Single Adult Rabbit and Rat Cardiac Myocytes Retain the Ca\(^{2+}\)- and Species-dependent Systolic and Diastolic Contractile Properties of Intact Muscle

MAURIZIO C. CAPOGROSSI, ARTHUR A. KORT, HAROLD A. SPURGEON, and EDWARD G. LAKATTA

From the Laboratory of Cardiovascular Science, Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, Maryland 21224

ABSTRACT The systolic and diastolic properties of single myocytes and intact papillary muscles isolated from hearts of adult rats and rabbits were examined at 37°C over a range of stimulation frequencies and bathing [Ca\(^{2+}\)]\(_o\) (Ca\(_o\)). In both rabbit myocytes and intact muscles bathed in 1 mM Ca\(_o\), increasing the frequency of stimulation from 6 to 120 min\(^{-1}\) resulted in a positive staircase of twitch performance. During stimulation at 2 min\(^{-1}\), twitch performance also increased with increases in Ca\(_o\) up to 20 mM. In the absence of stimulation, both rabbit myocytes and muscles were completely quiescent in <15 mM Ca\(_o\). Further increases in Ca\(_o\) caused the appearance of spontaneous asynchronous contractile waves in myocytes and in intact muscles caused scattered light intensity fluctuations (SLIF), which were previously demonstrated to be caused by Ca\(^{2+}\)-dependent spontaneous contractile waves. In contrast to rabbit preparations, intact rat papillary muscles exhibited SLIF in 1.0 mM Ca\(_o\). Two populations of rat myocytes were observed in 1 mM Ca\(_o\): ~85% of unstimulated cells exhibited low-frequency (3–4 min\(^{-1}\)) spontaneous contractile waves, whereas 15%, during a 1-min observation period, were quiescent. In a given Ca\(_o\), the contractile wave frequency in myocytes and SLIF in intact muscles were constant for long periods of time. In both intact rat muscles and myocytes with spontaneous waves, in 1 mM Ca\(_o\), increasing the frequency of stimulation from 6 to 120 min\(^{-1}\) resulted, on the average, in a 65% reduction in steady state twitch amplitude. Of the rat myocytes that did not manifest waves, some had a positive, some had a flat, and some had a negative staircase; the average steady state twitch amplitude of these cells during stimulation at 120 min\(^{-1}\) was 30% greater than that at 6 min\(^{-1}\). In contrast to rabbit preparations, twitch performance during stimulation at 2 min\(^{-1}\) saturated at 1.5 mM Ca\(_o\), in both intact rat muscles and in the myocytes with spontaneous waves. We conclude
that the widely divergent, Ca\textsuperscript{2+}-dependent systolic and diastolic properties of intact rat and rabbit cardiac muscle are retained with a high degree of fidelity in the majority of viable single myocytes isolated from the myocardium of these species, and that these myocytes are thus a valid model for studies of Ca\textsuperscript{2+}-dependent excitation-contraction mechanisms in the heart.

**INTRODUCTION**

The functional properties of intact cardiac muscle must, to a substantial extent, reflect those of the individual myocytes. However, the pluricellular arrangement of cells per se and the intercellular space and its elements have a significant impact on muscle function and may interfere with the assessment of some cell properties. Recent refinements in methodology have produced a new genre of single myocytes that are described as “Ca\textsuperscript{2+} tolerant” (Powell et al., 1980; Dow et al., 1981a, b; Farmer et al., 1983; Silver et al., 1983), in that they retain their elongated shape, high resting membrane potential, and excitability when bathed in millimolar [Ca\textsuperscript{2+}]\textsubscript{o} (Ca\textsubscript{o}). Thus, these single adult cardiac myocytes have tremendous appeal as a model system in which to study the mechanisms of excitation-contraction coupling that underlie myocardial function.

The extent to which single cardiac myocytes are useful for studies of the contractile process in muscle, however, depends on the fidelity with which they retain both the systolic and diastolic Ca\textsuperscript{2+} tolerance exhibited by intact muscle, and this varies markedly among species. For example, intact isolated bulk muscle from species such as rat and rabbit exhibit markedly different systolic properties (Sutko and Willerson, 1980; Lakatta and Yin, 1982; Bers, 1985). Species-specific systolic Ca\textsuperscript{2+} tolerance of single myocytes from these species can be addressed by comparing the interval-strength relationship between cells and intact muscle from each species in a given Ca\textsubscript{o} and by comparing their twitches at a given stimulation rate across a range of Ca\textsubscript{o}.

More recently, Ca\textsuperscript{2+}-dependent diastolic properties of intact cardiac muscle have been discovered. Studies in intact rat cardiac muscle have demonstrated the presence of spontaneous, Ca\textsuperscript{2+}-dependent, microscopic cell motion that occurs in the interstimulus interval during low-frequency stimulation and in the unstimulated state (Lakatta and Lappe, 1981; Stern et al., 1983). This motion, which is generated by localized myofilament-Ca\textsuperscript{2+} interaction, scatters a laser beam passed through the tissue, and thus can be detected as intensity fluctuations in the scattered field. The fact that these light oscillations are abolished by caffeine and ryanodine and are not present when Ca\textsubscript{o} in the myofilament space is buffered at a constant level suggested that they are caused by spontaneous Ca\textsuperscript{2+} oscillations that require an intact sarcoplasmic reticulum (SR) (Stern et al., 1983). Subsequent studies have directly documented spontaneous fluctuations in aequorin luminescence, a monitor of cell free [Ca\textsuperscript{2+}] (Ca\textsubscript{a}), which are also abolished by caffeine or ryanodine (Orchard et al., 1983; Wier et al., 1983). Cinemicrographic studies of intact muscle indicate that the microscopic myofilament motion caused by the spontaneous SR Ca\textsuperscript{2+} release occurs as propagating contractile waves within cells (Kort et al., 1985a). These waves depend on the Ca\textsuperscript{2+} load of the preparation (Stern et al., 1983; Kort and Lakatta, 1984), and
when the frequency of intensity fluctuations is shifted by changing the extent of cell Ca\textsuperscript{2+} loading or abolished by high concentrations of caffeine or ryanodine, the contractile wave frequency varies accordingly (Stern et al., 1983; Kort et al., 1985a). A striking feature of studies in muscle is that the threshold Ca\textsuperscript{2+} loading required for these contractile waves to occur has a marked species-tissue dependence. At one extreme, rat muscles exhibit spontaneous Ca\textsuperscript{2+} oscillations in the unstimulated state even when bathed in 1–2 mM Ca\textsubscript{o}; at the other extreme, unstimulated rabbit ventricular muscle does not exhibit these oscillations until Ca\textsubscript{o} is >10 mM (Kort and Lakatta, 1984).

Spontaneous SR Ca\textsuperscript{2+} release can have profound effects on cardiac muscle function. The localized Ca\textsuperscript{2+}-myofilament interaction alters tissue compliance and sarcomere and cell length and causes a Ca\textsuperscript{2+}-dependent tone (Lakatta and Lappe, 1981; Stern et al., 1983; Allen et al., 1985; Kort et al., 1985a, b). The resulting spontaneous SR Ca\textsuperscript{2+} release–induced inhomogeneity of sarcomere length, myofilament Ca\textsuperscript{2+} activation, SR Ca\textsuperscript{2+} loading, and altered tissue compliance can negatively affect twitch force during systole (Kort and Lakatta, 1984). Additionally, it can compromise force production by reducing SR Ca\textsuperscript{2+} release in response to a subsequent action potential (Kort and Lakatta, 1984; Allen et al., 1985; Valdeolmillos and Eisner, 1985). The Ca\textsuperscript{2+} modulation of cation channels or the Na/Ca carrier ion flux that results from spontaneous Ca\textsuperscript{2+} release into the myoplasm affects sarcolemmal membrane potential and produces a depolarization (Kass and Tsien, 1982) that can initiate arrhythmias.

However, since spontaneous SR Ca\textsuperscript{2+} release is neither spatially nor temporally homogeneous among cells within the bulk isolated cardiac muscle (Stern et al., 1983; Kort and Lakatta, 1984; Kort et al., 1985b), a study of its specific functional effects on a given cell, which will lead to a mechanistic understanding of the impact of this phenomenon on cell function, cannot be obtained in bulk tissue but requires single myocytes. While some aspects of spontaneous Ca\textsuperscript{2+} release have been examined in markedly Ca\textsuperscript{2+}-overloaded myocytes (Matsuda et al., 1982), a definition of its functional effects about the threshold level of Ca\textsuperscript{2+} loading required for it to occur is presently lacking. Thus, a study on the impact of “low-grade” spontaneous SR Ca\textsuperscript{2+} release on cell function could be accomplished in Ca\textsuperscript{2+}-tolerant single myocytes in the absence of marked Ca\textsuperscript{2+} overload. However, in order for single Ca\textsuperscript{2+}-tolerant myocytes to be useful for mechanistic studies of this type of spontaneous Ca\textsuperscript{2+} release, it must be determined whether they exhibit a dependence on Ca\textsubscript{o} for spontaneous contractile waves to occur similar to that of the bulk muscles from their respective species.

The present study tested whether single myocytes isolated from adult rat and rabbit hearts retain the species differences in Ca\textsuperscript{2+}-dependent systolic and, particularly, in Ca\textsuperscript{2+}-dependent diastolic properties over a wide range of cell Ca\textsuperscript{2+} loading. The results demonstrated that these single cardiac myocytes do in fact retain these properties of bulk muscle to a considerable degree. In the following article (Capogrossi et al., 1986), we address some specific interactions of spontaneous diastolic SR Ca\textsuperscript{2+} release and electrically stimulated twitches.

Some aspects of this work have been previously presented in abstract form (Capogrossi et al., 1984a, b, 1985b; Lakatta and Capogrossi, 1985).
Methods

Myocytes

Isolation procedure. Single myocytes were prepared by a modification of a technique previously described (Silver et al., 1983). Adult (6–8-mo-old) male Wistar rats (500–600 g) from the Gerontology Research Center Colony and rabbits (3–4 kg) were killed by decapitation or cervical dislocation, respectively. The heart was quickly removed, weighed, and placed in a beaker containing Spinner salt solution (Gibco Laboratories, Grand Island, NY) (composition in mM: 116.4 NaCl, 26.2 NaHCO₃, 10.1 NaH₂PO₄·H₂O, 5.4 KCl, 0.8 MgSO₄·H₂O, 5.5 D-glucose). The aorta was then cannulated and perfused with 25 ml of the same medium, 37 ± 1°C, non-recirculating, in order to wash out the blood from the myocardium. Perfusion was then switched to a recirculating Spinner salt solution supplemented with collagenase type II (Worthington Diagnostic Systems, Freehold, NJ) and CaCl₂ to reach final concentrations in the buffer of 160 U/ml and 60 μM, respectively. Perfusion pressure was 70 cm H₂O and the perfusate was continuously gassed with 95% O₂ and 5% CO₂ to keep the pH at 7.35 ± 0.05. The perfusion was terminated when the heart became flaccid, which occurred after 30–50 min.

The left ventricle was then isolated, placed in a beaker with 10 ml of the same perfusate at 37°C, minced, and pipetted several times using a glass pipette with an enlarged and flamed tip. This suspension was then filtered through a 200-μm mesh (Tetko, Inc., Elmsford, NY) into a conical tube that was immediately transferred to an incubator (Queue Systems Inc., Parkersburg, WV) providing an atmosphere with 5% CO₂, 95% air, and 95% humidity at 37°C. Within 3–5 min, a pellet was formed by gravity, the supernatant was removed, and the pellet was resuspended in Spinner buffer with 0.25 mM Ca²⁺. The procedure was repeated once more and the new pellet was resuspended in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco Laboratories) (composition in mM: 109.6 NaCl, 44.0 NaHCO₃, 0.9 NaH₂PO₄·H₂O, 5.4 KCl, 0.7 MgSO₄·7H₂O, 25 D-glucose, 1.8 CaCl₂) supplemented with 5% fetal bovine serum (Hyclone Laboratories, Logan, UT). 1 ml of this cell suspension was transferred to each of several 35-mm plastic petri dishes (Falcon, Becton, Dickinson & Co., Oxnard, CA), which were incubated for 120–180 min with a medium of the same composition; the medium was removed just before the addition of the cell suspension. Finally, the dishes were placed again in the incubator, for at least 60 min, before being used for experiments. The pretreatment of the plastic dishes and the resuspension of cells in DMEM containing 5% fetal bovine serum represented the critical steps of a previously described (Piper et al., 1982) differential attachment procedure that favors the attachment of only viable myocytes to the dish. This permitted repeated measurements of the contractile properties of the same myocyte in the presence of continuous bathing solution changes. 15–30 min after resuspension in DMEM buffer with 5% fetal bovine serum, 500 cells were observed to determine the yield of viable myocytes, which ranged between 45 and 90% in 1.8 mM Ca₀. Cells were considered viable when, in millimolar Ca₀, they had a rod-shaped appearance, clear striations, sharp edges, and no evidence of granulations or blebs (Fig. 1). After 60 min of incubation, the dishes were washed with fresh HEPES buffer (composition in mM: 137 NaCl, 1.2 MgSO₄·7H₂O, 5 KCl, 20 HEPES, 16 D-glucose; CaCl₂ was varied between 0.25 and 30; pH 7.4 ± 0.05). When the yield of viable cells was determined again, it usually exceeded 90% because almost exclusively viable myocytes attached to the dish and dead cells were removed during solution changes.

Contractile measurements. The myocytes used for contractile measurements had the appearance of those in Fig. 1. Experiments were typically performed 60–360 min after resuspension in millimolar Ca₀. Measurements in a given cell were made within a 90-min period unless otherwise indicated. The cells selected for study were those where the
Contact between the myocyte and the dish was limited to only a few microns in the central region of the cell. A 35-mm petri dish was placed in a chamber on the stage of an inverted Diavert microscope (E. Leitz, Inc., Rockleigh, NJ) (Fig. 2). The HEPES buffer was circulated through the dish at 3 ml/min and temperature was maintained constant by a heat exchanger (Haake Buchler Instruments, Inc., Saddle Brook, NJ). The temperature used in all the experiments was 37 ± 0.5°C. The image of a single cell was projected on a video monitor (model WV-5200, Panasonic, Matsushita Communication Industrial Co., Ltd., Yokohama, Japan) through a TV camera (Panasonic) adapted to the microscope. To quantify cell movement along its longitudinal axis, the camera was rotated to align the myocyte A-I pattern perpendicularly to the video monitor raster lines, and a video dimension analyzer (model 303, Instrumentation for Physiology and Medicine, Inc., San Diego, CA) monitored a selected raster line for differences in light intensity between the cell and the surrounding field. The time constant of the video analysis system response was 32 ms, as verified by analysis of an instantaneous length step simulated by the activation of a light-emitting diode in the microscope field. The signal was then transmitted to a chart recorder (Brush 220, Gould, Inc., Cleveland, OH) and to a computer (RD5500, Raytheon, Inc., Santa Ana, CA) for on-line analysis. Two-point platinum electrodes placed in the bathing fluid and connected to a stimulator (SD9, Grass Instrument Co., Quincy, MA) were used to field-stimulate the cell. Typically, pulses of 2–4 ms were used and a cell responded to stimulation of suprathreshold intensity with a synchronous contraction, i.e., a twitch. In addition, cells were monitored in the absence of stimulation to detect any spontaneous displacement such as that reported to occur in cells within intact rat muscles (Stern et al., 1983; Kort and Lakatta, 1984; Kort et al., 1985a) in the absence of stimulation. Additional subsets of myocytes were monitored for spontaneous contractile

FIGURE 1. Typical appearance of rabbit (top) and rat (bottom) myocytes defined as viable (see Methods) and used for contractile measurements. The differential interference contrast images were photographed on Kodak TP 2415 (Eastman Kodak Co., Rochester, NY). The pictures were obtained with an inverted microscope (IM-55, Carl Zeiss Inc., New York) using a Zeiss Planapochromat 63X (1.4 NA) oil immersion DIC objective and DIC condenser (0.63 NA). Cell lengths, measured in an unselected population of unstimulated rat and rabbit myocytes bathed in 1.5 mM Ca, and used for the present studies, averaged 131.3 ± 4.9 (n = 30) and 113.8 ± 3.0 μm (n = 30), respectively (x ± SEM).
activity in the absence of stimulation at 30 and 210 min after the addition of Ca, and a similar subset was followed for up to 24 h via time-lapse video microscopy.

Membrane potential measurement. Transmembrane potential recordings were made in an unselected subset of viable myocytes as depicted in Fig. 1 using microelectrodes filled with 3 M KCl, which were of 30–40 MΩ resistance. A high-input impedance amplifier (model M-707, W-P Instruments, Inc., New Haven, CT) equipped with a bridge circuit allowed voltage measurement and stimulation through the same microelectrode. Electrical contact was achieved via Ag/AgCl pellets. Cells were impaled in the center by gentle tapping of the table surface after making light contact with the microelectrode tip or by

![Diagram of apparatus for contractile measurements in single myocytes](image)

**Figure 2.** Apparatus used for contractile measurements in single myocytes. See text for details.

overcompensating the capacity neutralization circuitry of the M-707. The signal was amplified fivefold and fed to an FM tape recorder (model 3960, Hewlett-Packard Co., Palo Alto, CA) at 15 ips (frequency response, DC to 5 kHz) for later analysis and display purposes. Impalements were considered successful if, after a stabilization period (1–5 min), the resting potential did not vary more than 5–10 mV and if no alterations in cell morphology occurred; these requirements were satisfied in ~20% of the trials. Most impalements could be maintained for up to 120 min.

Intact Muscle

Thin right ventricular papillary muscles were removed from the hearts of rats and rabbits killed as described above, mounted in a chamber that permitted measurements of force
and light-scattering properties (Stern et al., 1983; Kort and Lakatta, 1984), and perfused with HEPES buffer at 37°C identical in composition to that used for contractile studies in myocytes.

After an equilibration period (120–300 min), muscles were stimulated to contract via 5-ms square-wave pulses delivered by a Grass stimulator via platinum electrodes mounted within the chamber. Twitch force was then measured over the same range of Ca, and stimulation frequencies used for myocytes.

In the absence of stimulation, light-scattering measurements (Lakatta and Lappe, 1981; Stern et al., 1983; Kort and Lakatta, 1984) were made over a range of Ca values. The rationale for these measurements derives from the findings of previous studies, which had demonstrated the presence of Ca-dependent, wave-like myofilament oscillatory motion occurring in the absence of stimulation in a variety of intact mammalian cardiac preparations (Kort and Lakatta, 1984). This microscopic myofilament displacement, which is asynchronous within and among cells, phase-modulates coherent light (e.g., a laser beam) as it passes through the preparation, producing intensity fluctuations in the scattered field, the frequency of which varies with the underlying myofilament motion (Kort et al., 1985a). The measurements in the present study permitted a comparison of the Ca dependence and species differences of these light fluctuations in the intact tissue with that of any spontaneous wave-like motion in the myocytes.

The method used to quantitate these scattered light intensity fluctuations (SLIF) has been published in detail elsewhere (Stern et al., 1983). Briefly, the light scattered at 30° from an incident He-Ne laser beam was collected on a photomultiplier tube (RF 313, Malvern Instruments, Malvern, England) and then analyzed by a Malvern K7025 digital autocorrelator. The autocorrelation function was characterized by its half-decay time, t0, and expressed as \( f_0 = \frac{\lambda}{2 \pi t_0} \). Values of \( f_0 \) less than ~0.55 Hz were defined as being below the system threshold (Kort and Lakatta, 1984). For the case of single-order scattering, \( f_0 \) is proportional to the root mean square velocity of the light scatterers within the illuminated tissue. Thus, \( f_0 \) is not equatable with the actual frequency of the underlying mechanical oscillatory motion observed by light microscopy, but is the approximate product of that frequency and the amplitude of its mechanical displacement in wavelengths of light (Kort et al., 1985a).

RESULTS

Rabbit Preparations

Regular stimulation of intact rabbit muscle from the quiescent state results in a progressive increase in the force developed in each successive twitch until a steady state is achieved (Fig. 3A). This “positive staircase” or “Bowditch Treppe” is a well-known characteristic of intact rabbit muscles (Bers, 1983). Fig. 3B depicts the effect of repetitive stimulation at increasing frequencies on steady state twitch force in rabbit muscles. Rabbit muscles exhibit a “positive,” steady state twitch strength–stimulation frequency relationship; i.e., as the stimulation frequency increases, the amplitude of the twitch increases.

In rabbit myocytes, the effect of stimulation on the twitch is qualitatively similar to that in muscles. Stimulation from rest produces a positive staircase in the extent of cell shortening in successive beats (Fig. 4A). In the steady state, greater cell shortening during the twitch occurs as the stimulation rate is increased (Fig. 4B). The steepness of the steady state twitch strength–stimulation frequency relationship, however, is steeper in muscles than in cells.
Fig. 5 illustrates the effect of an increase in $[Ca]$ on the average extent (A) and velocity (B) of cell shortening during the twitch in rabbit myocytes and of the twitch force (A) and the maximum rate of force development (B) during the twitch in rabbit papillary muscles. The twitch parameters in myocytes and muscles have been co-scaled so that the shapes of the $[Ca]$ dose-response curves in muscles and cells can be compared. The main point of the figure is that the $[Ca]$ dependence of the extent and velocity of shortening during the twitch in myocytes is qualitatively the same as that for twitch force and the maximum rate of force development in intact muscles. Both parameters in myocytes increased progressively with an increase in $[Ca]$ to values up to 20 mM.

In the absence of stimulation (Fig. 6B), in <10 mM $[Ca]$, rabbit myocytes were strictly quiescent and the intact muscles did not exhibit SLIF. With further increases in $[Ca]$, although spontaneous synchronous twitches were not observed in the cells used for study, spontaneous contractile waves that propagated the length of the myocytes did occur. Fig. 6A illustrates the contrast between the myocyte length change that results from a stimulated twitch and that resulting from a spontaneous wave. The stimulated twitch exhibits a more rapid rate of

**Figure 3.** (A) Regular stimulation of a representative quiescent (for 10 min) intact rabbit muscle at 120 min⁻¹ results in a positive staircase in the twitch force. $[Ca]$ was 1.0 mM. (B) Steady state twitch force development in intact rabbit muscles ($n = 3$) measured at various rates of regular stimulation in 1.0 mM $[Ca]$. Stimulation began at 6 min⁻¹ and was increased to the next higher rate after a steady state was achieved. As the stimulation frequency increased, twitch performance increased. The value at each stimulation rate in each preparation has been normalized to the maximum level obtained in that preparation. The maximum twitch force in muscles (at slack length) was $0.80 ± 0.064$ g/mm² ($\bar{x} ±$ SEM).
change of cell length, a greater extent of shortening of cell length, and a faster time course compared with the spontaneous contractile wave. The frequency of these spontaneous contractile waves increased with increasing Ca\(_2\) (Fig. 6B); at 30 mM Ca\(_2\), the average frequency of these waves did not exceed 4 min\(^{-1}\) (Fig. 6B). Note also that SLIF measurements in the intact muscle (Fig. 6B) exhibit a Ca\(_2\) dependence strikingly similar to that of the spontaneous contractile wave frequency in myocytes. Previous studies in rabbit muscles have shown that SLIF occur when Ca\(^{2+}\) loading reaches some threshold level and increase in frequency with further increases in Ca\(^{2+}\) loading (Kort and Lakatta, 1984). More recent studies have shown that in response to a change in Ca\(_2\), the SLIF frequency in muscle varies directly with spontaneous contractile wave frequency in myocytes (Kort et al., 1985a). The results presented thus far (Figs. 1–6) demonstrate that the Ca\(^{2+}\)-dependent properties of rabbit myocytes, both during regular electrical stimulation and in the absence of stimulation, closely parallel those of the intact muscle.

![Figure 4](image-url)
Rat Preparations

Two populations of rat myocytes could be distinguished when Ca\textsubscript{w} was initially raised to millimolar levels after cell isolation: those that were perfectly quiescent, and those that exhibited the contractile waves described in rabbit cells bathed in high Ca\textsubscript{w}. Fig. 7 illustrates the proportion of rod-shaped rat cells that exhibited spontaneous contractile waves and the distribution of wave frequencies during 1 min of observation at 30 and 210 min after the addition of 1.0 mM Ca\textsubscript{w}. Note that both at 30 and 210 min after the addition of 1 mM Ca\textsubscript{w}, a minority (~15%) of cells were perfectly quiescent; i.e., they did not exhibit spontaneous contractile waves during the observation period. The average number of waves per minute at 210 min after Ca\textsubscript{w} addition did not differ markedly from that at 30 min: 3.92 ± 0.41 vs. 3.14 ± 0.50, respectively (\overline{x} ± SEM). When the cells that did not
exhibit waves were excluded from the analysis, the average frequency at 210 min was 4.56 ± 0.40. Thus, the average wave frequency was <0.1 Hz, and very few cells exhibited spontaneous waves at frequencies >0.25 Hz. It is noteworthy that none of 100 viable rabbit cells from each of three hearts, which were prepared in the same manner as rat cells, exhibited these spontaneous contractile waves for up to 210 min after exposure to 1.0 mM Ca\textsubscript{o}. Thus, these cells served as an internal standard for the method used to prepare myocytes in the present study.

The wave frequency in a given cell remained relatively constant with time during exposure to 1 mM Ca\textsubscript{o} at 37°C. Examples of this are illustrated in Fig. 8, which depicts wave frequency in three cells monitored for 360 min by time-lapse video microscopy. Using this technique to follow cells for an even longer...
period of time and observing them for 3 min every hour for 24 h permitted recording of pre-morbid events. In five cells whose initial average frequency was $6.4 \pm 0.51$ min$^{-1}$ ($\bar{x} \pm$ SEM), an increase to $423 \pm 64\%$ control occurred in that observation period just before the appearance of a "rounded-up," nonviable cell in the next observation period. Thus, a rapid and marked acceleration of spontaneous wave frequency in unstimulated myocytes bathed in a given Ca$_o$ heralds cell death. This behavior is distinctly different from the waves of low and stable frequency in viable cells.

Since some isolated rat myocytes exhibit spontaneous contractile waves and some do not, it becomes necessary to determine which type, if either, exhibits the contractile behavior that is characteristic of intact rat cardiac muscle. This typical behavior of intact rat cardiac muscle is illustrated in Fig. 9. On initiation of stimulation from rest, in contrast to what occurs in rabbit, a "negative force staircase" or "Woodworth Treppe" (Sutko and Willerson, 1980) occurs in rat muscle, and, in the steady state, twitch force decreases with increasing stimulation rate (Fig. 9B).

**Figure 7.** The percentage ($\bar{x} \pm$ SEM) of unselected, rod-shaped rat myocytes that exhibit spontaneous contractile waves in the absence of stimulation, and the distribution of the frequencies at which these oscillations occur during a 1-min period of observation at 30 min (striped bars) and 210 min (solid bars) after exposure to 1.0 mM Ca$_o$. 100 rod-shaped myocytes from each of seven hearts were observed.

**Figure 8.** The stability of wave frequency in three representative rat myocytes monitored for 360 min after exposure to 1 mM Ca$_o$. 
Stimulation of rat myocytes that exhibit spontaneous contractile waves in the unstimulated state always resulted in a negative twitch staircase (Fig. 10A). Additionally, in these myocytes, the steady state twitch amplitude–stimulation frequency relationship was, as in the intact muscle, always negative (Fig. 10B). It is noteworthy that the twitch amplitude at 120:6 min⁻¹ was nearly identical to that in the rat papillary muscles depicted in Fig. 9B. Thus, increasing the rate of stimulation of these preparations appears to decrease the SR Ca²⁺ release with each twitch, as shown in studies that have used aequorin in rat muscle (Kurihara and Allen, 1982; Orchard and Lakatta, 1985).

In contrast, rat myocytes that did not exhibit spontaneous contractile waves responded in a variable manner when stimulation was started from rest: most exhibited a positive staircase (Fig. 11), whereas others had either a blunted negative staircase or a negative staircase comparable to that of cells exhibiting waves in the unstimulated state. Fig. 12 depicts the ratio of steady state twitch cell shortening at 120 min⁻¹ to that at 6 min⁻¹ in cells with and without
spontaneous waves in the unstimulated state. The average (± SEM) ratio of twitch shortening at 120:6 min⁻¹ of all cells that did not exhibit spontaneous waves (n = 13) was 1.3 ± 0.11 vs. 0.34 ± 0.04 in those that did exhibit spontaneous contractile waves in the unstimulated state (n = 12, p < 0.001). At 6 min⁻¹, cells that exhibited waves had a stronger steady state twitch shortening than those that did not exhibit waves: 5.13 ± 0.69 (n = 12) vs. 2.68 ± 0.36% of

![Diagram A](image1)

**Figure 10.** (A) A typical example of the effect of regular stimulation (120 min⁻¹) after a 10-min rest on the extent of cell shortening during the twitch in a rat myocyte that exhibited spontaneous contractile waves at rest in 1 mM Ca²⁺. Note the negative twitch staircase. (B) Cell shortening as a function of stimulation frequency in 1.0 mM Ca²⁺ in seven rat myocytes that exhibited spontaneous contractile waves at rest at a frequency of 6.4 ± 2.1 min⁻¹ (± SEM). Cells were stimulated at a given frequency, and after steady state twitch shortening occurred, stimulation was stopped for 1 min. The cells were then stimulated at the next higher frequency.

the resting cell length (n = 13, p < 0.005). At 120 min⁻¹, steady state twitch shortening was similar in both types of cells. An additional feature of Fig. 12B is the wide variance of the ratio of twitch shortening at 120 min⁻¹ to that at 6 min⁻¹ in cells that do not exhibit spontaneous contractile waves. Although on the average this ratio is positive, some of the cells do in fact exhibit a negative ratio.

Continued stimulation of the cells that did not exhibit prior spontaneous contractile waves appeared to load the cell with Ca²⁺ because de novo sponta-
neous contractile waves were observed in all of these cells upon cessation of stimulation. The spontaneous waves persisted in the majority of these cells and, upon subsequent stimulation at 6 and 120 min$^{-1}$, the cells exhibited a negative twitch amplitude–stimulation frequency relationship (Fig. 13). Occasionally, the appearance of waves after stimulation in cells that did not originally demonstrate waves was transient (15 min). Subsequent stimulation of such a cell, after disappearance of the waves, produced a flat staircase, i.e., one similar to that in response to the initial stimulation of that cell.

The results of the experiments shown in Figs. 7–13 indicate that a large majority of rat cells exhibit spontaneous waves after exposure to millimolar Ca$^{2+}$ and that, upon stimulation, the twitch characteristics in these cells, and not those of the minority population of perfectly quiescent cells, more closely resemble the intact rat muscle.

The next series of experiments was undertaken to determine whether, at a given frequency of stimulation, a variation in Ca$^{2+}$ changed the twitch in myocytes in a manner similar to that in the intact muscle. In intact rat muscle (Fig. 14), the twitch force ($A$) and maximum rate of force development ($B$) differed from those of rabbit in that saturation occurred at a Ca$^{2+}$ of $\sim$1.5 mM, whereas in rabbit muscle stimulated at the same frequency (2 min$^{-1}$), a Ca$^{2+}$ of $>$20 mM was typically required for saturation of twitch performance (Fig. 5). The main point of Fig. 14 is the demonstration that myocytes retain a Ca$^{2+}$ response of twitch performance (extent and velocity of shortening; Fig. 14, $A$ and $B$, respectively) qualitatively similar to the intact rat muscles and that this differs markedly from that in rabbit preparations (Fig. 5). It is noteworthy that, in rat myocytes, which in physiologic Ca$^{2+}$ have an average sarcomere length of 1.82 $\mu$m (data not shown), the estimated average maximal velocity of sarcomere shortening, in 1.5 mM Ca$^{2+}$, was 5.14 $\mu$m/s, not markedly different from the 9.07 $\mu$m/s previously reported in a similar Ca$^{2+}$ (Kreuger et al., 1980).

The Ca$^{2+}$ dependence of diastolic properties of rat cells also resembled that of
intact muscles. The Ca_o dependence of the spontaneous wave frequency in the absence of stimulation and that of SLIF measured in unstimulated intact muscles were identical (Fig. 15A). A comparison of Figs. 15A and 6B indicates that in both rat myocytes and muscles, spontaneous waves and SLIF occurred in a Ca_o substantially below that required for their presence in rabbit preparations. A plot of SLIF vs. contractile wave frequency from both species across the range of Ca_o studied in both (Fig. 15B) indicates that rat and rabbit cardiac preparations fall on different parts of a continuum that relates the Ca_o dependence of SLIF and wave frequency. However, f_s, although reported in hertz, is not the actual
FIGURE 13. A representative myocyte that did not exhibit spontaneous contractile waves when bathed unstimulated in 1.0 mM Ca++. An initial bout of stimulation at 6 and 120 min⁻¹ produced a twitch amplitude of similar size at both stimulation frequencies (upper tracings). After cessation of stimulation, spontaneous contractile waves appeared and persisted at a constant frequency of 4 min⁻¹ (lower left tracing). During a subsequent bout of stimulation 60 min later, the twitch shortening at 6 min⁻¹ was greater than that at 120 min⁻¹.

FIGURE 14. (A) The effect of Ca⁺⁺ on the extent of twitch shortening in rat myocytes (n = 9; dotted line) and twitch force in intact rat papillary muscles (n = 4; solid line) stimulated at 2 min⁻¹. (B) The effect of Ca⁺⁺ on shortening velocity in myocytes and maximum rate of force production in intact muscles studied in A. Muscle cross-sectional area was 0.19 ± 0.03 mm² (x ± SEM).
frequency of the underlying contractile waves that cause SLIF in muscles (Kort et al., 1985a). Rather, the actual frequency of these waves that underlie SLIF in intact muscle, as recently determined from displacement analyses, is $<0.1$ Hz in physiologic $C_{a}$, as it is in these myocytes (Kort et al., 1985a).

**FIGURE 15.** (A) The $C_{a}$ dependence of spontaneous wave frequency in single rat myocytes (dotted line) and SLIF frequency in intact rat muscles (solid line). SLIF and wave frequency have been co-scaled to facilitate comparison of the $C_{a}$ effect in each. The preparations studied were the same as in Fig. 14, except that there was no stimulation. (B) Spontaneous wave frequency in myocytes and SLIF frequency in muscles measured across a range of $C_{a}$ in rats (solid symbols) and rabbits (open symbols). The rabbit data are from the studies in Fig. 6B and the rat data are from the experiments reported in A of this figure.
The data in Fig. 15B indicate that the presence and Ca\(^{2+}\) dependence of the frequency of spontaneous contractile waves in the absence of stimulation in myocytes of either species are virtually identical to those in unstimulated intact muscles of the respective species, as is manifest in SLIF measurements. Rat myocytes that exhibit spontaneous contractile waves maintain a normal membrane potential. In a random subset of 16 rat myocytes bathed in 1.8 mM Ca\(_o\), which exhibited an average wave frequency corresponding to the cells in Fig. 15A that were bathed in a similar Ca\(_o\), the resting membrane potential averaged 84 ± 4.4 mV (± SEM). In addition, a small transient depolarization accompanied the spontaneous contractile wave (Fig. 16). Ryanodine (1 µM) or caffeine (10 mM) abolished the contractile waves in myocytes (data not shown) and SLIF in muscles as reported in previous studies (Capogrossi et al., 1984a; Kort and Lakatta, 1984; Kort et al., 1985a).

**DISCUSSION**

The results clearly indicate that isolated single adult rat and rabbit myocardi
cells, as prepared and studied in the present experiments, retain the Ca\(^{2+}\)-
dependent properties exhibited by the intact muscles of their respective species. This is the case not only for the change in twitch parameters in response to
alterations in cell Ca\(^{2+}\) effected by different rates of stimulation or by changes in 
Ca\(_o\), but also for the Ca\(^{2+}\)-dependent properties in the unstimulated state. Specifically, the rat and rabbit myocytes exhibit spontaneous contractile waves in the absence of stimulation only under conditions in which these are observed in their respective intact muscles and are manifest as SLIF, and the wave
frequency in myocytes exhibits a Ca\(^{2+}\) dependence similar to SLIF in intact
muscle. It is important to note, however, that the functions describing the frequency and Ca\(^{2+}\) dependence of twitch amplitude or those describing the Ca\(^{2+}\)
dependence of diastolic phenomena in cells and muscles are not strictly super-
imposable. This may indicate some participation of extracellular spaces in the
determination of these functional properties.

Models of excitation-contraction coupling in the heart have usually considered
the myocardium to be strictly quiescent when unstimulated. The present and
previous results (Lakatta and Lappe, 1981; Kort and Lakatta, 1984; Kort et al.,
1985a) indicate that this is not the case for rat cardiac preparations and, under
certain experimental conditions, need not be the case in other species. The
localized contractile activity that constitutes the contractile wave and its reversible
Ca\(^{2+}\) dependence was first described in cells from chick embryo hearts
(Olivo, 1924). It is now known that this phenomenon is due to localized Ca\(^{2+}\)
release within the cell (Fabiato and Fabiato, 1972, 1973; Dani et al., 1979; Rieser
et al., 1979; Chiesi et al., 1981; Cobbold and Bourne, 1984; Fabiato, 1985a). Since
inhibition of SR function by high concentrations of caffeine or ryanodine
abolishes the oscillations in both intact muscles (Stern et al., 1983; Kort et al.,
1985a; Lakatta et al., 1985) and “skinned” cells (Fabiato and Fabiato, 1972,
1973, 1975; Dani et al., 1979; Rieser et al., 1979; Chiesi et al., 1981), these
oscillations have been attributed to spontaneous Ca\(^{2+}\) release from the SR.

The presence of spontaneous oscillations in the intact rat muscle and Ca\(^{2+}\)-
tolerant myocytes, as demonstrated in the present study, is expected even in the
absence of experimental Ca\(^{2+}\) overload. This follows from the observation that
the bathing Ca\(_o\) required for the appearance of spontaneous contractile waves in
cells devoid of sarcolemmal function is at or below the estimated free myoplasmic
[Ca\(^{2+}\)] in unstimulated intact preparations, i.e., between 150 and 250 nM (Blinks
et al., 1982; Cobbold and Bourne, 1984; Sheu et al., 1984). Specifically, me-
chanically skinned rat cardiac cells exhibit spontaneous repetitive contractile
waves when bathed in a Ca\(_o\) as low as 100 nM (Fabiato and Fabiato, 1975;
Fabiato and Baumgarten, 1984). Similarly, in rat cells devoid of sarcolemmal
function that have been studied under conditions in which mitochondria are not
Ca\(^{2+}\)-loaded, spontaneous contractile waves were observed in the majority
of myocytes bathed in 100 nM Ca\(_o\) (Chiesi et al., 1981). A correction for the effect
of Donnan osmotic forces related to the absence of sarcolemmal barrier would
only increase the estimate for free Ca\(^{2+}\) in the myofilament space in these studies
by 35%, i.e., to ~135 nM (Fabiato, 1985a). When adjusted for the temperature
effect, the oscillation frequencies that we report are in a range comparable to
that in mechanically or chemically skinned rat myocytes bathed in 100 nM Ca\(_o\)
(Fabiato and Fabiato, 1975; Chiesi et al., 1981; Fabiato and Baumgarten, 1984).

The species difference for the conditions required for spontaneous oscillations
to occur in the present study is also expected because rabbit cells devoid of
sarcolemmal function require a higher bathing Ca\(_o\) for their occurrence, i.e., a
pCa of \(\geq 6.8\) (Chiesi et al., 1981). The present results indicate that the same is
true for intact rabbit muscle. Thus, the rat and rabbit left ventricular cardiac
myocytes reflect the two extremes of a mammalian species difference that has
previously been demonstrated to occur in muscle with presumably intact sarco-
lemmal function. However, whether all the cells of the multicellular preparations
are intact has not been established.

It is noteworthy that the occurrence and Ca\(^{2+}\) dependence of spontaneous
contractile waves in both the intact muscle and myocytes with intact sarcolemmal function exhibit a Ca\(^{2+}\) species dependence that appears to be identical to that for the phenomenon referred to as \textquotedblleft Ca\(^{2+}\)-induced Ca\(^{2+}\) release\textquotedblright{} from the SR described in myocytes without sarcolemma (Fabiato and Fabiato, 1978; Fabiato, 1983; Kort and Lakatta, 1984). However, it is not certain whether the precise mechanisms that govern both types of release are identical (Fabiato, 1985a, b).

The present and previous studies (Lakatta and Lappe, 1981; Kort and Lakatta, 1984; Kort et al., 1985a) indicate that the extent of Ca\(^{2+}\) loading of unstimulated single myocytes or of cells within an intact muscle cannot be inferred solely from the presence or absence of spontaneous Ca\(^{2+}\) oscillations. Rather, this requires a knowledge of the oscillation frequency and the species under study. The present results show that unstimulated rabbit cells or muscles bathed in 1–2 mM Ca\(_o\) do not exhibit contractile waves or SLIF. Similarly, more recent studies of the intact rabbit heart have indicated that SLIF are not present under these conditions (Stern et al., 1985). In contrast, unstimulated intact rat muscles and \(\sim 85\%\) of myocytes do exhibit spontaneous contractile waves under these conditions but their frequency is low, i.e., \(<0.1–0.2\) Hz (present study and Kort et al., 1985a).

The intact unstimulated rat heart also exhibits SLIF in 1.5 mM Ca\(_o\) (Stern et al., 1985).

We therefore do not consider the presence of SLIF or low-frequency contractile waves in unstimulated rat cardiac cells or tissue to be indicative of Ca\(^{2+}\) overload. The oscillatory waves in myocytes, whose frequency is low and stable for long periods of time, need to be differentiated from rapid oscillations in cells, which subsequently round up within several minutes and cease to function. We too have observed these rapid oscillations before cell death in nonviable cells. Similarly, recent studies (Lehto et al., 1983) have shown that while rapid oscillations occur concomitantly with a drop in membrane potential, the wave frequency was as low as that observed in this study when resting membrane potential was normal.

Rat cardiac cells, like some of those in the present study, that do not exhibit Ca\(^{2+}\) oscillations might be considered \textquotedblleft Ca\(^{2+}\)-underloaded\textquotedblright{}, as suggested by their atypical twitch response to field stimulation from rest and to an increase in the stimulation rate, i.e., a positive or flat staircase. In this regard, it is noteworthy that in mechanically skinned fragments, a negative staircase in SR Ca\(^{2+}\) release occurs in response to repetitively applied Ca\(^{2+}\) triggers, which simulate electrical stimulation when this preparation is bathed at a pCa of 7.0 (Fabiato, 1985c). However, when the bathing [Ca\(^{2+}\)] is lowered (pCa 7.75), the first release after rest is not potentiated and repetitive Ca\(^{2+}\) triggers do not result in diminished Ca\(^{2+}\) release, i.e., the negative staircase is abolished. Similarly, in intact rat papillary muscles, a reduction in Ca\(_o\) changes the twitch strength-stimulation frequency relation from a negative to a flat one (Forester and Mainwood, 1974), and blocking SR function with ryanodine, caffeine, or strontium causes a rest decay of twitch force in rat muscles (Sutko and Willerson, 1980; Kort and Lakatta, 1983) and thus a positive staircase with ensuing repetitive stimulation. The observation that some cells that do not exhibit waves have a negative staircase upon stimulation (Fig. 12B) suggests that it may be possible to obtain cells that are sufficiently loaded with Ca\(^{2+}\) to present a negative staircase but not
loaded enough to present a spontaneous cyclic Ca\(^{2+}\) release from the SR. A difference in Ca\(^{2+}\) loading between rat cells that exhibit spontaneous waves, and even between those that do not, could be due to the isolation procedure or to a true difference in Ca\(^{2+}\) loading among cells that comprise the myocardium in vivo (Watanabe et al., 1983). In the present study, all myocytes that did not exhibit spontaneous contractile waves when bathed unstimulated in millimolar Ca\(_o\), did so after a period of stimulation. This could be explained by an effect of stimulation to increased intracellular \([\text{Ca}^{2+}]\) (Bers, 1985; Hilgemann, 1985).

Because sarcolemmal depolarization during stimulation leads to Ca\(^{2+}\) release from the SR, which is also the apparent source of Ca\(^{2+}\) that generates spontaneous oscillations (Capogrossi et al., 1985c), regular electrical stimulation might be expected to modulate the occurrence of spontaneous oscillations. Conversely, diastolic waves might be expected to affect twitch function (Allen et al., 1985; Capogrossi et al., 1985a). In the following article (Capogrossi et al., 1986), we address the effects of electrical stimulation on spontaneous Ca\(^{2+}\) oscillations in single cardiac myocytes. These studies show that spontaneous contractile waves do not occur in Ca\(^{2+}\)-tolerant rat myocytes during electrical stimulation at rates >0.5 Hz when these cells are bathed in physiologic Ca\(_o\), in the absence of drugs. Additionally, these studies have defined the experimental conditions that permit spontaneous Ca\(^{2+}\) oscillations to occur between stimulated twitches, and the effect of their appearance in the "diastolic" interval on the subsequent twitch.

We thank Mr. Don J. Pelto and Ms. Nadine Fontan for excellent technical assistance, Mrs. Joan C. Griffin for typing, and Dr. Michael D. Stern for a critical reading of the manuscript.

Original version received 10 March 1986 and accepted version received 22 June 1986.

REFERENCES


characteristics in rat cardiac myocytes depend on spontaneous contractile waves in the absence of stimulation. *Federation Proceedings.* 44:829. (Abstr.)


Fabiato, A. 1983. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *American Journal of Physiology.* 245:C1–C14.


CAPOGROSSI ET AL.  Contractile Properties of Rabbit and Rat Cardiac Myocytes  613


