words: calcium transient, fura-2, myocyte

Introduction

A transient increase in intracellular Ca\(^{2+}\) concentration is an important link in the excitation-contraction coupling mechanism in heart muscle. The time course and magnitude of the Ca\(^{2+}\) transient has been examined using a bioluminescent protein, aequorin, or Ca\(^{2+}\) indicator dyes such as fura-2, indo-1 or quin-2. The results indicate that many positive inotropic drugs or pathological conditions modify the Ca\(^{2+}\) transient. In the above studies, however, changes in Ca\(^{2+}\) concentrations were observed as those averaged from many cells, and events occurring within a single cell have not been studied because of limitations of systems used to record Ca\(^{2+}\) concentrations. When intracellular Ca\(^{2+}\) distributions were examined, studies were limited to that in a resting cell or during oscillatory aftercontractions.

To advance our understanding of the excitation-contraction coupling mechanism, and to understand pathophysiological changes that occur in hypertrophied or Ca\(^{2+}\) overloaded myocytes, it is necessary to examine the Ca\(^{2+}\) transient, especially its origin and the pattern of spreading, within a cell. Recently, Takamatsu and Wier recorded the Ca\(^{2+}\) transient within a single myocyte, and reported that Ca\(^{2+}\) concentrations within the cell were uniform in normal cells. They did observe non-uniform distribution of Ca\(^{2+}\) in a Ca\(^{2+}\) overloaded dying myocyte in which spontaneous slow waves of Ca\(^{2+}\) repeatedly travelled from one end to the other.

Because the time course of the Ca\(^{2+}\) transient in heart muscle is rapid, the time resolution of 67 msec used by the above investigators may be insufficient to record the initial events that occur in heart muscle. The pictures should be taken at about 10-msec intervals. We have constructed a system based on fluorescence microscope with ultraviolet pulse illumination and a digital image analyzer. We now report the description of the system and results obtained with myocytes isolated from rat heart.

Materials and Methods

Myocytes

Ca\(^{2+}\)-tolerant myocytes were isolated from cardiac ventricular muscle of Wistar rats (approximately 200
grams) using collagenase during low Ca\(^{2+}\) perfusion as described by Stemmer et al.\(^{14}\) Cells were loaded with fura-2 by incubating in the presence of 1 \(\mu\)M fura-2/AM (Molecular Probes, Eugene, Oregon) at 37°C for 15 min in a HEPES buffer solution containing 130 mM NaCl, 5.8 mM KCl, 1 mM Na\(_2\)HPO\(_4\), 1 mM MgCl\(_2\), 1.2 mM CaCl\(_2\), 10 mM glucose and 10 mM HEPES. The pH value was adjusted to 7.4. Subsequently, cells were suspended in a Krebs-Henseleit bicarbonate buffer solution containing 118 mM NaCl, 27.1 mM NaHCO\(_3\), 4.8 mM KCl, 1 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 1.2 mM CaCl\(_2\) and 10 mM glucose. The solution was saturated with a 95% O\(_2\), 5% CO\(_2\) gas mixture yielding the final pH value of 7.4. Cells were placed in an open chamber with a cover-glass bottom on the stage of an inverted epifluorescence microscope (Nikon Diaphot TMD, Nihon Kogaku, Tokyo). The volume of the chamber was approximately 0.5 ml, and the medium was continuously replaced by a flow of fresh buffer solution (0.3 ml/min) saturated with a 95% O\(_2\), 5% CO\(_2\) gas mixture. Cells were dispersed so that no more than four cells could be seen in a field with the 40X objective lens of the microscope. Most viable (rod-shaped) cells were attached to the glass bottom, whereas many rounded cells did not attach to the glass. Floating rounded cells were removed by occasionally increasing the flow rate. Cells were stimulated at room temperature (27–28°C) with an electrical field of 1 Hz with 4-msec pulses using a pair of platinum wire electrodes.

Recording system

Fluorescence microscopy and digital image analyzer system was constructed using a modified xenon lamp and an electrical stimulator (modified ARGUS 100/Ca, Hamamatsu Photonics, Hamamatsu, Japan and model SEN3301 stimulator with SS201J stimulus isolation unit, Nihon Kohden Kogyo, Tokyo). A 300-watt xenon lamp (Hamamatsu Photonics, type L-2480–01) was lit with two power supplies in parallel; one provided a minimal DC current necessary for maintaining stable glowing of the xenon arc, and the other supplied a large current of short duration for pulse illumination (Fig. 1). Ultraviolet illumination of samples resulting from stable firing of the lamp was further reduced by using a series of neutral density filters. A microcomputer controls the timing for the video camera (C2400–08 SIT camera, Hamamatsu Photonics), the electrical stimulator, and the pulse illumination (Fig. 1). The intervals between the electrical stimulation and the pulse illumination can be set within the range between 5 msec after the electrical stimulation and 50 msec before the next stimulation (Fig. 2). The preparation is illuminated with high intensity for 1.5 msec. The resulting fluorescence picture is deposited on the light-sensitive screen of the SIT camera which subsequently converts the picture into electrical signals. The electrical charge representing the image on the photosensitive screen of the SIT camera is stable until it is scanned within 30 msec by the electron beam of the camera for recording. Even though the camera needs 30 msec to collect data, the picture represents the 1.5 msec illumination period preceding the scan.

With a given time delay following stimulation, fluorescence pictures at 500 nm were taken with an excitation wavelength of 340 nm. On every other cycle, the back-
ground picture was taken without the pulse-illumination but with electrical stimulation (Fig. 2, lower panels). The values of background pictures were subtracted from those obtained with pulse illumination at corresponding pixels. Digital accumulation of five to eight pictures representing ten to sixteen cardiac cycles produced clear pictures with a relatively low level of fura-2 loading used in the present study. Immediately after a set of pictures was taken at 340 nm excitation, the wavelength was switched to 380 nm, and the process was repeated. The entire sequence was repeated 10 to 15 times while altering the time delay between electrical stimulation and pulse illumination. Approximately 8 min were needed to obtain pictures representing fifteen time points during the cardiac cycle. After all pictures were obtained and digitized, free Ca²⁺ concentration was calculated using the method described by Grynkiewicz et al. from the ratio of fluorescent intensity observed with 340 and 380 nm excitation at each pixel on the screen. Calibration curves for Ca²⁺ concentrations were obtained using EDTA-Ca²⁺ buffer system and with calculated Ca²⁺ concentrations of 10 to 10,000 nM.

**Results**

The cardiac myocytes isolated from rat heart maintained a well striated, rod shape appearance for several hours under the microscope. These cells were quiescent unless electrically stimulated. No signs of Ca³⁺ overloading, such as oscillatory aftercontractions following electrical stimulation were observed (data not shown). During an early phase of the study, however, those myocytes which were continuously monitored under the microscope started to contract spontaneously after 30 to 40 min. Fluorescence pictures showed that very slow Ca²⁺ waves repeatedly moved from one end of the cell to the other end in these cells (data not shown, but similar results were reported by Takamatsu and Wier). These contractions and Ca²⁺ movements were not time-locked to electrical stimulation and could be observed even when electrical stimulation was stopped. A phase of spontaneous movements and Ca²⁺ waves was followed by a sudden rounding of the cell associated with a marked and uniform increase in intracellular Ca²⁺ concentrations. Slow Ca²⁺ waves observed in Ca²⁺ overloaded cells probably represent artifact. The results from these cells were not reported in this paper.

The spontaneous Ca²⁺ waves were not observed with other myocytes in the chamber at the time when the cell under observation was rounded. This would indicate that these spontaneous movements and Ca²⁺ waves were caused by cell injury resulting from exposure of the cell to ultraviolet light used for observation. Subsequently, the level of the ultraviolet exposure resulting from continuous firing of the xenon lamp was reduced using a series of neutral density filters, and the spontaneous movements of the myocytes were completely prevented. In order to compensate the absorption of excitation light by the neutral density filters, the voltage of the xenon lamp for pulse illumination was increased to the maximum for the system, and several frames were accumulated to obtain clear pictures. With this new system, the cells maintained rod shape, and were quiescent unless electrically stimulated, for up to 3 hours.

Fig. 3 shows the wave of intracellular Ca²⁺ concentration, or the movement of the Ca²⁺ transient, which was triggered by and time-locked to the electrical stimulation. Apparently, intracellular Ca²⁺ does not increase uniformly within a myocyte when the cell is stimulated with electrical field. In the cell shown in Fig. 3, initial increase in intracellular Ca²⁺ occurred at the far right as well as at the mid-point of the cell (10-msec panel). The area of relatively low Ca²⁺ concentrations shown in pale blue color rapidly spread throughout the entire cell volume, whereas areas of higher Ca²⁺ concentrations shown in white color slowly spread around the point of origin (20-msec panel). Subsequently, the areas of high Ca²⁺ concentration spread to the entire cell volume reaching the peak Ca²⁺ concentration at about 80 msec. After 100 msec, the intracellular Ca²⁺ concentration uniformly decreased returning to the resting value at about 300 msec in the cells stimulated at 1 Hz. These results clearly demonstrate that intracellular Ca²⁺ concentration is not uniform during the initial 40-msec period when the Ca²⁺ concentration was increasing within a myocyte.

The shortening of the myocyte was slightly delayed compared to the Ca²⁺ transient. The maximal shortening of the cell shown in Fig. 3 occurred at about 100 msec after electrical stimulation when average intracellular Ca²⁺ concentration was already declining.

**Discussion**

The present system consisting of Hamamatsu Photonics ARGUS 100/Ca with modified software and a xenon lamp with the power supply for pulse illumination was capable of recording the distribution of intracellular Ca²⁺ concentrations with temporal and spatial resolution sufficient to analyze the initial spreading of the Ca²⁺ transient following electrical stimulation of an isolated myocyte. The software for ARGUS 100/Ca was modified so that consecutive pictures could be taken for signal accumulation, and the Ca²⁺ concentration was calculated from the ratio of fluorescent intensity using the accumulated data. The preparation was illuminated with 1.5-msec pulse of high intensity light delivered from a xenon lamp during the blanking period of the SIT camera. During the illumination period, the picture was deposited on the photo-sensitive screen of the camera and then
Fig. 3 Changes in intracellular Ca\(^{2+}\) concentrations triggered by electrical stimulation of a myocyte isolated from rat heart. An isolated myocyte was incubated at 27–28°C, and stimulated with electrical field at 1Hz. The number at upper left corner of each panel represents the time in milliseconds after electrical stimulation. Typical pictures from many cells.

Because signals were accumulated over several cycles, and pictures at 340 and 380 nm excitation were taken from separate cycles, there is a possibility of motion artifact in calculated ratios and hence Ca\(^{2+}\) concentrations. In fact, there are several places with seemingly high “Ca\(^{2+}\)” concentrations in Fig. 3, especially around the edge of the cell. These artifacts, however, are relatively isolated, and the general pattern of Ca\(^{2+}\) distribution can be observed with myocytes which were contracting in a regular and stable manner responding to electrical stimulations using the current technique.

Transient increases in intracellular Ca\(^{2+}\) ion concentration, the Ca\(^{2+}\) transient, following the excitation of myocardial cells, were demonstrated using the Ca\(^{2+}\)-sensitive bioluminescent protein, aequorin,\(^3\)–\(^5\) or the Ca\(^{2+}\)-sensitive fluorescent dye, fura-2,\(^6\) indo-1\(^7\)–\(^8\) or quin-2.\(^2\) The signal, however, was recorded from many cells, and the spatial distribution of the transient within a cell could not be observed. Recently, Takamatsu and Wier\(^13\) observed the fluorescence of indo-1 loaded cells with the use of a microscope and a video camera. Video cameras, however, do not have a sufficient time resolution to record the increasing phase of the Ca\(^{2+}\) transient. For example, Takamatsu and Wier\(^13\) recorded Ca\(^{2+}\) concentrations and reported that increases in intracellular Ca\(^{2+}\) concentrations were uniform within the cell. The present system, which is capable of recording the intracellular Ca\(^{2+}\) concentrations at 10 msec intervals, clearly demonstrated that the distribution of Ca\(^{2+}\) is not uniform during the initial 60-msec period.

It is possible to set intervals shorter than 10 msec:
however, the reaction of fura-2 with Ca\textsuperscript{2+} may take several milliseconds\textsuperscript{18,19} and this reaction time is the limitation for the temporal resolution of the current system when fura-2 is used. The delay in Ca\textsuperscript{2+}-fura-2 reaction, however, was relatively insignificant because the time between the Ca\textsuperscript{2+} transient and the contraction was about 20 msec, consistent with reported values\textsuperscript{2,3,5,8}. The changes in Ca\textsuperscript{2+} concentrations may be delayed by Ca\textsuperscript{2+} buffering activity of fura-2. During the preliminary studies, the time course of the observed Ca\textsuperscript{2+} transient was significantly slower. Reducing the level of fura-2 loading decreased the time to peak Ca\textsuperscript{2+} concentration. No further decrease in the time to peak Ca\textsuperscript{2+} concentration was observed when fura-2 loading was reduced below the current level (data not shown).

In summary, a modified ARGUS 100/Ca system with pulse illumination is capable of recording the origin and spreading of the intracellular Ca\textsuperscript{2+} transient in isolated myocytes. The Ca\textsuperscript{2+} concentration was not uniform within a myocyte during the initial 60-msec period.

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References

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