Brief Communication

Cytosolic Calcium Staircase in Cultured Myocardial Cells

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To elucidate the mechanism of the tension staircase, chick embryonic myocardial cell aggregates were loaded with the fluorescent cytosolic calcium indicator, indol. Fluctuations in indol fluorescence were compared with recordings of cell edge movement during spontaneous beating and during stimulation by intracellular current pulses. Indol-loaded aggregates exhibit fluorescence transients during each transmembrane action potential. The rising phase of the transients is rapid, but the decaying phase is slow (several hundred msec) and is similar in time course to the pandiastolic relaxation seen in the optical recordings of cell edge movement. Acceleration of beat frequency by brief depolarizing current pulses produces an ascending staircase in both edge movement and systolic \([Ca^{2+}]\). There is a similar staircase in the diastolic \([Ca^{2+}]\), which is also reflected by diastolic cell edge movement. The existence of a diastolic \([Ca^{2+}]\) staircase may provide new insight into the mechanism of the force-frequency relation in the heart. (Circulation Research 1987;61:934–939)

The effect of beat frequency on the contractility of myocardial cells is well established. Abrupt changes in the interstimulus interval produce stepwise changes in the strength of contraction, a phenomenon commonly referred to as “staircase.” It is widely accepted that the staircase arises from a progressive change in the peak intracellular calcium level. However, the behavior of the \([Ca^{2+}]\) transient during staircase has not been directly studied. Much of our understanding of excitation-contraction coupling derives from recordings of aequorin luminescence. The aequorin signal is sufficiently sensitive to detect \([Ca^{2+}]\) transients in cardiac tissue, but it suffers from two drawbacks. First, to achieve satisfactory signal-to-noise ratios, it is usually necessary to signal-average many individual transients; hence, beat-to-beat changes in \([Ca^{2+}]\) transients may not be appreciated. Second, the aequorin light response varies with the 2.5 power of \([Ca^{2+}]\), which makes small fluctuations in diastolic calcium potentially difficult to study.

The advent of a new generation of fluorescent calcium indicators may obviate some of the limitations inherent in previous methodologies. These indicators bind to calcium with a one-to-one stoichiometry and are most sensitive to fluctuations in \([Ca^{2+}]\), in the submicromolar range. In the present study, \([Ca^{2+}]\) transients have been measured during rapid pacing of chick embryonic myocardial cells loaded with the fluorescent indicator indol. Staircase can be observed in mechanical recordings from these cells and is accompanied by a progressive increase in contractile deformation at end-diastole, which suggests that residual calcium accumulates in the myoplasm between beats. The indol recordings confirm that the peak level of the \([Ca^{2+}]\) transient increases during staircase and that there is an accompanying increase in diastolic \([Ca^{2+}]\). This is the first report in which the postulated fluctuation of diastolic \([Ca^{2+}]\), is directly demonstrated.

Materials and Methods

Chick embryonic myocardial cell aggregates were prepared from 9–12 day embryos as described previously. Dissociated cells were cultured in Sykes Moore chambers (Bellco Glass, Vineland, N.J.) at a density of 10^6/ml. The bottom of each chamber was coated with Sylgard resin (Dow Corning, Midland, Mich.), which prevented attachment of cells. Spontaneously beating aggregates formed within 3 days and were maintained in culture until the time of the experiment when they were transferred to a resin-free glass surface. Experiments were performed in physiological saline containing (in mM) NaCl 137, NaH2PO4 0.4, KCl 2.7, MgCl2 1.0, CaCl2 1.8, dextrose 5.5, and HEPES 6.0 at pH 7.4 and 37 ± 1°C.

Cell aggregates were loaded with indol by incubation with indol AM (20 μM), the acetoxymethyl ester of indol, in tissue culture medium at 37°C. Extracellular indol AM was removed by washing with physiological saline. The cells were then transferred to a temperature-regulated nonfluorescent chamber on the stage of an inverted microscope. Illumination was provided by a 100 W ultraviolet source (Leitz, Rockleigh, N.J.), filtered at 360 ± 5 nm (Corion, Holliston, Mass.). Fluorescence emissions were filtered at 400 ± 20 nm or 500 ± 20 nm (Corion) before reaching a photomultiplier tube (R268 Hamamatsu, Bridgewater, N.J.) placed in the focal plane of a camera port.
The photomultiplier output was filtered at 50 Hz by a low pass electronic filter. Fluorescence signals were calibrated as described by Tsien et al.\textsuperscript{10} with modifications according to Hesketh et al.\textsuperscript{11} Maximum (F\textsubscript{max}) and minimum (F\textsubscript{min}) fluorescence of the indol-loaded cell aggregates at 400 ± 20 nm emission were determined at the end of the experiment. Each aggregate was first exposed to a solution containing 20 mM CaCl\textsubscript{2} and the calcium ionophore ionomycin (150 nM). Ionomycin allowed calcium to enter the cells and saturate the indol to give F\textsubscript{max}. The aggregate was then exposed to a solution containing 150 nM ionomycin and 20 mM MnCl\textsubscript{2}. Entry of Mn\textsuperscript{2+} into cells was also facilitated by ionomycin, resulting in quenching of indol fluorescence. F\textsubscript{min}, the indol fluorescence in the absence of Ca\textsuperscript{2+}, was then given by the equation

\[
F\textsubscript{min} = F\textsubscript{M}\textsuperscript{e} + 0.18(F\textsubscript{max} - F\textsubscript{M}\textsuperscript{e})
\]

where F\textsubscript{M}\textsuperscript{e} is the fluorescence measured in the presence of MnCl\textsubscript{2} and 0.18 is a factor determined from measurements performed in cuvettes.\textsuperscript{11} The concentration of intracellular free calcium, [Ca\textsuperscript{2+}], was then obtained as

\[
[Ca^{2+}] = K_0(F - F\textsubscript{min})/(F\textsubscript{max} - F)
\]

where F was the cellular fluorescence measured and K\textsubscript{0} was 250 nM.

Contractile activity of indol-loaded cell aggregates was studied by recording cell edge movements with a photoresitive diode placed in the image plane of an eyepiece lens. The photodiode output has been shown to be linearly correlated with edge displacement.\textsuperscript{8} Intracellular recordings were performed using glass microelectrodes (tip resistance of 20–60 MΩ) filled with 2 M KC\textsubscript{l}. Current pulses were generated by a WPI stimulator (model 850A, World Precision Instruments, New Haven, Conn.). Signals were displayed on a dual-beam oscilloscope and recorded by a strip-chart recorder.

**Results**

Indol loads easily into chick embryonic myocardial cells, leading to an increase in fluorescence intensity. Indol-loaded cell aggregates have normal action potentials, with a maximum diastolic potential of near −75 mV. The amplitude and temporal characteristics of the edge movement signals are also similar in loaded and unloaded cells, which indicates that buffering of intracellular free calcium by indol is not significant in spheroidal aggregates of chick embryonic myocytes (c.f., Peeters et al\textsuperscript{13}). When illuminated at 360 nm, indol-loaded aggregates exhibit fluorescence transients during each transmembrane action potential. Fluorescence emissions at 400 nm increase during the transients (Figure 1, top trace), whereas those at 500 nm decrease during the transients (Figure 1, bottom trace). No fluorescence transients are observed in unloaded aggregates. Transients are also absent if fluorescence emissions are recorded from indol-loaded cells at 450 nm, which is the isosbestic point for calcium-dependent fluorescence in vitro.\textsuperscript{9} These observations demonstrate that the signals are calcium specific and do not arise from motion artifacts.

Many of the features of the indol signals are consistent with the results obtained by other methods. Calibration of the recordings with ionomycin (Figure 2) gives an end-diastolic [Ca\textsuperscript{2+}], of 208 ± 24 nM (SEM, n = 15), which is similar to values obtained with ion-selective electrodes.\textsuperscript{12,13} The systolic transients rise abruptly (t\textsubscript{1/2} = 5–15 msec) and follow the upstroke of the action potential by 20 ± 1 msec (Figure 3). The calculated peak systolic [Ca\textsuperscript{2+}], is 612 ± 49 nM (SEM, n = 15), which is within the range of values obtained in ferret myocardium with aequorin.\textsuperscript{14} Comparable values of diastolic and systolic [Ca\textsuperscript{2+}], have been obtained in monolayers of embryonic cardiac cells with indol.\textsuperscript{9} The most novel feature of the indol transients is their relatively slow decay. During spontaneous beating, the decline in calculated [Ca\textsuperscript{2+}], can be fitted by an exponential (R = 0.9) with a time constant of 96 ± 21 msec. A slow component of calcium removal has been predicted from the slow return of edge displacement, which is also exponential, and has a similar time constant.\textsuperscript{8}

The behavior of [Ca\textsuperscript{2+}], during staircase is illustrated in Figures 4 and 5, where indol fluorescence emissions are monitored at 400 and 500 nm, respectively. When beat frequency is accelerated by application of a train of depolarizing current pulses, "stairstep" increments in systolic and diastolic [Ca\textsuperscript{2+}], are clearly evident. Figure 6A shows results from a similar experiment in which the peak systolic (○) and end-diastolic (○) [Ca\textsuperscript{2+}], are determined. The increase in systolic [Ca\textsuperscript{2+}], is at
FIGURE 2. Calibration procedure for determination of 
[Ca\textsuperscript{2+}] in an indol-loaded cell aggregate. Fluorescence emissions are recorded at 400 ± 20 nm. Simultaneous exposure of the aggregate to 150 nM ionomycin and 20 mM CaCl\textsubscript{2} (top trace) leads to a sustained increase in fluorescence to a maximum value called F\textsubscript{\text{max}}. Subsequent exposure to 20 mM MnCl\textsubscript{2} in the continued presence of 150 nM ionomycin completely quenches indol fluorescence to give a value called F\textsubscript{\text{uj}} (bottom trace). Determination of these values allows conversion of fluorescence measurements to [Ca\textsuperscript{2+}] which is indicated in nM in the right-hand scales (see equations in text). Dotted lines at 20 and 80 seconds represent periods in which the traces are obscured by fluid turbulence artifact.

least partly due to the fact that the transients arise from a higher baseline. On termination of pacing, the spontaneous cycle length is prolonged because of overdrive suppression. During the long interbeat interval, diastolic [Ca\textsuperscript{2+}], continues to decay until the previous baseline level is reached and the next spontaneous beat takes place (Figures 4 and 5).

Fluctuations in [Ca\textsuperscript{2+}], during staircase are closely paralleled by the mechanical response, as reflected by optical recordings of cell edge movement (Figure 6B). Rapid pacing induces a progressive increase in both peak systolic and end-diastolic edge displacement. Cessation of pacing leads to overdrive suppression, during which there is gradual return of cell edge position to the original baseline. Return of the edge from a given displacement takes longer during the pause than is required during spontaneous beating. Examination of the indol signals (Figures 4 and 5) confirms that this behavior is due to protracted decay of [Ca\textsuperscript{2+}]. This phenomenon does not result from buffering of intracellular calcium by indol because it can also be observed in the absence of indol. In fact, initial descriptions of the edge movement staircase led to the proposal that the instantaneous shape of myocardial cell aggregates during diastole might, by itself, be an informative index of [Ca\textsuperscript{2+}].\textsuperscript{8,9,15}

Discussion

The force-frequency relation in the heart was recognized more than a hundred years ago by Bowditch, who coined the term "Treppe" to describe the progressive increase in the strength of contraction with increasing stimulus frequency. In their classic review, Koch-Weser and Blinks\textsuperscript{1} suggested that the "positive inotropic effect of activation" is due to an increase in the amount of calcium entering the muscle with each beat. Using canine papillary muscles, Langer demonstrated that an increase in beat frequency leads to an increase in the rate of calcium uptake.\textsuperscript{16} Blinks and his collaborators\textsuperscript{3} have characterized the relation between [Ca\textsuperscript{2+}] and aequorin bioluminescence in cardiac muscle and have confirmed that contraction is initiated by an increase in [Ca\textsuperscript{2+}]. Although beat-to-beat variation has not been studied, the aequorin results demonstrate that the amplitude of the [Ca\textsuperscript{2+}], transient determines contraction strength.

In this study, the [Ca\textsuperscript{2+}], staircase in embryonic myocardial cell aggregates has been directly recorded. The favorable fluorescence properties of indol permit beat-to-beat calcium transients to be resolved without signal-averaging. Both systolic and diastolic [Ca\textsuperscript{2+}], staircases are demonstrated and closely resemble the mechanical responses seen in recordings of cell edge movement.

Do Diastolic Fluorescence Changes Reflect Genuine Fluctuations in [Ca\textsuperscript{2+}]?

A major insight provided by the indol recordings is the fact that [Ca\textsuperscript{2+}], decays more slowly in diastole than could be appreciated from earlier calcium measurement techniques. Slow decay of [Ca\textsuperscript{2+}], was first inferred from the relaxation of cell edge displacement in chick embryonic myocardial cells\textsuperscript{8,9,13} and has now been corroborated by the slow decay of fluorescence transients recorded with three tetracarboxylate indicators in a variety of myocyte preparations.\textsuperscript{5,6,17,19} However, there
are potential artifacts in the fluorescence recordings that must be excluded before the diastolic [Ca^{2+}] fluctuations can be considered as proven.

First, it is possible that the slow fluorescence decay represents the time required for dissociation of the Ca^{2+}-indol complex. Quast et al.\(^20\) have studied the in vitro kinetics of quin2 and found that the "on" reaction is unmeasurable (faster than 2 msec), while the "off" reaction has a half-time of 13–20 msec under a variety of conditions. This observation confirms that the much slower decay of quin2 fluorescence in electrically stimulated myocyte suspensions\(^9\) is not an artifact of reaction kinetics. To examine the kinetics of indol, similar measurements have been performed in a stirred fluorometer chamber with an exponential mixing curve (\(T_{1/2} = 50\) msec). With this instrument, the increase in \(F_{500}\) produced by addition of EGTA to a solution containing Ca^{2+} and indol is superimposable on the mixing curve. Thus, the in vitro off reaction of indol also has a half-time \(<50\) msec. It is concluded, therefore, that the very prolonged fluorescence tails seen in myocardial cell aggregates (up to 2 seconds in duration) are unlikely to reflect the reaction kinetics of indol.

A second possible source of artifact is buffering of [Ca^{2+}] by indol. Ashley et al.\(^21\) have shown that excessive loading of crustacean muscle fibers with quin2 can artificially prolong the decay of fluorescence transients. However, in this situation, the amplitude of the contraction is markedly reduced, and its time course is converted to a "sawtooth," with a slow and nearly constant rate of decay. In our experiments, the grossly normal amplitude of the contraction and its brisk initial relaxation confirm that buffering is not significant, at least during the period when the recordings are made (c.f., Peeters et al.\(^5\)).

A third possible source of artifact would be the measurement of [Ca^{2+}]-dependent fluorescence from the interior of organelles into which the indicator might...
penetrate prior to de-esterification. In our experiments, the similar behavior of the mechanical response and the [Ca\textsuperscript{2+}], transients is the strongest evidence that the fluorescence arises from the myofilament space. Recordings obtained using other indicators and cell types support this interpretation. [Ca\textsuperscript{2+}], transients obtained by direct injection of fura2 free acid into rat myocytes have a slow decay, which is similar to that observed with fura2 AM\textsuperscript{17} or other cell-permeant indicators.\textsuperscript{5,6,9} Quin2 fluorescence in rat myocyte suspensions is decreased by stimulation of \(\beta\)-adrenoceptors,\textsuperscript{22} which enhances uptake of calcium by the sarcoplasmic reticulum. In electrically stimulated cells of the rabbit ventricle, adrenaline accelerates the decay of indo1 transients (H. Lee and W.T. Clusin, unpublished data). This observation supports the myoplasmic origin of the transients and also confirms that their rate of decay is not limited by the kinetics of indo1.

**Mechanisms of [Ca\textsuperscript{2+}], Staircase and Its Role in Regulation of Contraction Strength**

The mechanism for the stepwise increase in [Ca\textsuperscript{2+}], with increased beat frequency is not fully understood. It is generally acknowledged that the intracellular sodium concentration affects contraction strength through regulation of calcium transport across the sarcolemma.\textsuperscript{23-24} An increase in [Na\textsuperscript{+}], during repetitive stimulation would then increase net calcium influx during each action potential. An alternative explanation is the so-called “two-compartment model.”\textsuperscript{25-26} Here, the calcium entry during each action potential could remain constant, but an increase in the frequency of action potentials would increase the availability of a releasable calcium store. A third possibility is suggested by the residual calcium hypothesis that has been formulated for synaptic transmitter release.\textsuperscript{27} Here, the total increment in [Ca\textsuperscript{2+}], during each impulse is constant, but as the frequency of impulses increases, residual calcium accumulates in the cytoplasm. Transmission is facilitated because of the nonlinear relation between peak calcium and quantum release probability. This concept is potentially applicable to the heart, owing to the nonlinear, i.e., sigmoidal, relation between myoplasmic calcium and contraction strength.\textsuperscript{28}

The possibility that variation of diastolic [Ca\textsuperscript{2+}], regulates systolic force has not always been recognized. Aequorin transients in adult mammalian fibers decay rapidly and show no diastolic variation except during "calcium overload."\textsuperscript{29,30} There are two possible explanations for this discrepancy. First, it is possible that [Ca\textsuperscript{2+}], decays more slowly in embryonic myocytes than in adult fibers because of the role of calcium...
channels in pacemaking. Our data do not support this idea since overdrive suppression, which should delay the opening of pacemaker calcium channels, does not accelerate the decline in [Ca\(^{2+}\)]

Furthermore, recordings obtained in adult myocytes with quin2, fura2, or indo1 do not show a noticeably faster decay. A more likely explanation for the discrepancy is that diastolic [Ca\(^{2+}\)] does vary in adult cardiac fibers but that this variation escapes detection by aequorin because of the relative insensitivity of the photoprotein at low levels of [Ca\(^{2+}\)]. This idea is supported by experiments in molluscan neurons, where the linear indicator arsenazo III shows a stairstep increase in the baseline during a train of action potentials that is nearly absent with aequorin.

It is possible that other interventions besides overdrive affect contraction strength through modulation of diastolic [Ca\(^{2+}\)]. Diastolic edge movements in chick embryonic myocardial cells are slowed by reduction of external sodium or by an increase in external calcium. These maneuvers can increase both systolic and diastolic edge displacement if beat frequency is held constant. Veratrine and ouabain also increase diastolic edge displacement if beat frequency is held constant. Veratrine and ouabain also increase diastolic edge displacement in conjunction with their positive inotropic effect. It is possible that a corresponding increase in diastolic [Ca\(^{2+}\)] can be demonstrated in these conditions and that similar phenomena occur during inotropic stimulation of adult myocardium.

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