THE ROLE OF THE SODIUM ION IN CALCIUM-FREE SOLUTION ON THE MECHANISM OF THE CALCIUM PARADOX IN THE GUINEA PIG HEART

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We examined the effect of low Na concentrations (25 mM) in Ca-free solutions on the myocardial damage induced by the Ca paradox in guinea pigs using electron microscopy and electrophysiological methods. Ultrastructural damage consist of contraction bands, swelling of mitochondria and disappearance of major part of contractile apparatus induced by the Ca paradox was prevented by reducing Na from 145 to 25 mM in the Ca-free solution. The penetration of lanthanum into the myocardial cells, which was induced by the Ca paradox, was also prevented by reducing Na concentration during the Ca depletion period.

Perfusion with Ca-free solution containing normal Na (145 mM) for 10 min caused a significant prolongation in the action potential duration (APD) of left ventricular papillary muscle, and reperfusion with Ca containing solution caused depolarization. The perfusion with Ca-free solution containing low Na concentration (25 mM) significantly reduced APD, but APD returned almost to control values without depolarization after reperfusion with Ca-containing solution for 5 min.

These results suggest that the Na concentration in a Ca-free solution plays an important role in the pathogenesis of the Ca paradox phenomenon in guinea pig hearts.
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PERFUSION of an isolated heart with Ca-free solution followed by reperfusion with a Ca-containing solution results in the rapid occurrence of severe cellular damage, enzyme leakage and the development of contracture. This phenomenon is called "Ca paradox" and it occurs in many species^{1–5}

Several studies have shown that reducing the Na concentration of the Ca-free solution protects from myocardial damage during the Ca paradox^{4,5}. However, Busselen⁶ reported that no protection was observed in

isolated rat hearts when the Na concentration was reduced in the Ca-free solution.

We have used electron microscopic techniques with or without utilizing lanthanum (La) as a tracer, and electrophysiological methods to study the effect of low Na (25 mM) in the Ca-free solution on the myocardial damage induced by the Ca paradox.

MATERIALS AND METHODS

Hearts from adult male guinea-pigs, weighing about 300 g, were used in this study.

This study was carried out in accordance

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with the Guide for animal experimentation, Faculty of Medicine, Kagoshima University. After inducing anesthesia with an intraperitoneal injection of sodium pentobarbital (40 mg/kg), the chest was opened under artificial respiration (140 ml air/min), and the heart was perfused with Krebs Henseleit solution by cannulating the aorta. The heart was then isolated and suspended on a noncirculating Langendorff apparatus at a perfusion pressure of 100 cm of water and electrically paced at a frequency of 5 Hz.

The composition in mM of the control Krebs-Henseleit (K-H) solution (pH 7.4) was NaCl: 120; KCl: 4.7; NaHCO_3 : 25; KH_2PO_4 : 1.2; MgCl_2 : 1.2; CaCl_2 : 2.5, glucose: 11. The solution was maintained at 37 °C and continuously gassed with a mixture of 95% O_2 and 5% CO_2 .

In order to decrease the Ca concentration in the K-H solution, CaCl_2 was simply omitted (Ca-free solution), and in some of the experiments the Na concentration in the Ca-free solution was decreased by replacing NaCl with equimolar choline chloride (low Na and Ca-free solution). The experimental protocol was as follows. The control K-H solution was perfused for 20 min. Then the perfusate was changed from the K-H solution to the Ca-free solution or low Na and Ca-free solution and the perfusion was continued for 10 min. Ca was then reintroduced by returning to perfusion with the K-H solution.

In preliminary experiments, a 10 min Ca-free period induced the Ca paradox constantly and the low Na and Ca-free solution (25 mM) clearly protected ultrastructural damage induced by the Ca paradox, but 75 & 50 mM Na in Ca-free solution did not protect constantly. We used 10 min for Ca-free period and 25 mM Na for low Na and Ca-free solution.

After repletion of Ca for 5 min, hearts in each experiment were examined by electron microscopy using lanthanum staining according to the method of Hoffstein et al.⁷ The hearts were perfused with 4% glutaraldehyde in 0.1 M cacodylate buffer containing 1.3% $\text{La}(\text{NO}_3)_3$, pH 7.4 for 10 min through the side arm of the aortic cannula. The left ventricular free walls were cut into small cubes in the fixative, and then fixed for 2 h

at room temperature with occasional gentle shaking. After 2 h incubation the tissues were trimmed and cut into smaller pieces. The tissues were washed 3 times within 1 h in a mixture of 1 volume of 10% sucrose in 0.1 M cacodylate buffer, pH 7.4, and 2 volumes of 4% $\text{La}(\text{NO}_3)_3$, then they were rinsed in distilled water and dehydrated in acetone. The tissue samples were embedded in Epon and polymerized at 70 °C for 48 h. The tissue blocks were cut with a diamond knife on a Porter-Blum ultramicrotome and examined with or without staining with uranyl acetate and lead citrate. Ten blocks of left ventricle from each experiment were examined with a JEOL 100B electron microscope.

Five hearts for each experiment were fixed with glutaraldehyde by perfusion with 4% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) for 10 min through the side arm of the aortic cannula. The hearts were cut further into small pieces and kept in fresh fixative for 2 h at 4 °C, postfixed with 1% osmium tetroxide in the same buffer for 1 h, dehydrated in ethanol and embedded in Epon 812. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate, and examined with JOEL 100B electron microscope. Ten blocks of left ventricle from each experiment were examined.

Electrophysiological methods

The effect of Ca depletion and Ca repletion on the action potentials of the left ventricular papillary muscle of guinea pig were examined. Guinea pigs were anesthetized with an ip injection of sodium pentobarbital (40 mg/kg). The heart was quickly removed and dissected in oxygenated K-H solution and the left ventricular papillary muscles were excised. The preparations were mounted in a 2 ml volume tissue chamber perfused with K-H solution, gassed with 95% O_2 , and 5% CO_2 , at a constant flow rate of 2 ml/min, using a micro-tube pump (Atto, SJ-1211). The temperature of the chamber was maintained at 36–37 °C. Rectangular pulsed currents of 2 msec in duration and twice diastolic threshold in intensity were delivered at a cycle length of 1000 msec through bipolar silver wires. The transmembrane potentials were measured by means of microelectrode penetration. All microelec-

Fig.1. Electron micrograph of cardiac cells of an isolated guinea pig heart which had been perfused for 20 min with a Ca containing solution, for 10 min with Ca-free solution, and for 5 min with Ca containing solution. The cells have completely lost their normal organization pattern. Severe contraction bands are observed and mitochondria are distributed at random within the cytoplasm. Mitochondria appear spherical and are often swollen.

Fig.2. Electron micrograph of cells of isolated guinea pig heart which had been perfused for 20 min with Ca containing solution, for 10 min with Ca-free and low Na solution, for 5 min with Ca containing solution. Normal organization has been maintained. Contraction bands and swollen mitochondria are not observed. Only dilated T tubules are observed.

trodes were filled with 3 M KCl and had a resistance of 10–30 M. The microelectrode was coupled to the input of an amplifier (Nihon Kohden, ME28201) with neutralized input capacity. The recorded potentials were

Fig.3. Lanthanum staining. Electron micrograph of cardiac cells of an isolated guinea pig heart which had been perfused for 20 min with Ca containing solution, for 10 min with Ca-free solution, 5 min with Ca containing solution. Lanthanum is observed in intracellular areas especially around the mitochondria (arrows).
La: lanthanum.

Fig.4. Electron micrograph of cells of an isolated guinea pig heart which had been perfused for 20 min with Ca containing solution, for 10 min with Ca-free and low Na solution and for 5 min with Ca containing solution. Lanthanum is not observed in intracellular area.

displayed on an oscilloscope (Nihon Kohden, VC-9) and were recorded on 35 mm film by camera (Nihon Kohden PC-213).

Each preparation was driven for 20 min to ensure equilibration. After obtaining control recordings, the preparations were superfused with Ca-free solution or low Na and Ca-free solution for 10 min and reperfed with Ca

Fig.5. Action potentials at control, Ca depletion with 145 mM Na (upper panel) or with 25 mM Na (lower panel), and Ca repletion period. By reducing Na from 145 mM to 25 mM during Ca depletion period, the action potential returns to near control value without depolarization.

TABLE I EFFECT OF CA REPLETION ON THE ACTION POTENTIAL OF LEFT VENTRICULAR PAPILLARY MUSCLE AFTER 10 MIN OF CA DEPLETION WITH (B) OR WITHOUT NA REDUCTION (A)

		Control	Ca depletion	Ca repletion
OS	A	26±3	25±4	*14±9
	B	27±3	*18±6	26±2
RP	A	82±3	78±3	*62±14
	B	81±4	80±2	81±4
APD ₃₀	A	159±9	*177±23	*79±57
	B	165±22	*132±39	143±29
APD ₉₀	A	225±13	*242±24	*152±46
	B	239±26	*193±41	215±42

A:n=6, B:n=6

*indicates significant difference from control ($p<0.01$)

OS: over shoot, RP: resting potential, APD₃₀: 30% duration of action potential, APD₉₀: 90% duration of action potential

containing K-H solution for 5 min.

RESULTS

Considerable damage to the ultrastructure of the myocardial cells were observed in all of 5 hearts, which had been perfused successively with control K-H solution for 20 min with Ca free solution (Na=145 mM) for 10 min and with control K-H solution for 5 min. The major part of the contractile component was abnormal and broad contraction bands were observed. Mitochondria revealed the presence of aggregates of electron-dense material, which varied in number, size and shape (Fig. 1). These ultrastructural changes were prevented by reducing Na to 25 mM in

the Ca-free solution in all 5 hearts. Dilated T tubules and slightly separated intercalated discs were observed, but contraction bands and electron-dense materials in the mitochondria were not observed in all blocks (Fig. 2).

La was observed in the intracellular space, especially in outer membrane of mitochondria and myofilaments, in all of the 5 hearts which had been perfused successively with K-H solution for 20 min, Ca free solution (Na=145 mM) for 10 min and with K-H solution for 5 min (Fig. 3).

However the La penetration into the myocardial cells was prevented by reducing Na to 25 mM in Ca-free solution. La was observed only in the T-tubules and the ex-

tracellular space in each of the 5 hearts in this group (Fig. 4).

Fig. 5 shows the representative recording of action potential under control conditions and under Ca depletion and Ca repletion. When the solution was changed from the normal K-H solution to Ca-free solution, the action potential duration was prolonged. In addition, the action potential was shortened and was depolarized progressively on returning the Ca concentration to the normal level. Finally, the electrical activity was suppressed if the superfusion with the K-H solution was continued. In contrast to these changes induced by Ca depletion, the electrical activity remained almost intact when the Na concentration was decreased in the Ca-free solution (lower traces). In the Ca-free and low Na solution (25 mM) the action potential was significantly shortened and returned to near control value without showing depolarization when the Ca concentration was changed to 2.5 mM for 5 min. Similar results were observed in each of hearts in this group and summary of the action potential parameters is given in Table I.

DISCUSSION

Our results of electron microscopic findings (with or without La staining) of the hearts which had been perfused successively with K-H solution for 20 min, Ca-free solution for 10 min and with K-H solution for 5 min, confirm the results of previous reports¹⁻³

Yates and Dhalla⁴ reported that reducing the Na concentration of the Ca-free medium prevented the separation of the intercalated discs. In this study, a reduction in the Na concentration from 145 mM to 25 mM during the perfusion with Ca-free solution was able to prevent not only the ultrastructural damage but also the penetration of La into myocytes induced by the Ca paradox.

La has been used as a tracer of the extracellular space at the ultrastructural level? In normal myocytes, La remains confined to the extracellular space, but it has been shown to penetrate into myocytes with sarcolemmal injuries.⁷ Our results suggest that a reduction in Na concentration in Ca-free solution can protect ultrastructural damage and sarcolemmal injuries induced by the Ca

paradox.

The APD of the left ventricular papillary muscle was prolonged with Ca-free solution. This result also confirms a previous report? The prolongation of APD may be due to enhanced Na current through the inward current because the prolonged APD was decreased by Ca-channel blockers, such as diltiazem⁸ or verapamil⁹. Recently, Hess and Tsien¹⁰ reported that slow inward current was carried by Na at $[Ca]_o < 0.1$ mM. It is well recognized that myocardial Na content increases with Ca-free perfusion.¹¹⁻¹³ These data suggest that Na may enter into the myocardial cell through slow inward current during the Ca-free period.

Goshima et al¹¹ reported that Ca uptake by cultured myocardial cells during reperfusion with Ca-containing medium depended upon $[Na]_i$ and suggested that Ca is taken up by myocardial cells by the mechanism of Na-Ca exchange. Recently, Bonvallet et al¹⁴ reported that Ca paradox of frog auricular trabeculae was due to an entry of Ca²⁺ through the Na-Ca exchange mechanism. They held the membrane potential at -60 mV, far from the activation range of the slow inward current, throughout the experiment and found that the Ca paradox phenomenon developed with ouabain in frog auricular trabeculae. However, Nayler et al¹² reported that verapamil and nifedipine reduced the early gain in Ca by rat myocardium during the Ca repletion period by approximately 80% and they suggested that the contribution of the Na-Ca exchange to Ca gain might be small. The difference between the two results may be due to differences in experimental animals and to the presence or absence of ouabain in the medium.

Recently, Ruigrok¹⁵ summarized several studies about the Ca paradox and reported that an increase in $[Na]_i$ during the Ca-free period was not essential for the occurrence of the Ca paradox. However, Chapman and Bhojani^{5,16} have studied the Ca paradox using ion-sensitive microelectrodes and reported that the intensity of the Ca paradox was primarily determined by the $[Na]_i$ during the Ca-free period. They speculated that the principal pathway of Na during Ca-free period is by a slow inward current, and thought that the discrepancy might be due to

the differences between preparations and species.

Perfusion with Ca-free and low Na (25 mM) solution decreased the prolongation of the APD which was observed during perfusion with Ca-free and normal Na (145 mM) solution.

Linden & Brooker⁹ reported that verapamil, a Ca channel blocker, produced a 90% reduction in the duration of the very long action potential of the frog ventricular strip in the Ca-free period. Ca channel blockers may protect against the myocardial damage produced by the Ca paradox due to reducing the entry of Na into the myocardial cells during Ca-free period⁸.

APD is determined by several types of channels, mainly a slow inward current and outward K current. Thus we cannot completely rule out the possibility that the outward K current could affect the decrease of APD, however our results suggest that a reduction in Na concentration (from 145 to 25 mM) in the Ca-free solution reduce the entry of Na into the myocardial cells through the slow inward current during Ca-free period. The reduced $[Na]_i$ pass through this pathway may reduce the subsequent influx of Ca into cytosol via Na-Ca exchange during Ca-repletion.

In conclusion, the present study suggests that a reduction of Na concentration in a Ca-free solution protects myocardium against the damages of the Ca paradox by reducing the entry of Na into myocardial cells through the Ca channel during the Ca-free period.

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