

Intracellular Membranes as Boundaries for Ionic Distribution. *In Situ* Elemental Distribution in Guinea Pig Heart Muscle in Different Defined Electro-Mechanical Coupling States*

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Using x-ray microanalysis and cryoultramicrotomy, calcium and other diffusible elements were localized in heart muscle strips which had been shock frozen under different, defined conditions of electromechanical coupling. Guinea pig papillary muscles were shock frozen: 1) 1 1/2 seconds after paired stimulation, 2) 5 minutes after rest in normal bath medium and 3) 5 minutes after rest in bath medium to which noradrenaline was added. In 1) high calcium concentrations of 11.5 mmol/kg d.w. were regularly detected *in sites* at the level of Z-lines, which probably correspond to the Z rete of SR. In 2) in which the mechanogram of the first contraction after rest normally showed a small and retarded peak, the cell stores seemed to be nearly empty, with exception of a few regions between the mitochondria which revealed calcium accumulations of 77 mmol/kg d.w. These regions included JSR and/or T-tubuli. In 3) in which the mechanogram of the first contraction after rest normally showed a retarded peak with high tension, calcium was found in several cell structures. The highest amount, 25 mmol/kg d.w., was detected over the cell membrane. Measurable amounts were also detected over Z-lines and sarcomeres.

In the present experiments, the respective rate of rise of tension, and time to peak tension, were extremely different. Possible correlations between different contraction patterns and different calcium stores involved in the various experiments have been discussed.

Introduction

Unlike skeletal muscle, the development of tension in cardiac muscle is not thought to be essentially modulated by calcium movements between different compartments within the cell. During the action potential in cardiac muscle, there are significant movements of calcium from the extracellular space into the cell [1, 2]. To date it has been difficult to evaluate the actual contribution of each cellular compartment to the events of electromechanical coupling in cardiac muscle [3].

With the aid of cryotechniques and electron probe x-ray microanalysis we are studying the *in situ* distribution of calcium and other diffusible elements in chemically untreated heart muscle under different electromechanical coupling conditions. We have chosen types of experiments in which a) the freeze

trapping of the physiological state is easy and b) at the same time the functional state is extremely different from one experiment to another. We therefore can expect to detect differences in elemental compartmentalization.

In the experiments we will describe and discuss we have taken advantage of the well known fact that rise of tension and time to peak tension in some mammalian cardiac muscles depends on the stimulation rate and pattern [4, 5]. In the case of stimulation at short intervals or after paired pulses 1), the mechanogram reveals a predominant early component (time to peak tension ca. 400 ms) and the developed tension is high. In the case of contraction after several minutes rest 2) the time to peak tension is retarded and the mechanogram reveals a predominant late component (time to peak tension ca. 550 ms). Because the tension developed after rest in 2) is extremely low, we chose a third case 3) in which the muscle rested in bath solution to which 10^{-5} M noradrenaline was added [5, 6]. Under these conditions the first contraction after rest is of the late

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type (as in 2)), but the peak of the mechanogram is high as in 1). Thus, these different pattern of contractions suggest that different calcium stores working in a different mode are involved in the respective situations. We have therefore looked for qualitative and quantitative differences in the storage of calcium and the distribution of other elements in the compartments of the heart muscle cell under the above conditions.

Materials and Methods

Papillary muscles and trabeculae (2–4 mm long, 0.45–0.8 mm \varnothing) from the right ventricle of young guinea pigs (200–250 g body weight) were dissected in oxygenated tyrode solution of the following composition (in mmol/l): NaCl 130; KCl 4.9; $MgCl_2$ 1.2; $CaCl_2$ 2.1; Glucose 24; NaH_2PO_4 0.85; $NaHCO_3$

20, pH 7.4; 22 °C. Each end of the muscle strips was pierced with stainless steel or gold-plated hooks which, in turn, were linked to stainless steel hooks, one of which was fixed to a micromanipulator connected to the force transducer, while the other was directly fixed on the inductive force transducer. This arrangement permits stretching of the muscle to l_{max} in small steps.

Stimulation was carried out either via two platinum plate electrodes in the bath chamber, or (in the last seconds before freezing in experiment 1)) via the gold-plated hooks which served as electrodes when the chamber was removed. Stimulus and tension development were recorded simultaneously. When the strips reached steady state at 95% of l_{max} (after ca. 1–1 1/2 h), we started with the experiments, as shown in Fig. 1.

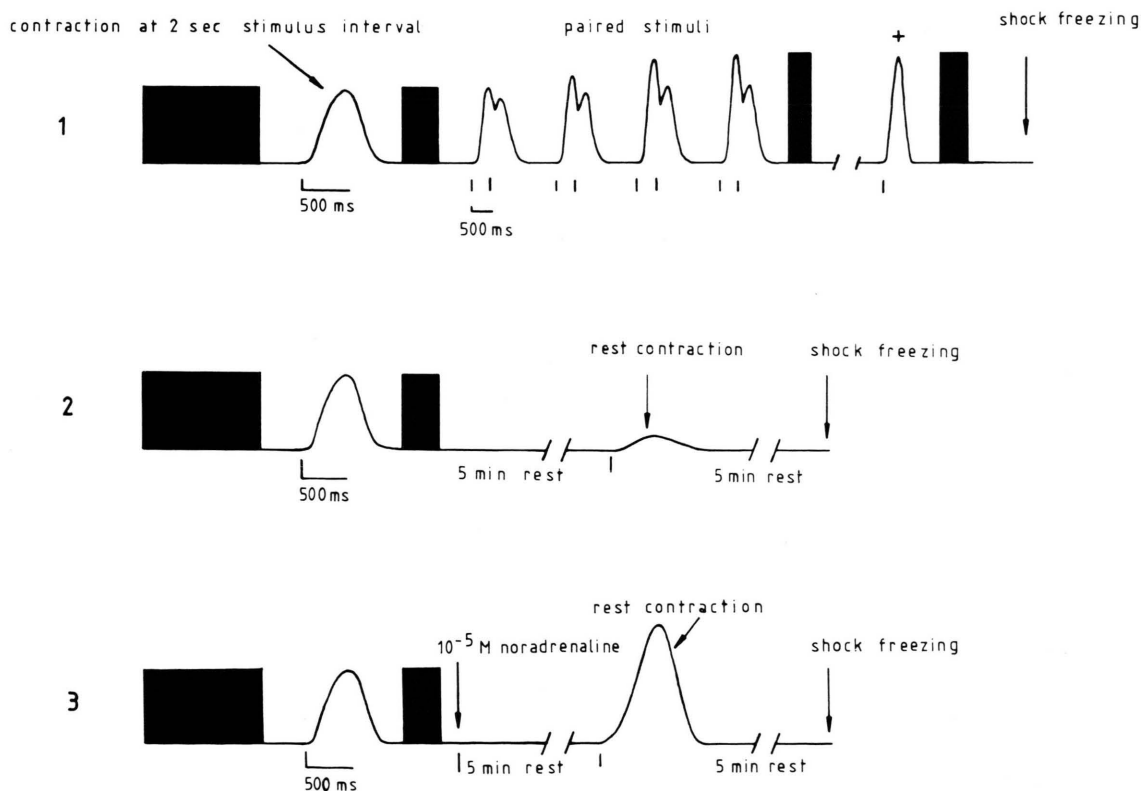


Fig. 1. Design of the experiments. In 1): The muscle is stimulated at 2 s intervals and 1 contraction (1st arrow) is recorded at high paper velocity. Thereafter, paired stimuli are applied (slower paper velocity) till maximal peak tension is achieved. Stimulation is interrupted for 5 s and 1 contraction is recorded in order to check if some force decay occurs in a time longer than that we need for freezing (+). Thereafter paired stimulation is continued and the coolant is brought into contact with the muscle (2nd arrow). In 2): Contractions at 2 s stimulus interval are followed by 5 min rest in normal bath medium, 1 test contraction, 5 min rest again, and following shock freezing. In 3): The same procedure as in 2 but noradrenaline is added to the bath medium at the beginning of the 1st rest period.

A single contraction at the "base" stimulation rate of 0.5 Hz was recorded at high paper velocity in order to check the functional state of each single muscle (peak of developed tension/mm² and time to peak tension). Then (in experiment 1)) paired stimuli were started and, as soon as the maximum tension was reached (normally after 4–8 beats), stimulation was interrupted for 5 seconds and 1 stimulus (+) was applied. The tension record shows that after 5 seconds rest, normally no important tension decay occurs. Thereafter, the muscle was stimulated again with paired stimuli. The chamber was quickly removed and the muscle strips were shock frozen at defined length 1 to 2 seconds after the last stimulus (now applied via the hooks). In experiment 2) (Fig. 1) the muscles were shock frozen after 5 minutes rest in normal bath solution. In experiment 3) the strips were frozen after 5 minutes rest in a bath solution which additionally included 10⁻⁵ M nor-adrenaline.

All those muscle strips were rejected which showed effects of damage during any step of the preparation: low tension per mm² at 95% I_{\max} , abnormal configuration of the mechanogram during paired stimulation, or physical damage of the strips upon contact between muscle and cooling medium. In order to visualize the possible accidents we recorded the behaviour of the muscle during freezing at high paper velocity (Fig. 2). This procedure was carried out in order to exclude any spontaneous contraction during freezing.

The metal holder of the cryoultramicrotome was gently brought into contact with the muscle stretched in the muscle bath while recording force. After a few minutes the muscles normally recover from the disturbance and return to the original level of tension.

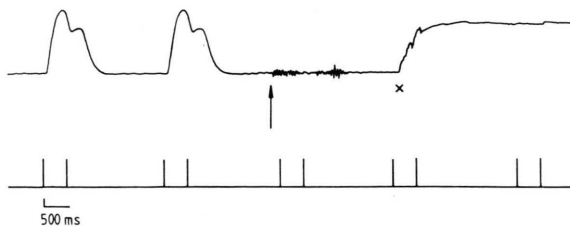


Fig. 2. Tension record during freezing in one experiment of type 1. The record shows that spontaneous contractions do not occur at the moment of contact between muscle and coolant. At X, the arm of the inductive force transducer is becoming cold. The vibrations shown in the record after freezing result from accidental mechanical disturbances of the experimenter after the muscle was already frozen.

Freezing procedure

A container with undercooled liquid propane was then quickly shoved up around the muscle, which was frozen in the stretched condition with the hooks in place. After 2 or 3 minutes the hooks were removed under undercooled propane, and the muscle was transferred in liquid N₂ to the cryochamber of an LKB ultramicrotome [7].

Ultrathin cryosections were cut at chamber temperatures of maximally -130 °C. A detailed description of the cryoultramicrotomy has been published previously [8]. The cryosections were then freeze dried at 10⁻⁶ Torr in a cold heavy brass container which warmed up to room temperature during the night. Subsequently the grids were carbon coated. Some muscle strips were occasionally freeze substituted in order to check the freezing procedure [8].

x-Ray microanalysis

The observation and analysis of the preparations was carried out with a Siemens Elmiskop 102 electron microscope equipped with an energy dispersive system (Kevex 5100). The analyzing conditions were extensively described in [8]. We worked at 80 KV acceleration voltage and 6 µA beam current. During measurements (100 s) point analysis is made at magnifications of 10 000 or 20 000. Alternatively analysis through the cytoplasm was taken with reduced area of 0.25 µm². As described previously [12] for quantitation of elements we used the continuum method of Hall [9]. As standard for quantitation we used mixtures of albumin and phosvitine and binary crystals according to Shuman *et al.* [10].

For each type of experiment we utilized three muscle strips which showed optimal behaviour during all the preparative steps. We analyzed two longitudinally oriented and one cross-oriented muscle from each experiment. In one region we carried out at least 30 measurements per element of adjacent organelles. At least 10 cells were analyzed per section. Data was evaluated with Student's *t*-test, and $p < 0.05$ was taken as the limit of significance.

Results

Only those muscles were prepared for analysis which showed optimal functional response to stimulation. Particular attention was given to the configuration of the mechanogram after paired stimulation.

Morphology. During this study it became necessary to carefully examine the peculiar morphology of our conventionally fixed and embedded guinea pig preparations under the electron microscope in order to interpret the analytical data. The muscle cells of the papillary muscle of our guinea pigs are relatively small in diameter (6–10 μm), at least compared with those of rats of the same body weight. The bundles of myofilaments are also small (about 1 μm) and each bundle is enveloped in a rete of free SR with a dense Z-rete (Fig. 3), showing frequent fenestrations. At the level of each Z-line, external to the SR Z-rete, the T-tubuli occur in periodic rings

situated between mitochondrial rows and generally connected with one, occasionally two elements of collapsed junctional SR.

After shock freezing, the external 15–20 μm of the strips are generally free of large ice crystals. Cryosections of this well frozen region reveal poor contrast, and identification of most cell compartments is difficult (Fig. 4). Nevertheless, mitochondria, the nucleus, Z-lines and t-tubuli are recognizable. Experience collected during the past three years of analysis also facilitated the identification of cell compartments on the basis of their characteristic spectra [12].



Fig. 3. Longitudinal section of guinea pig heart muscle cell. The muscle strip was conventionally fixed (2% glutaraldehyde) and embedded (araldite). Note the dense rete at the level of the Z-lines. Z=lines, M=mitochondria.

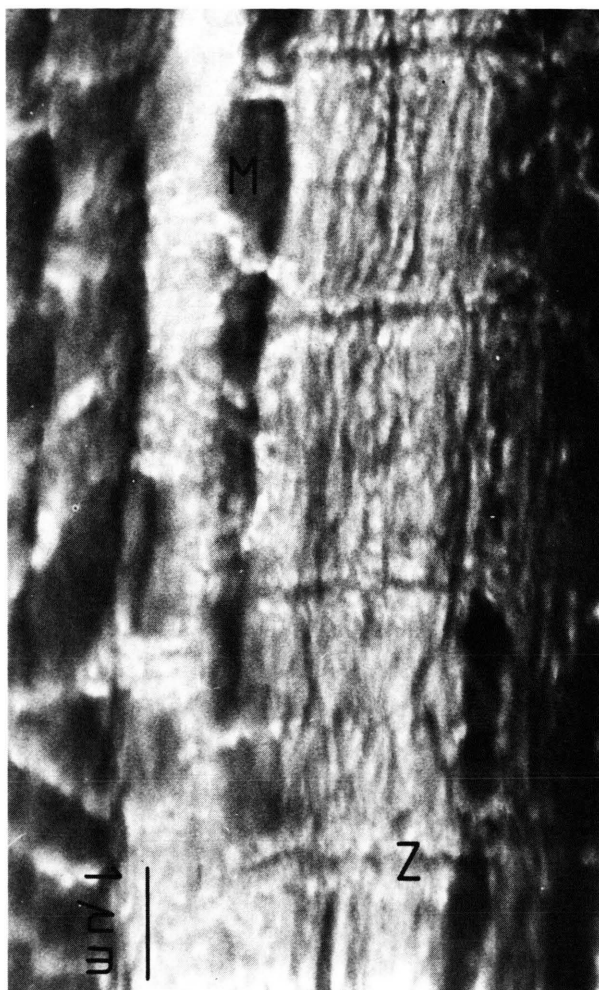


Fig. 4. Longitudinal cryosection of guinea pig papillary muscle. Note the absence of larger ice crystals and the poor morphology. M = mitochondria; Z = Z-lines.

X-Ray microanalysis

Experiment 1)

In this experiment the developed tension was high (Fig. 1, 5) and the time to peak tension "short" (ca. 400 ms) when the low temperature is considered. Elemental analysis of papillary muscle frozen maximally 1–2 seconds after the last stimulus shows a characteristic distribution (Fig. 6). In the sarcomeres, calcium is present in amounts too small to be detected clearly by the analyzing beam. Potassium reaches mass fractions of about 550 mmol/kg

d.w., which correspond to a concentration of ca. 130–150 mm when we assume the water content of this compartment to be ca. 75%. Chlorine is present in a mass fraction of ca. 125 mmol/kg d.w., corresponding to a concentration of about 30 mm. We have found similar high intracellular chlorine concentrations in all heart muscle preparations examined to date. We cannot completely exclude that this finding is an artifact, although the very sharp jumps in Cl concentration between adjacent compartments would seem to do so. Sodium is present in a low mass fraction of about 30 mmol/kg d.w. corresponding to ca. 7 mm. Mg is present in mass fraction of ca. 50 mmol/kg d.w. We found ca. 220 mmol/kg d.w. P in the sarcomeres. Sulphur is low; it is conceivable that some S evaporated in the microscope (see discussion of this point in [8]). If we compare the elemental distribution over the sarcomeres with that over the Z-lines (Fig. 6), it is evident that calcium occurs in significantly higher amounts over the Z-lines than over the sarcomeres. Differences in Na and K concentrations were found, but they are not statistically significant with the current number of analyses.

When we move the analyzing beam along the cell membrane in the cross or longitudinal sections it is evident that the Na concentration over this structure is higher than in the sarcomeres or Z-lines; and Cl is also significantly higher than in sarcomeres and Z lines.

The calcium concentration indicated by the signal over the cell membrane is higher than over the cytoplasm, but it is not higher than that of the bath medium. In this experiment 1) calcium seems to be clearly localised in structures situated at the level of the Z-lines.

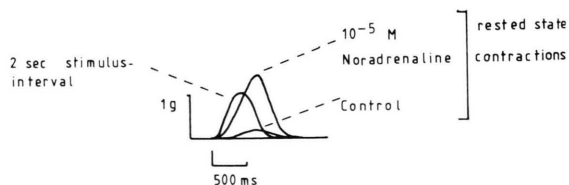


Fig. 5. Superimposed contractions of a guinea-pig papillary muscle (0.7 mm diameter, 3 mm length) under different stimulation pattern; at 2 s stimulus interval, the peak tension is reached 360 ms after stimulus (early type of contraction). In rested state contractions in normal bath medium or in bath medium supplemented with 10^{-5} M noradrenaline peak tension is reached 550 ms after stimulus.

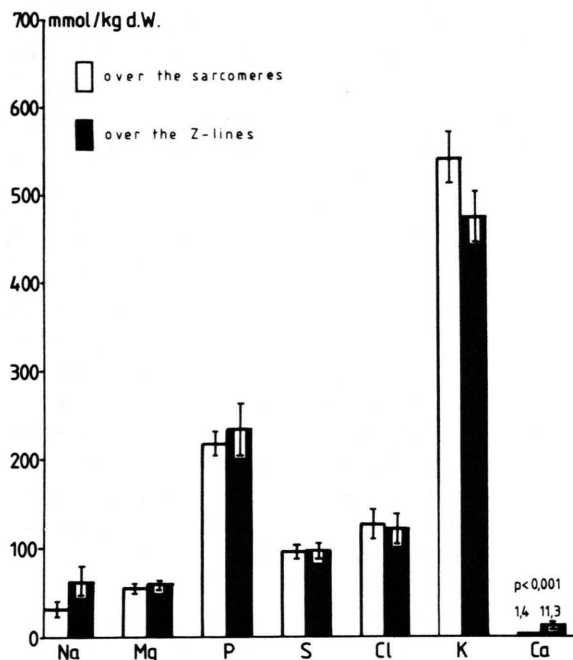


Fig. 6. Comparison of elemental mass fractions in different cell compartments in experiment 1): data of measurements over the sarcomeres are compared with data of measurements over the Z-lines.

Experiment 2)

In the second experiment, the muscle strips were frozen after five minutes rest in normal bathing solution. At this time, the first contraction would generate very low tension, being of the "late" type (Fig. 5). Time to peak tension is about 550 ms. In this situation we only found calcium in regions (corresponding to junctional SR?) between the mitochondria at the level of the Z-lines. The calcium concentrations in such regions are the highest we have ever found in normal heart muscle preparations (Fig. 7). Yet when moving the electron beam over the cell we found that these periodical regions are mostly "empty". The calcium concentration over the sarcomeres is 1.7 mmol/kg d.w. and there is no significant difference from the calcium concentration over the sarcomeres of experiment 1). Calcium is neither accumulated in the mitochondria nor over the Z-lines. In this experiment most of the membrane compartments seem to be empty, with the exception of a few regions between the mitochondria.

Experiment 3)

In the third experiment the muscle strips were frozen after five minutes of rest in a solution containing 10^{-5} M noradrenaline (Fig. 1). At this time the contraction produced by a single stimulus would be of the "late" type, but the tension would be very high (Fig. 5). In this situation calcium was not detected in the regions in which it occurred in experiment 1) or 2). Over the sarcomeres a slightly higher calcium signal is detectable in comparison to experiment 1) and 2) (Fig. 9). The mitochondria do not accumulate any substantial amount of calcium; at the level of the Z-lines more calcium is detectable than in experiment 2, and about as much as in experiment 1) (Fig. 9). But the signal over the cell membrane revealed the highest calcium accumulation in this experiment. The concentration of 25 mmol/kg d.w. detected over the cell membrane is significantly

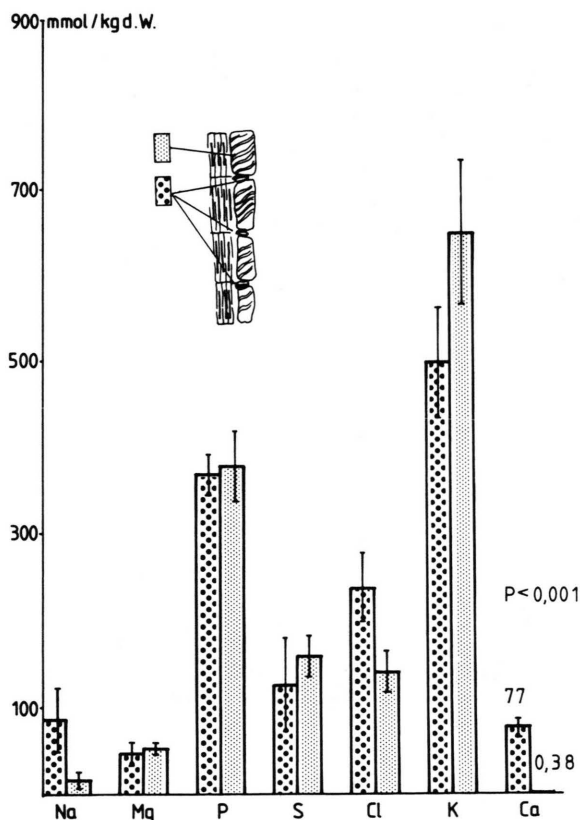


Fig. 7. Elemental distribution over mitochondria and adjacent structures (JSR, t-tubuli) in experiment 2). Note the high calcium mass fraction in the latter structures.

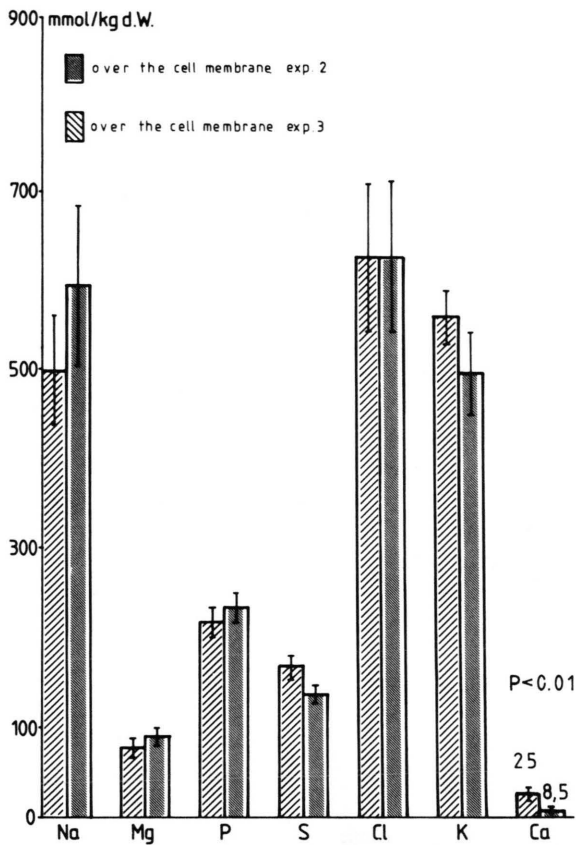


Fig. 8. Comparison of elemental distribution over the cell membrane in experiment 2) and 3).

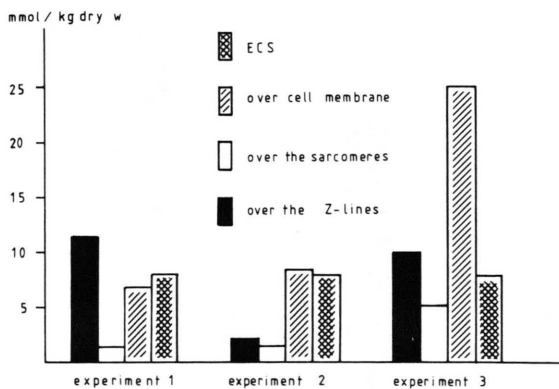


Fig. 9. Calcium mass fraction in different cell compartments in the 3 experiments. On the left, experiment 1), with freezing 1 1/2 seconds after the last stimulus. In the middle (experiment 2)), with freezing after five minutes rest in normal bath medium. On the right, with freezing after 5 minutes rest in noradrenaline-containing bath medium (experiment 3)).

higher than over the sarcomeres and also than over the extracellular space (8 mmol/kg d.w.). The Na and the Cl concentrations detected over the cell membrane are significantly higher than those detected over the sarcomeres, as is the case for P (Fig. 8).

Discussion

At the present time, three cell compartments could conceivably be capable of regulating intracellular calcium, *i.e.*, modulating force, in cardiac muscle. It is assumed that these three compartments: the sarcoplasmic reticulum, mitochondria and surface membrane, are able to transport calcium ions between tissue phases at substantial rates. Any intracellular store which functions in activating contraction must satisfy the following criteria: 1) the stored amount of calcium must be sufficient, 2) the calcium release must be rapid enough to produce the observed rate of rise of force, and 3) the release must be triggered in response to depolarization of the surface membrane. Whereas sarcoplasmic reticulum is considered to be a labile store, the calcium content of which determines the inotropic state of the muscle [3], cardiac mitochondria [11] only seem to play an important role in calcium storage in certain pathological conditions [8, 12, 14]. Some authors suggest that the surface membrane, with its calcium binding site, plays the most important role in the recycling of calcium whereas recycling between different intracellular stores is negligible [15].

It is known that developed tension decreases with increasing duration of the beat interval applied to cat [3] or guinea pig [6] papillary muscle. The tension minimum is reached at minimum beat intervals of 3–4 minutes. The peak of tension is retarded and the developed tension is lower compared with contractions at higher frequencies. Depending on the duration of the stimulus interval in guinea pig papillary muscle, we observe two extreme types of contraction in which either the early or the late component is predominant. The early type, which appears at a short stimulus interval or after paired stimuli, seems to derive its calcium from a store which releases it easily. In the case of rested state contraction calcium release seems to be considerably delayed. Moreover, changes in the composition of the bath solution elicit a full mechanical response in the first contraction after rest, whereas the positive or

negative staircase is regularly observed at normal beat rates. Thus it seems reasonable to look for evidence of another kind of calcium store which could be responsible for the late component of contraction.

In experiment 1) we froze the muscle ca. 1.5 seconds after the last contraction. This was the moment in which a stimulus would cause a maximal mechanical response, *i.e.*, when the calcium stores responsible for the next contraction must have been filled. The contraction which would appear under these conditions would be of the early type (Fig. 1). In all examined samples we found a significant calcium accumulation only in sites corresponding to the Z-lines. Because the bundles of myofilaments in the muscle strips are very thin, regions corresponding to Z-lines in the cryosections certainly also contain part of the dense Z rete of free SR; the latter structure is superimposed on the Z-lines and regularly wrapped around the thin myofilament bundles at this level. The elemental composition, particularly the low chlorine and the high potassium concentrations of these regions permit us to assume that we are analyzing intracellular structures. We are tempted to consider these structures, which appear in dense rete at the level of the Z-lines, to be responsible for the calcium accumulation required for the maximal mechanical response of the muscle to the paired stimuli or stimuli at short intervals, *i.e.*, the case in which contraction shows a predominant "early" component. This store is nearest to the myofilaments and has an intermediate location between T-tubuli and contractile material. The calcium diffusion distance from this store to the myofilaments is the shortest possible in the cell, although one might also say that the diffusion distance is likewise the shortest from the myofilaments to these stores. This finding alone obviously does not permit us to state whether these structures, as a part of SR, accumulate calcium on its way into or out of the cell. Nevertheless, if the plasmalemma is the site of calcium accumulation on its way into the cell, whereas SR is the site of calcium accumulation on its way out of the cell, then we can expect to find a detectable amount of calcium over the plasmalemma. But the calcium signal over the plasmalemma in this experiment is similar to that of the extracellular space (about 7 mmol/kg d.w.) and corresponds to the concentration of 2 mM calcium in the bath medium.

Another possible interpretation of this finding is that the Z-lines themselves or other structures con-

nected with the Z-lines play the role of calcium store in this case. For this notion we have no support from any information available at this time, which obviously does not exclude that such a possibility exists.

In experiment 2) we froze the muscles 5 minutes after rest in normal bath, *i.e.*, a point in time at which an applied stimulus would produce a very small contraction of the "late" type. In this experiment we found most of the cellular stores "empty" of calcium, *i.e.*, the calcium amounts were not significantly different from that of the surrounding cytoplasm. Merely a few sites between the mitochondria and lateral to the Z-lines contained the highest calcium accumulation of normal heart muscle which we have observed to date. In conventional EM pictures, T-tubuli and JSR are situated at these sites. At this time we cannot determine whether these structures consist of transverse tubuli, adjacent JSR, or both. The sodium signal of the sites is higher than that of the adjacent mitochondria but as low as that of the sarcomeres. The potassium concentration over the sites is relatively high and also corresponds to that of intracellular structures. This fact supports the notion that the structures corresponding to the sites are part of an intracellular membrane system. During prolonged rest in normal bath solution, calcium seems to be accumulated in stores which are not directly in contact with the myofilaments bundles. At the first AP after rest the small activation can be produced either by Ca inflow through the cell membrane during the relatively long action potential [1, 3], or by triggered release of the calcium previously sequestered in the few stores which we found filled during rest. The stores involved in the latter case should release calcium slower than those of experiment 1). In any case, the finding that calcium is accumulated in a site different from that in muscles frozen 1–2 seconds after the last contraction (experiment 1)) supports the notion that a different store is responsible for the "late" contraction.

In experiment 3) 10^{-5} M noradrenaline was added to the bath solution, which caused a high first contraction of the "late" type after 5 minutes rest. In this experiment we found relatively high calcium signals over several cellular structures, *i.e.*, sarcomeres, Z-lines, cell membrane – but not over the mitochondria. The highest signal was found over the cell membrane; at this time we cannot establish whether this calcium is located on the outer or inner or both sides of the membrane. The high chlorine and sodi-

um concentrations suggest that we are analyzing the outer side of the membrane, whereas the high potassium and phosphorus reflect intracellular concentrations. Further analyses with a maximally focussed electron beam in the STEM mode may clarify this point.

In experiment 1) and 3) the high peak of the developed tension is not extremely different, but the retarded start of rise of tension and time to peak tension differs markedly. In experiment 3) the activating calcium accumulated on several cellular structures, including surface membrane, seems to require a longer time to reach the myofilaments after the beginning of the AP. In experiment 1), however, calcium seems to derive from a membrane system which can release it quickly to the myofilament bundles. It is evident that the calcium accumulation in structures near the cell membrane and/or its diffusion through the membrane is facilitated in some way by the incubation in noradrenaline bath (experiment 3)). An increased calcium inward current through the plasma membrane has been found from electrophysiologists during the AP under noradrenaline [5]. Our results give evidence that substantial calcium accumulation occurs on several cellular structures under noradrenaline during rest as well. In our opinion, it would be worthwhile to reexamine the question of whether calcium coming from the cell membrane can couple directly to the myofilaments under the influence of noradrenaline. Planned experiments with drugs which selectively block Ca entry into the cell may clarify this point.

The preliminary results of the present study provide first information about the *in situ* distribution

of calcium and other elements in guinea pig heart muscle in different states of electromechanical coupling. In some cases, however, the correct attribution of the data to specific organelles is rendered difficult by the uncertain morphological identification of some structures. Nevertheless, quantitative data has been obtained on the elemental composition of different compartments involved in calcium accumulation in heart muscle under different conditions of electromechanical coupling.

In describing and discussing the present results we do not pretend to confirm or contradict any of the current models of electromechanical coupling. But we are convinced that one cannot ignore information regarding *in situ* elemental distribution for full understanding of such processes. The methods for obtaining such data are difficult, but certainly not more destructive than those currently used for studying EMC events.

We would like to conclude with a comment. Cryotechniques and x-ray microanalysis are methods requiring high caution in the interpretation of data and above all critical experience in detecting preparative artifacts. Moreover we cannot expect these methods to give us information which they are unable to provide, such as quantitation of calcium in the micromolar range. When preparations suitable to microanalysis have been obtained (and for certain tissues this is the case today in some laboratories), we can begin to conduct more sophisticated experiments; provided diffusible elements are sequestered in high concentrations in small volumes, they will not escape detection by the analyzing beam.

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