Scanning Microscopy

Volume 1994
Number 8 *The Science of Biological Microanalysis*

Article 1

1-7-1994

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Scanning Microscopy Supplement 8, 1994 (pages 1-11) 0892-953X/94$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

MEASUREMENT OF SUBCELLULAR CA^{2+} REDISTRIBUTION IN CARDIAC MUSCLE IN SITU: TIME RESOLVED RAPID FREEZING AND ELECTRON PROBE MICROANALYSIS

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(Received for publication October 29, 1993, and in revised form January 7, 1994)

Abstract

To directly assess the physiological roles of sarcoplasmic reticulum (SR) and miitochondria (MT), we have utilized energy dispersive electron probe microanalysis (EPMA) on ultrathin freeze-dried cryosections from isolated papillary muscles, rapidly frozen at precise time points of the contractile cycle. Using this approach, we can detect redistribution of subcellular Ca^{2+} during the cardiac contractile cycle. Changes in Ca^{2+} of less than 1.0 mmol/kg dry wt can be detected. By determining the variability of the Ca^{2+} measurements in preliminary experiments, we have also demonstrated that it is possible to optimize experimental design, i.e., to predict the number of animals per treatment group and the number of X-ray spectra per animal that are required in order to detect a specified Ca^{2+} difference. Quantitative EPMA of rapidly frozen contracting papillary muscle has also allowed us to correlate the Ca^{2+} content of SR and MT with the contractile state of the muscle. Our results show a decrease of 40% in the amount of Ca^{2+} stored in the junctional SR during a cardiac muscle twitch, thus providing direct evidence for a role of the SR as a primary site of Ca^{2+} release. In addition, we have demonstrated dissociation between MT Ca^{2+} uptake and activation of regulatory enzymes, such as pyruvate dehydrogenase, indicating that MT Ca^{2+} uptake is not required for activation of MT metabolism.

Key Words: Electron probe microanalysis, junctional sarcoplasmic reticulum, calcium, mitochondria, cryosection, time-resolved rapid freezing, heart, papillary muscle, contractile force, analysis of variance.

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Introduction

Since the early experiments of Ringer at the end of the 19th century, it has become a basic tenet of cardiac physiology that an increase in cytoplasmic Ca^{2+} is the most important regulator of cardiac muscle contraction. However, before the advent of high resolution analytical methods, especially quantitative electron probe microanalysis (EPMA), for the direct measurement of the Ca^{2+} content of intracellular stores (Hall, 1971; Shuman *et al.*, 1976; LeFurgey *et al.*, 1988), the sources and sinks of Ca^{2+} in the cardiac muscle cell could only be inferred by indirect methods.

Over the years, attention has focused on the roles of two potentially important organelles, the sarcoplasmic reticulum (SR) and mitochondria (MT), which have been proposed to play an essential role in Ca^{2+} regulation in the cardiac muscle cell. While much evidence has accumulated to support the hypothesis that activator Ca^{2+} has its origin in the SR, a direct measurement of the release of SR Ca^{2+} had not been obtained, largely due to limitations of methods for measuring subcellular Ca^{2+} redistribution in the intact tissue. Similarly, as described in more detail below, the extent of Ca^{2+} uptake and release by MT in cardiac muscle cells has, for many years, been controversial (Hansford 1985; Wan *et al.*, 1989).

In order to directly assess the physiological roles of SR and MT, we have utilized energy dispersive EPMA on freeze-dried cryosections obtained from isolated contracting papillary muscles which are rapidly frozen at precise and predetermined time points of the contractile cycle (Moravec and Bond, 1991, 1992). This unique experimental approach has allowed us to directly correlate changes in the contractile state of the muscle with Ca^{2+} cycling by subcellular organelles.

Background - Regulation of Ca^{2+} Cycling in the Heart

In contrast to many other cells and tissues, cytoplas-

mic Ca^{2+} in heart muscle cells *in vivo* is constantly changing: an increase of cytoplasmic Ca^{2+} always precedes the muscle twitch (Yue, 1987; Spurgeon *et al.*, 1990). Following depolarization of the cardiac cell membrane, Ca^{2+} influx via voltage sensitive Ca^{2+} channels and Ca^{2+}-induced Ca^{2+} release from intracellular stores, there is a transient increase in cytoplasmic Ca^{2+} to approximately 500-700 nM (O'Rourke *et al.*, 1990) . This is the final and necessary step in the activation of myofibrillar contractile proteins and in force generation. Following this release of Ca^{2+} from intracellular stores into the cytoplasm, cytoplasmic Ca^{2+} then rapidly returns to resting levels. This occurs largely via Ca^{2+} uptake into the SR by the Ca^{2+}ATPase, an ATP dependent Ca^{2+} pump which traverses the SR membrane. Other processes, notably the Na^{+} Ca^{2+} exchanger of the cardiac cell membrane, are also believed to play an important role in restoring free cytoplasmic Ca^{2+} to low resting levels (approximately 100 nM free Ca^{2+}) (Bers, 1991).

The kinetics of the periodic cycling of free cytoplasmic Ca^{2+} on a beat to beat basis in the cardiac muscle cell have been measured by a number of investigators using Ca^{2+} selective fluorescent indicators, such as fura 2 and indo 1 (O'Rourke *et al.*, 1990; Spurgeon *et al.*, 1990). However, whereas use of Ca^{2+} sensitive probes has provided important information about the time-course of changes in free cytoplasmic Ca^{2+}, the free Ca^{2+} actually represents only a very small proportion of the total Ca^{2+} that redistributes within the cardiac muscle cell during a cardiac muscle contraction (Moravec and Bond, 1991). In fact, most of the Ca^{2+} that enters the cytoplasm during stimulation of the cardiac muscle cell is invisible to these fluorescent indicators, as it is immediately bound to regulatory binding sites on the contractile proteins (e.g. troponin C) as well as to a number of other Ca^{2+} binding sites in the cell. It has been calculated that the increase in total Ca^{2+} in the cytoplasmic compartment (based on total available Ca^{2+} binding sites) during a cardiac muscle twitch is on the order of 100 μmol/l cell H_2O (Hove-Madsen and Bers, 1993), as compared with an increase of only 0.5 to 0.7 μM free Ca^{2+}. In other words, only approximately 0.5% of the Ca^{2+} that enters the cytoplasm immediately prior to the twitch contraction is free in the cytoplasm. Thus, in order to determine the total amount of Ca^{2+} required to activate a cardiac contraction, it is essential to measure the total Ca^{2+} content within the pool of activator Ca^{2+}, as well as the proportion of this pool which is released into the cytoplasm following stimulation.

This raises the question as to the source of the increase in cytoplasmic Ca^{2+} that is responsible for activation of the cardiac contraction. Considerable evidence, albeit mainly indirect, has accumulated over the years pointing to the SR as a source as well as a sink for regulatory Ca^{2+} in the cardiac muscle cell. For example, Fabiato's experiments were the first to elegantly show release of Ca^{2+} from a non-mitochondrial intracellular Ca^{2+} store in permeabilized single cardiac muscle cells (Fabiato, 1983); more recently, the Ca^{2+} release channel of the SR, commonly referred to as the ryanodine receptor, has been shown to correspond to the periodic connections or "feet" structure which appear to span the gap between the junctional SR and the T-tubule membrane (Inui *et al.*, 1987). Furthermore, the probability of opening of the SR Ca^{2+} release channel to permit efflux of SR Ca^{2+} into the cytoplasm is also significantly increased when the Ca^{2+} concentration at the outer face of the SR membrane suddenly increases (Rousseau *et al.*, 1987); additional evidence which implicates the SR as a Ca^{2+} storage organelle is the presence of the low affinity high capacity, Ca^{2+} binding protein, calsequestrin, within the junctional SR of cardiac muscle, as detected by immunoelectron microscopy (Jorgensen *et al.*, 1988). Recent definitive evidence that the junctional regions of cardiac SR serve as Ca^{2+} storage sites, was provided by energy dispersive EPMA measurements in cryosections of rapidly frozen preparations of cardiac muscle (Jorgensen *et al.*, 1988; Wheeler-Clark and Tormey, 1987). These results are comparable to the demonstrations by Somlyo and co-workers of high concentrations of Ca^{2+} in the terminal cisternae of the SR in skeletal muscle (Somlyo *et al.*, 1981) as well as evidence that the SR in smooth muscle also plays a role in Ca^{2+} storage and release (Bond *et al.*, 1984b).

Thus a large body of evidence had accumulated over the years to suggest that the SR is a primary source of activator Ca^{2+} for cardiac muscle contraction. However, the crucial piece of evidence implicating the SR, not only as a Ca^{2+} storage organelle, but also as a Ca^{2+} release site for activation of cardiac muscle contraction, was lacking : this was the direct measurement of release of Ca^{2+} from the SR during a cardiac muscle twitch. We therefore set out to obtain these measurements of SR Ca^{2+} release using EPMA on freeze-dried cryosections of hamster papillary muscles rapidly frozen both during relaxation and during contraction.

Before these goals could be achieved, two important criteria needed to be satisfied: (1) the quality of freezing of the cardiac muscle preparation needed to be sufficiently good to be able to readily resolve the small (30 - 50 nm diameter) tubules of junctional SR in the freeze-dried cryosections of the rapidly frozen muscle, and (2) since the Ca^{2+} distribution within the cardiac muscle cell

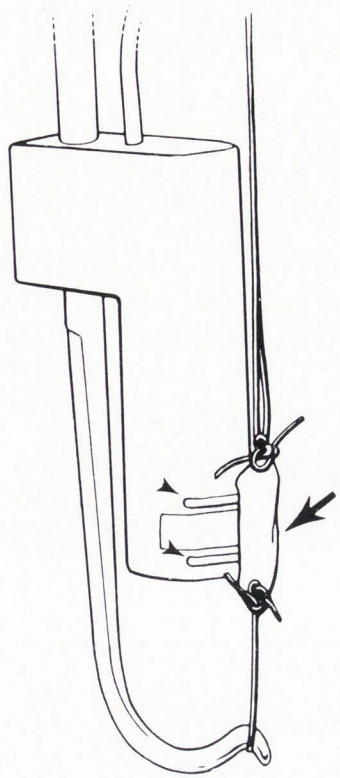

Figure 1: Illustration of the arrangement of isolated papillary muscle (arrow) attached via a silk thread to force transducer (not shown). The two parallel Pt electrodes used to stimulate the muscle are indicated by arrowheads.

is constantly changing as a function of the contractile state of the cell, it was essential to be able to program in advance the precise time-point in the cardiac cycle at which the contracting muscle was frozen. The methods which we have developed and which permit us to obtain time-resolved rapid freezing of contracting cardiac muscle preparations are presented below.

Rapid Freezing of Isolated Papillary Muscles

Time-resolved rapid freezing of isolated cardiac muscle preparations at defined time-points of the contractile cycle (Moravec and Bond, 1991) is based on a method first developed in the Somlyos' laboratory for the rapid freezing of bundles of skeletal muscle either during relaxation or during a tetanic contraction (Somlyo *et al.*, 1977; Somlyo *et al.*, 1981). Our goal, in extending this approach to contracting cardiac muscle preparations, was to obtain from the same muscle both functional information and measurements of sub-cellular Ca^{2+} distribution. For these experiments, we chose to use isolated left ventricular papillary muscle from hamster or rat heart (Moravec *et al.*, 1994a). For high resolution EPMA analyses of rapidly frozen tissue, the isolated papillary muscle offers several advantages over the intact heart *in vivo* or *in vitro*, although as discussed later, there are instances where the isolated perfused Langendorff heart is also a useful experimental model. The principal advantages of the isolated papillary muscle are as follows (1): the contractile state can be readily controlled and, in comparison with the intact heart *in vivo*, force development can be recorded in the absence of extraneous effects of changes in afterload or altered sympathetic stimulation. (2): because of the relatively small size of the tissue preparation (0.7 to 0.8 mm^2 cross-sectional area for hamster and rat left ventricular papillary muscles), excellent ultrastructural preservation can be achieved in the rapidly frozen tissue. Even small structures such as T-tubules and junctional SR (30-50 nm in diameter) can be readily visualized, (3) freezing of electrically stimulated, isometrically contracting papillary muscles can be readily achieved at precise, predetermined, timepoints of the contractile cycle. (4) both contractile measurements and microanalysis on the freeze-dried cryosections are simplified because all the muscle fibers run longitudinally, in contrast to the three dimensional arrangement of the fibers in the intact heart. This novel approach has provided us with a unique opportunity to correlate contractile parameters with subcellular Ca^{2+} content in the same cardiac preparation (Moravec and Bond, 1991, 1992).

In order to achieve this goal, we have arranged our rapid freezing set-up such that the muscle will continue to contract right up to the moment that it is frozen by rapid immersion in liquid N_2 cooled ethane. This is achieved by (1) incorporating the platinum electrodes used to stimulate the muscle into the muscle holder (figure 1) and (2) by maintaining the muscle in a warm humid environment, right up to the instant that it is frozen (see below). Freezing of the isolated contracting muscle then occurs by rapid propulsion of a beaker of liquid N_2 cooled ethane (-185°C), at a rate of approximately 3.5 m/s, up to the contracting muscle (Moravec and Bond, 1991). A similar strategy (taking the coolant up to the muscle, rather than *vice versa*), was originally used to freeze isolated skeletal muscle fibers (Somlyo *et al.*, 1977) and isolated contracting strips of vascular smooth muscle (Bond *et al.*, 1984a, 1984b). In order to ensure that the contracting muscle is frozen under physiological conditions and that no artefactual redistribution of diffusible elements occurs prior to the freeze, i.e. during the brief exposure of the muscle to air after removal of the water-jacketed muscle bath, we humidify the atmosphere immediately surrounding the muscle.

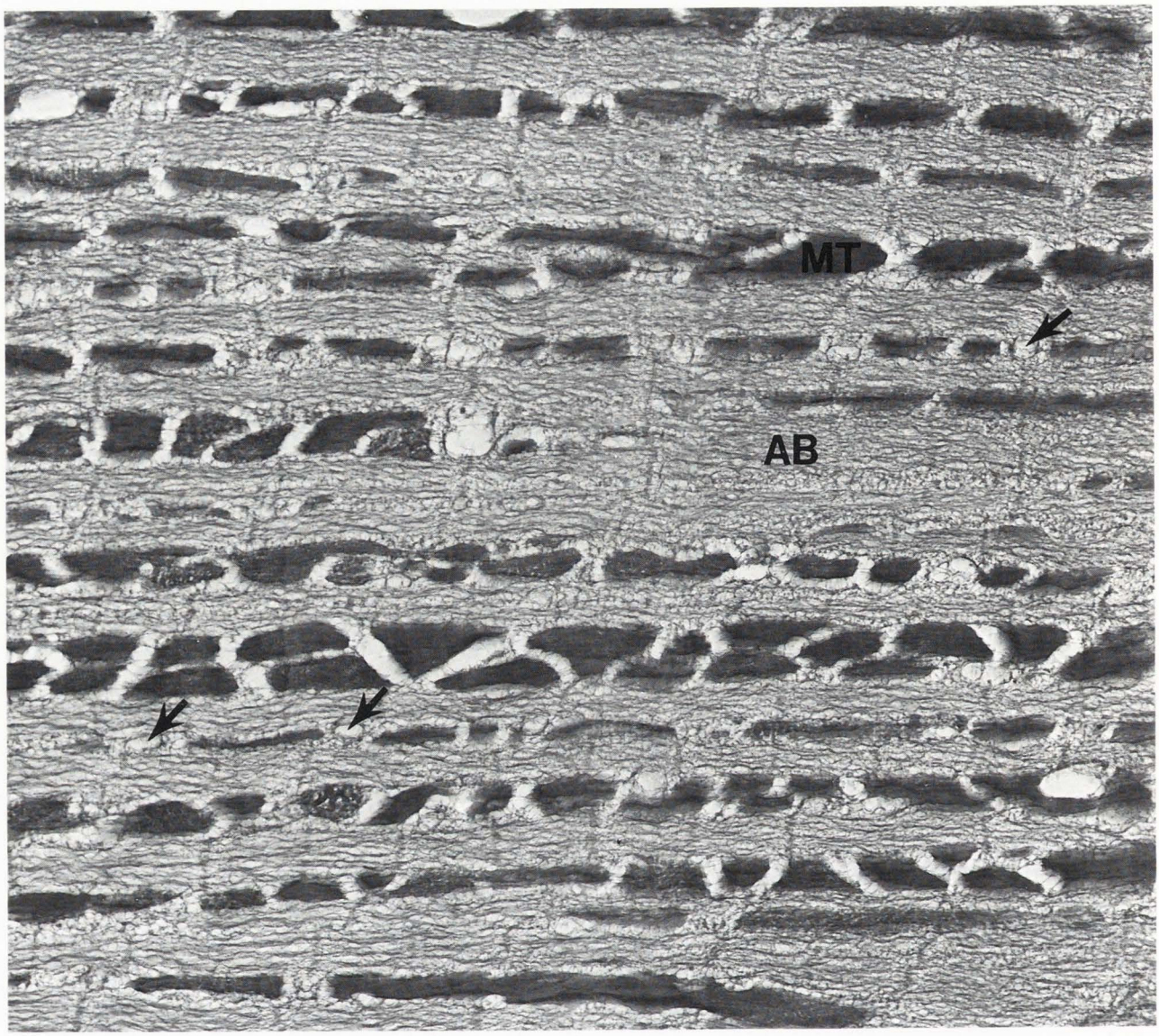

Figure 2: Freeze-dried cryosection obtained from the surface of a rapidly frozen hamster papillary muscle. T-tubules (arrow) with surrounding electron-dense rim of junctional SR are shown; MT: mitochondria; AB: A-band. Bar = 1 μm.

This is achieved by gently blowing warmed (28-29°C) humidified air over the surface of the contracting muscle; this arrangement is effective in preventing any significant changes in contractile function of the papillary muscle prior to the freeze.

The precise moment of the freeze is determined by programming a dedicated microprocessor to activate a pneumatic cylinder. Activation of the cylinder (which rapidly propels the beaker of ethane upward) occurs at a desired number of milliseconds after the previous stimulus, with a time resolution of approximately 10 ms (Moravec and Bond, 1991). The mechanical delay of seventy msec (the time for the beaker of ethane to travel up to the muscle) is also taken into consideration in the programming of the microprocessor. To confirm that the muscle is frozen at the intended time point in the cardiac cycle, an optical sensor has been installed in the freezing apparatus at the level of the muscle. When the light path is interrupted, an artifact is recorded on the trace, indicating the precise instant of the freeze.

Cryosections 100-150 nm thick are then cut from the surface of the rapidly frozen muscle in a Leica Ultracut E cryoultramicrotome at an ambient temperature of -130°C. Since the longitudinal axis of the

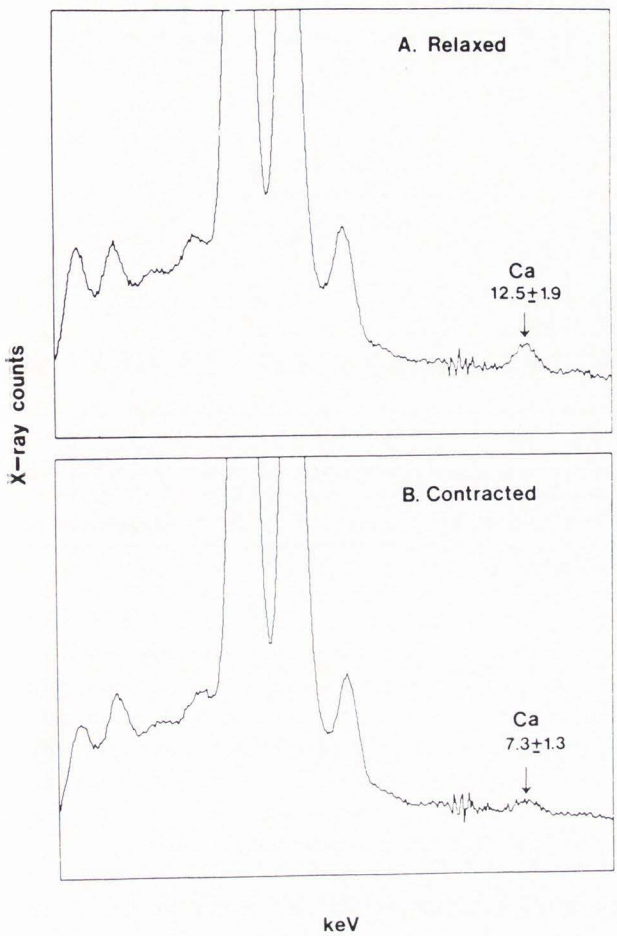

Figure 3: Averaged energy dispersive X-ray spectra of junctional SR from isolated contracting hamster papillary muscles frozen (A) during relaxation or (B) at peak rate of tension rise (peak +dT/dt), i.e. during contraction. In order to better demonstrate the amplitude of the Ca^{2+} K_α peaks, the potassium K_α and potassium K_β peaks have been stripped out by computer after fitting.

cardiac muscle cells is parallel to the long axis of the papillary muscle, it is a relatively easy task to obtain cryosections in which the sections cut are parallel to the myofibrils (figure 2). Obtaining longitudinally oriented cryosections greatly facilitates localization of transverse tubules and the surrounding electron-dense rim of junctional SR in the freeze-dried sections.

Electron Probe Microanalysis of Cryosections of Rapidly Frozen Hamster Papillary Muscles.

The rapid freezing methods described above have made it possible for us to obtain time-resolved quantitative measurements of the Ca^{2+} content of subcellular organelles (SR and mitochondria) in rapidly frozen

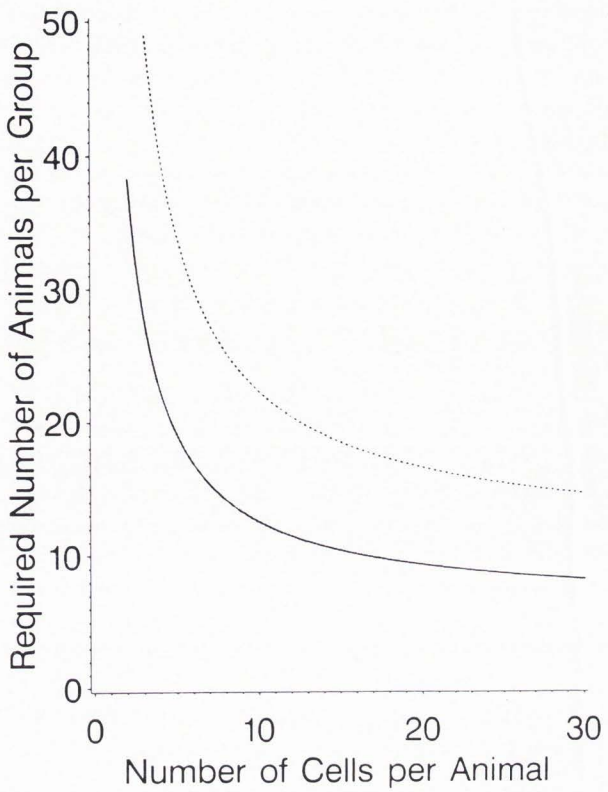

Figure 4. Relationship between number of animals, n, and number of X-ray spectra per animal, m, required to detect a difference between two treatment groups in MT Ca^{2+} content of 0.4 mmol/kg dry wt (solid line) or 0.3 mmol/kg dry wt (dashed line) with 80% power $(1 - \beta) = 80\%$ and $\alpha = 0.05$.

cardiac muscle and to correlate these measurements with the contractile state of the muscle. The cryosections were obtained from rapidly frozen hamster papillary muscles. These isolated contracting muscles were frozen either at peak +dT/dt, when free cytoplasmic Ca^{2+} should be maximal and thus the SR store Ca^{2+} depleted, or between contractions, when Ca^{2+} reuptake into the SR after release should be complete. The cryosections were analyzed as described below.

X-ray data were collected either in the transmission mode in a Philips 400T transmission electron microscope (TEM) or, more recently, in scanning transmission (STEM) mode in a Philips CM12 STEM. In order to minimize mass loss from the analyzed cryosections, specimens were routinely chilled to -100°C in a Gatan temperature-regulated specimen holder before turning on the beam. Average probe currents of 1 nA were used. In TEM, spectra were obtained from the A-band using stationary probes of average diameter 700 nm, from MT using an average probe size of 300 nm and from junc-

tional SR, using spots of approximately 65 nm in diameter. For the data collected in STEM, spectra were collected using rasters of average dimension 500 by 300 nm for the A-band, 500 by 200 nm for MT and an elongated probe of average dimensions 100 by 30 nm for the junctional SR.

Sarcoplasmic Reticulum Calcium Release in Rapidly Frozen Papillary Muscles

Analysis of X-ray data (collected in TEM) from a total of 8-9 SR from each of 5 papillary muscles frozen at peak +dT/dt and 5 muscles frozen during relaxation, (Table 1 and figure 3) revealed that the Ca^{2+} content of the junctional SR was 42% lower in the muscles frozen during contraction, a decrease of approximately 5.1 ± 4.3 mmol/kg dry wt of SR Ca^{2+}, as compared with muscles frozen during relaxation. Taking into account the relative volumes and hydration states of junctional SR and myofibrillar space (Page, 1978; Moravec and Bond, 1991), this would result in an increase of approximately 50 μmol Ca^{2+}/kg dry wt cytoplasm or 15 μmol Ca^{2+}/kg H_20. This is approximately 1/6 of the total Ca^{2+} increase in the cytoplasm calculated by Hove-Madsen and Bers (1993) to occur during a cardiac muscle twitch.

Our measurements may underestimate the total amount of Ca^{2+} within the junctional SR since the probed microvolume is likely to include, in addition to SR, regions of cytoplasm where Ca^{2+} content is much lower than in the SR. There is also likely to be "contamination" of the measured SR Ca^{2+} content by extracellular Ca^{2+} in the t-tubular space. However, we have estimated the inclusion of t-tubular space in the probed microvolume of the "SR" to be minimal since t-tubular Na^+ and Cl^- content were 7 and 10 fold higher, respectively, than the highest concentrations of these elements measured in the junctional SR. Furthermore, we have determined inclusion of t-tubular space in each of the experimental groups to be similar, as the Na^+ and Cl^- content of the junctional SR did not differ significantly between any of the groups (Moravec and Bond, 1991). We did not attempt to deconvolute overlap from t-tubule contributions within the plane of the section (Tormey and Walsh, 1989) as this procedure was shown by Tormey's group to have a relatively small effect on final SR Ca^{2+} values.

It should be noted that the increase in total cytoplasmic Ca^{2+} that has been calculated by other investigators is based on the assumption that Ca^{2+} binding to Ca^{2+}-specific binding sites in the cytoplasm reaches equilibrium during the cardiac muscle twitch (Hove-Madsen and Bers, 1993); however it is not clear whether this actually occurs during the brief cytoplasmic Ca^{2+} transient. In other words, the increase in total cytoplasmic Ca^{2+} at peak contraction is likely to be considerably less than predicted. Nevertheless, taking these caveats into considerations, our measurements directly demonstrate that the junctional SR of cardiac muscle serves not only as an important intracellular Ca^{2+} store but also, upon stimulation of the cardiac muscle cell, that the SR releases Ca^{2+} for activation of contraction.

Table 1. Subcellular Calcium Content in Rapidly Frozen Hamster Papillary Muscle (mmol/kg dry wt ± SEM)

	Relaxed	Contracted	Relaxed (+ Ryanodine)
A-Band	1.6±0.2	1.5±0.2	0.7±0.2**
Mitochondria	0.1±0.2	0.4±0.2	0.1±0.2
Junctional SR	12.5±1.2	7.4±1.1***	6.4±0.8***

n (number of spectra in each group) = 43-45. Number of animals per group = 5.
** $p < .02$ vs Relaxed, *** $p < .01$ vs Relaxed

We have also obtained additional evidence for release of a fraction of the Ca^{2+} stored in the SR by determining the effect of ryanodine on the size of the store in rapidly frozen, contracting papillary muscles. Consistent with the known action of ryanodine to deplete the SR of Ca^{2+} (Bers *et al.*, 1987; Marban and Wier, 1985), we found that a 20 minute treatment of the muscles with 10^{-7}M ryanodine decreased DT to 44.2 ± 3.5% of the initial value. Consistent with this action of the drug to decrease DT, we also showed that a 20 minute exposure of the contracting papillary muscles to ryanodine resulted in a 49% reduction of junctional SR Ca^{2+} content (as measured by EPMA) in muscles frozen during relaxation (Table 1). This decrease was similar to the reduction in junctional SR Ca^{2+} observed in muscles frozen without ryanodine but during contraction, again indicating that only a proportion of the Ca^{2+} stored in the SR is readily available for release. In summary, we have demonstrated release of approximately 40% of SR Ca^{2+} either by stimulation of contraction or by the release of the stored Ca^{2+} following opening of the Ca^{2+} release channels by treatment with ryanodine. The fate of the remaining 60% of Ca^{2+} in the SR has not been determined, however it is likely that this Ca^{2+} pool constitutes an "inotropic reserve" which is released upon stimulation by inotropic agents, such as β-adrenergic

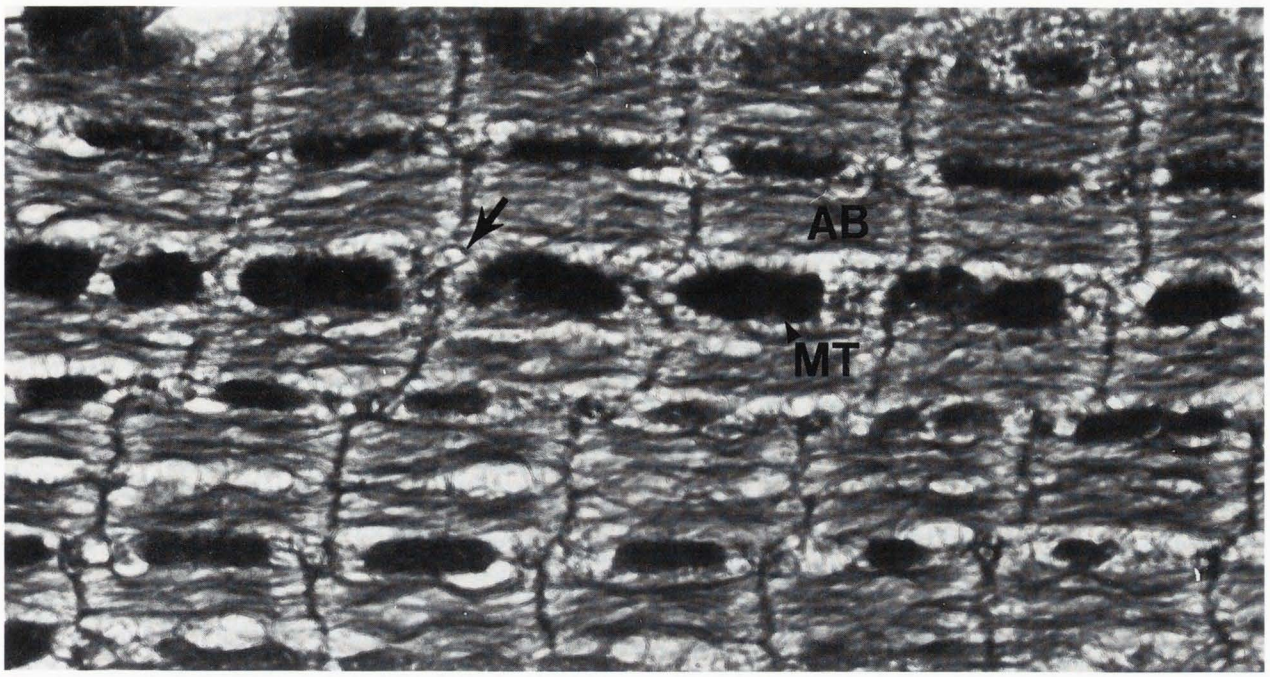

Figure 5. Freeze-dried cryosection obtained from the surface of a rapidly frozen isolated Langendorff perfused heart. The beating heart was frozen using a PS1000 metal mirror freezing device. MT: mitochondria; AB: A-band; arrow: T-tubule with electron-dense ring of junctional SR. Bar = 1 μm.

agonists.

In support of these observations, a depletion of SR Ca^{2+} content has also been measured by EPMA by Wendt-Gallitelli and Isenberg (1991) in isolated, voltage-clamped, guinea pig cardiac myocytes, rapidly frozen 15-25 ms after depolarization with the patch electrode. These investigators measured a statistically significant decrease in SR Ca^{2+} from 8.6 to 4.1 mmol/kg dry wt, again supporting the idea of a physiological role of the SR as a Ca^{2+} release compartment in cardiac muscle. Recently, using a very different experimental approach, Bers and co-workers (Bassani *et al.*, 1993) also demonstrated release of approximately 40% of Ca^{2+} from the SR during a twitch contraction. In these experiments, in isolated cardiac muscle cells loaded with the Ca^{2+} sensitive indicator, indo 1, the size of the caffeine releasable pool of Ca^{2+} (equivalent to the SR Ca^{2+} store) was found to be reduced by 40% during a twitch.

Mitochondrial Calcium Content as a Function of Inotropic State in Rapidly Frozen Cryosections

In addition to investigations of the role of the junctional SR in the regulation of Ca^{2+} cycling in cardiac muscle, we also wished to determine the role, if any, of MT in Ca^{2+} regulation in the cardiac muscle cell. It is now well accepted that MT do not constitute a large Ca^{2+} store in cardiac muscle under normal physiological conditions, although it is has been demonstrated that under conditions of Ca^{2+} overload, MT can take up very large amounts of Ca^{2+} (Scarpa and Graziotti, 1973; Bond *et al.*, 1989).

The contribution of MT to Ca^{2+} regulation during the contractile cycle was first examined in the absence of inotropic stimulation, by measuring MT Ca^{2+} content as a function of contractile state. When MT Ca^{2+} was measured in muscles frozen during relaxation, it was not found to differ significantly from the average MT Ca^{2+} value measured in the contracted muscles: our results revealed no significant uptake of Ca^{2+} during the contractile cycle and showed that, in contracting papillary muscles, MT Ca^{2+} ranged from only 0.1 to 0.4 mmol/kg dry wt. In contrast, Wendt-Gallitelli and Isenberg (1991) observed a three-fold increase in MT Ca^{2+} content following stimulation of voltage-clamped myocytes, from 1.3 $\pm$ 0.2 mmol/kg dry wt during diastole to 3.7 $\pm$ 0.5 mmol/kg dry wt 30-45 ms after stimulation. The explanation for these divergent results is not known, although the experimental protocols were very different in the two studies: in one case, isolated isometrically contracting hamster papillary muscles were used and in the other case, isolated, voltage-clamped

guinea pig myocytes were stimulated by patch pipette and then rapidly frozen.

In addition to the question of MT Ca^{2+} uptake during the cardiac contractile cycle, we have also investigated the proposal that MT Ca^{2+} uptake is intimately involved in the regulation of MT metabolism (Denton and McCormack, 1990; Hansford, 1985). Specifically, it has been suggested that the increase in peak cytoplasmic Ca^{2+} that occurs during a cardiac muscle twitch following hormonal stimulation leads to an increase in MT Ca^{2+} which, in turn, activates key Ca^{2+}-sensitive regulatory enzymes of the citric acid cycle, such as pyruvate dehydrogenase (PDH) (Moreno-Sanchez and Hansford, 1988; McCormack *et al.*, 1989). In short, it is hypothesized by these investigators that an increase in MT Ca^{2+} provides the signal which couples MT energy production to myofibrillar energy use.

We investigated this hypothesis by determining the effect of inotropic stimulation (using a β-adrenergic agonist, isoproterenol) on MT Ca^{2+} content and on PDH activity. Time resolved rapid freezing and quantitative EPMA of cryosections of the frozen muscle (described above), permitted us to measure not only the increase in developed tension (DT) in the isolated papillary muscle following inotropic stimulation, but also MT Ca^{2+} content and PDH activity in the same rapidly frozen papillary muscle. To confirm the sensitivity of our technique to small increases in MT Ca^{2+}, we also froze a group of muscles after incubation in low (46.5 mM) extracellular Na^+ solution (with Na^+ substituted by N-methyl-D-glucamine). This manipulation resulted in a statistically significant increase in MT Ca^{2+} content to 1.3 ± 0.3 mmol/kg dry wt, thus indicating that we are readily able to detect anticipated increases in MT Ca^{2+} content.

The results of these studies revealed a dissociation between activation of PDH activity and an increase in MT Ca^{2+}: specifically, PDH activity increased in response to β-adrenergic stimulation in the absence of an increase in MT Ca^{2+} content: MT Ca^{2+} was 0.5 ± 0.1 mmol Ca^{2+}/kg dry wt in the control muscles and 0.5 ± 0.2 mmol/kg dry wt in muscles frozen after isoproterenol stimulation. These results therefore demonstrated, for the first time that activation of key regulatory MT enzymes, such as PDH, does *not* require an increase in MT Ca^{2+}, thus refuting the hypothesis (Denton and McCormack, 1990; Hansford, 1985) that an increase in MT Ca^{2+} is a necessary step in the activation of MT metabolism. We would propose that, rather than an increase in Ca^{2+} being the necessary trigger for activation of MT metabolism, alternative mechanisms, such as regulation of respiratory rate by the NAD/NADH ratio or by the phosphorylation potential, may play a regulatory role.

In order to further demonstrate the physiological significance of these observations, we have recently investigated MT Ca^{2+} regulation in rapidly frozen Langendorff perfused hamster hearts. This latter approach offers advantages, as well as disadvantages, as compared with EPMA of rapidly frozen papillary muscles. The principal advantage is that contraction of the isolated perfused heart more closely models heart function *in vivo*, since the isolated Langendorff heart beats at physiological rates (4.0 Hz or 260 beats/min) and is also perfused at 37°C. In contrast, in order to maintain stable muscle function in isometrically contracting papillary muscles over 1-2 hrs, it is necessary to stimulate the muscle at a lower frequency (0.2 Hz) and at 28-29°C. A disadvantage of the intact heart preparation is that the increased tissue mass makes it is more difficult to obtain well-frozen preparations, free of ice-crystal damage. However, with the PS1000 metal-mirror cryofixation device (Delaware Diamond Knives, Wilmington, DE), we have been able to obtain adequate preservation of cellular ultrastructure such that MT Ca^{2+} content can be routinely measured in cryosections obtained from the surface of the rapidly frozen hearts (figure 5). Using this method, we have demonstrated, for the first time, that neither inotropic stimulation with the β-adrenergic agonist, isoproterenol, nor increased perfusion pressure causes any measurable increase in MT Ca^{2+} content (Moravec *et al.*, 1994b). Both of these interventions result in significant increases in the contractile performance of the isolated perfused heart (measured as dP/dt, rate of change of developed pressure). These studies therefore confirm and extend the results obtained in isolated contracting papillary muscles subjected to an inotropic stimulus (Moravec and Bond, 1992).

Ca^{2+} Sensitivity of EPMA

The power of quantitative energy dispersive EPMA to measure subcellular Ca^{2+} distribution and redistribution in cryosections of rapidly frozen tissue depends ultimately on the ability to obtain physiologically meaningful data. In this context, "meaningful" data is best described as data sets in which the minimal detectable differences in subcellular Ca^{2+} content are at least as small as the expected differences, based on biological considerations. The question of whether meaningful data can, in fact, be measured by EPMA under a particular set of experimental conditions can be readily determined at the outset of studies, such as those described above, from calculations based on the variability of the data.

The critical values that must first be determined are n, the number of animals per treatment group, and m, the number of X-ray spectra (of a predetermined counting time) per animal that must be collected in order to detect a specified difference in Ca^{2+}, using the appropriate statistical procedure, (i.e. nested analysis of variance, ANOVA). The number of animals, n, (as a function of m) can then be calculated using the values of the between-animal variance, σ_b^2 and the within-animal variance σ_w^2 obtained from the output of the nested ANOVA (Bond *et al.*, 1993). For example, if we wish to determine the values of n and m that are needed to have sufficient statistical power (1-β, typically 80% power), to detect a difference, D, in MT Ca^{2+} between two treatment groups, with $D = (\mu_1 - \mu_2)$, and with a significance level of α (typically 0.05), we perform the following calculation:

$$n = 2(\sigma_b^2 + \sigma_w^2/m)(Z_{\alpha/2} + Z_\beta)^2/ D^2, \qquad (1)$$

where $Z_{\alpha/2}$ and Z_β are the upper $\alpha/2$ and β percentiles, respectively, of the standard normal distribution (e.g. for $\alpha = 0.05$ and $\beta = 0.20$, $Z_{\alpha/2} = 1.96$ and $Z_\beta = 0.842$.). For the MT Ca^{2+} data used to obtain the relationship shown in figure 4, σ_b^2 and σ_w^2 were 0.06 mmol/kg dry wt and 0.66 mmol/kg dry wt respectively. As can be seen from the figure, for a given value of m, and with a power of (1-β), n can be determined for a specified Ca^{2+} difference. Thus, from figure 4, a difference in MT Ca^{2+} of 0.4 mmol/kg dry wt (solid line) can be measured by collecting either 10 X-ray spectra from muscles from each of 13 animals per treatment group or alternatively, 20 spectra from each of 10 animals per group. In general, the choice of the optimal value of n, as a function of m, should be based on practical considerations of efficiency and cost. It should also be noted that, in general, the Ca^{2+} sensitivity that can be achieved with energy dispersive EPMA of ultrathin cryosections in STEM or in TEM is less for very small organelles, such as the junctional SR, than for larger organelles, such as MT, because a smaller diameter electron beam (and thus less current) is used to excite the microvolume of interest (Bond *et al.*, 1993).

Summary

In summary, we have demonstrated that energy dispersive EPMA of ultrathin cryosections of rapidly frozen tissue is a powerful technique to examine physiological distribution and redistribution of Ca^{2+} at subcellular spatial resolution. In tissues such as cardiac muscle, where cytoplasmic Ca^{2+} is constantly cycling and regulating the contractile state of the muscle, the subcellular redistributions of Ca^{2+} that occur during each contraction can successfully be determined by an experimental set-up in which both tissue function (force or pressure development, the activity of regulatory enzymes such as PDH) and subcellular Ca^{2+} content can be measured in the same preparation. A final, but necessary, step in obtaining meaningful data is a preliminary analysis of the between-animal and within-animal variance components of the measurements, which then makes possible an optimal experimental design.

Acknowledgements

This work was supported by Grants HL 41883 (to M.B.), HL 49929 (to C.S.M), a Specialized Center of Research (SCOR) Grant in Hypertension HL 33713 (R.M.Graham, P.I.) from the NIH and by an Established Investigator Award from the American Heart Association (M.B.).

We would like to acknowledge the excellent technical assistance of Candis Disch and Russ Desnoyer in cutting cryosections; we also wish to thank Russ Desnoyer and Milorad Milovanovic for rapid freezing of the isolated perfused Langendorff hearts.

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Discussion with Reviewers

A. Warley: The authors refer to use of their cryofixation procedure with a Langendorff perfused heart preparation. Would the authors give further details of the type of preparation that they can achieve?

Authors: We have included a micrograph of a freeze-dried cryosection (figure 5) which provides an example of the quality of the freeze that we can routinely obtain using the DDK PS1000 metal mirror cryofixation device with freezing surfaces cooled to liquid N_2 temperature. As can be seen in the figure, the freezing is sufficiently good to readily distinguish mitochondria and A-band and, in some instances, the location of the T-tubules and surrounding dense rim of junctional SR. With further improvements in freezing, we expect to be routinely be able to visualize the small tubules of SR.

T. von Zglinicki: How large was the contribution from the surrounding cytoplasm? Were deconvolution methods used?

Authors: Because the tubules of junctional SR are narrower (40-60 nm) than the thickness of the freeze-dried cryosection (100-150 nm), there are likely to be contributions from both cytoplasm and t-tubular space throughout the depth of the irradiated microvolume of the cryosection. This should result in two opposing effects: the contribution from the cytoplasm (where Ca^{2+} is low) would result in the measured SR Ca^{2+} content being lower than the "true" value; in contrast, inadvertent inclusion of t-tubular space would tend to increase the SR Ca^{2+} above its "true" value. We have previously shown, however, that based on the Na^+ and Cl^- content of the SR, "contamination" of SR spectra by t-tubular space is minimal (Moravec and Bond, 1991); in addition, t-tubule contribution was found to be similar for all of the experimental groups, since the Na^+ and Cl^- content of the junctional SR did not differ significantly. Nevertheless, the experimental finding which we have tried to emphasize here, and which we believe is the most physiologically relevant observation, is the *difference* between the SR Ca^{2+} values measured under the different experimental conditions. Since we have previously shown (Moravec and Bond, 1991) that there is no significant increase (at least as detected by EPMA) of total cytoplasmic Ca^{2+} during a cardiac muscle twitch (see Table 1), we propose that *differences* in SR Ca^{2+} between relaxation and contraction should be unaffected by "contamination" of X-ray counts originating surrounding cytoplasm and T-tubule. Indeed, our demonstration of a decrease of 42% in SR Ca^{2+} during a cardiac muscle twitch has recently been confirmed by the work of Bers (Hove-Madsen and Bers, 1993) using a completely different experimental approach. A significant decrease in SR Ca^{2+} was also measured by Wendt-Gallitelli following stimulation of isolated ventricular myocytes (Wendt-Gallitelli and Isenberg, 1991). These two studies confirmed our observations of release of a proportion of SR Ca^{2+} during the cardiac muscle twitch, leaving a significant amount of Ca^{2+} remaining in the SR at peak contraction.

Deconvolution techniques (Tormey and Walsh, 1989), which correct for contributions from Ca^{2+} in the t-tubular space due to the spread of the electron beam outside the immediate area, but within the plane of the cryosection, were shown by these investigators to have a relatively small effect on SR Ca^{2+} values.

T. von Zglinicki: Were contraction-dependent changes in other ions than Ca found as well? In which compartments? How could these changes, if found, be understood?

Authors: In addition to the statistically significant decreases in SR Ca^{2+} content observed in the contracted and ryanodine-treated groups, as compared with the relaxed group of papillary muscles, the only other statistically significant changes observed were: (a) a decrease in A-band Ca in the ryanodine treated group, as compared with controls (see Table 1) and (b) a decrease in mitochondrial Mg in the ryanodine-treated group, as compared with controls from 42 $\pm$ 3 mmol/kg dry wt (n=45 spectra) in the relaxed group to 34 $\pm$ 2 mmol/kg dry wt after ryanodine treatment (n=45 spectra). The decrease in A-band Ca in the ryanodine-treated group has been observed by other investigators using different techniques (Marban and Wier, 1985) and may be explained by decreased steady state exchange of Ca^{2+} between the cytoplasm and a depleted SR Ca^{2+} pool. In regard to the observed decrease in mitochondrial Mg^{2+}, we have no specific explanation for this observation, although release and uptake of Mg^{2+} by mitochondria has been demonstrated in the heart (Romani and Scarpa, 1990) in response to inotropic stimulation, indicating that the mitochondrial Mg^{2+} pool in cardiac muscle cells is "labile".