ULTRASTRUCTURAL CHANGES IN THE SARCOPLASMIC RETICULUM IN ACUTE MYOCARDIAL ISCHEMIA

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We investigated the morphologic changes in the sarcoplasmic reticulum (SR) in acute myocardial ischemia, induced by occlusion of the coronary artery in the canine heart, by freeze-fracture of SR in situ and in vitro, and they were compared to the alterations in Ca^{++} -stimulated ATPase activity and protein composition of the isolated SR. Both SR in situ and the isolated SR exhibited typical intramembranous particles with diameters of 70 to 90 Å in freeze-fracture replicas. The intramembranous particle density of the P face in SR in situ was $3,319 \pm 75$ (mean $\pm$ SE) per μm^2 and that in the isolated SR was $2,667 \pm 60$; particles were more numerous on the concave (P) face than on the convex (E) face. In ischemia for 30 to 60 min a significant decrease in the number of particles was found in SR in situ, and the corresponding change was noted in the isolated SR. Simultaneous decreases in Ca^{++} -stimulated ATPase activity and the major ATPase protein of the SR were recognized. The close correlation of the changing pattern between the reduction in Ca^{++} -ATPase and that in intramembranous particle density during ischemia supports the suggestion that a large part of the intramembranous particles represent ATPase protein itself. Decrease in the SR membrane particles strongly suggests the degradation of ATPase protein in the process of ischemic myocardial injury.

THE sarcoplasmic reticulum (SR) is a highly specialized membrane system which regulates calcium ion fluxes during contraction and relaxation. The ATP-dependent calcium pump of the SR membrane establishes a concentration gradient for Ca^{++} across this membrane. Although many biochemical data indicate decreases in Ca^{++} -ATPase activity and Ca^{++} -binding rate of the SR after acute coronary artery occlusion,¹⁻³ their membrane structures appear to be relatively unchanged on ultrathin sections in transmission

electron microscopy, showing, for example, mere swelling.^{4,5} The freeze-fracture technique was introduced in order to characterize the alteration in membrane interiors during ischemic injury in correlation with the chronologic biochemical changes in the SR.

From gel electrophoretic analysis of the SR proteins, the Ca^{++} -pump ATPase protein constitutes 40 to 50% of the proteins of the SR membrane and its molecular weight is about 100,000.^{6,7} This ATPase is an intrinsic component of the SR, presumably located in the lipid phase of the membrane, and has been characterized ultrastructurally by freeze-fracture technique to be 70 to 90 Å particles of the P face of the fractured skeletal⁸ and cardiac⁹ SR membranes. In this article we estimated changes

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in the density of the intramembranous particles (IMP) of 70 to 90 Å in diameter in the freeze-fractured SR membrane and correlated them with alterations in Ca^{++} -stimulated ATPase activity and composition of SR proteins of the isolated SR in experimental ischemic myocardium in the dog.

MATERIALS AND METHODS

Surgical procedure. Twenty-six adult mongrel dogs, weighing 10 to 22 kg, were anesthetized with intravenous injection of sodium pentobarbital (Nembutal®) at 30 mg/kg body weight. A left-sided thoracotomy was performed under positive pressure respiration with room air using a Harvard type respirator. The anterior descending branch of the left coronary artery was isolated and ligated completely with a silk thread just below the first diagonal branch. The occurrence of transmural myocardial ischemia was confirmed by a continuous ST segment elevation of more than 0.5 mV in the leads near V_3 and V_4 . The dogs were sacrificed chronologically from 30 to 180 min after ligation and the heart was removed in the beating state. The subendocardial muscle at the central portion of the postischemic cyanotic region was excised immediately, with the non-ischemic portion of the left ventricle as a control.

Freeze-fracture technique. Myocardial tissue was cut into small blocks in 2% glutaraldehyde containing 7.5% sucrose buffered with 0.1 M cacodylate HCl (pH 7.2). Subsequently the blocks were fixed in the same solution for 2 hours and transferred to 0.1 M cacodylate (pH 7.2) containing 30% glycerol for 2 hours at 4°C. Small cubes of tissue were fitted in the tissue holders and immediately frozen in Freon 22 which was cooled with liquid nitrogen. The frozen tissues were transferred to the cold stage of an Eiko-FD-2A unit and fractured at -115°C, in a vacuum of 2×10^{-7} Torr. Fractured surfaces were immediately shadowed with platinum at an angle of 45°. The platinum replica was stabilized by deposition of pure carbon. The replicas were cleaned with bleach, rinsed in distilled water and mounted on uncoated copper grids.

Pelleted fractions of isolated microsomal vesicles were incubated in 30% glycerol with 0.1 M cacodylate buffer overnight to allow gentle resuspension of the pellet. Small droplets were placed on the tissue holders and frozen as

described above.

Stereological procedure. The replicas were photographed in an electron microscope (Hitachi H-300) at 30,000 fold. The negatives were projected on film for counting (final magnification, $\times 150,000$). A 8 mm test circle was placed randomly on the P and E face of intact SR membrane. The number of 70 to 90 Å particles enclosed by the circle was counted. The 8 mm test circle corresponded to an area of $2.2 \times 10^{-3} \mu\text{m}^2$. For the isolated SR vesicles, the same 8 mm circle was placed on concave and convex profiles free of cast shadow.¹⁰ All the IMP totally enclosed within the circle were counted. For intact myocardium, 100 circles (area: $0.22 \mu\text{m}^2$) were counted on the SR membrane. In the isolated SR 200 vesicles were counted at each stage of ischemia (area: $0.44 \mu\text{m}^2$).

Preparation of the sarcoplasmic reticulum. Isolation of the SR was performed by the modified method of Harigaya and Schwartz,¹¹ as described previously.¹ All the procedures were done at 4°C unless otherwise mentioned. Briefly, approximately 2 Gm of myocardial tissue were cut into small pieces and homogenized in 9 vol of a solution containing 0.01 M NaHCO_3 , 0.005 M NaN_3 and 0.0001 M EGTA with a Polytron homogenizer PT-3J 3 times for 5 sec with intervals of 20 sec. The homogenate was spun at $9000 \times G$ for 10 min twice, and the supernatant was centrifuged at $120,000 \times G$ for 30 min. The final pellet was suspended in 0.05 M KCl and 0.02 M tris (hydroxymethyl) methylaminoethane-sulfonic acid (TES)-tris (pH 7.0), the suspension was ultracentrifuged again, and the precipitate was saved as the SR fraction. Protein concentration was determined by the method of Lowry using 1 mg/ml bovine serum albumin in 0.05 M KCl and 0.02 M TES-tris (pH 7.0) as a standard.

Measurement of ATPase activity. Ca^{++} -stimulated ATPase activity of the isolated SR was examined by the modified method of Harigaya et al.¹² ATPase reaction was performed in a medium containing 0.1 M KCl, 0.005 M MgCl_2 , 0.005 M NaN_3 , 0.005 M ATP and 0.02 M tris-maleate (pH 7.2) in the presence of 2×0.00001 M CaCl_2 or 0.001 M EGTA. Reaction was started with the addition of ATP at 37°C for 5 min and stopped by the addition of 20% TCA. The inorganic phosphate liberated from ATP was determined by the method of Fiske and SubbaRow.¹³

Sodium dodecyl sulfate polyacrylamide gel

Fig.1. Freeze-fracture electron micrograph of a normal myocardial cell. Free sarcoplasmic reticulum (SR) shows a network of tubules and cisternae surrounding myofibrils. SRP = P face of sarcoplasmic reticulum. SRE = E face of sarcoplasmic reticulum. M = mitochondria. T = T-system. Bar indicates 1 μ m.

electrophoresis. SDS gel electrophoretic analysis of SR proteins was performed by the modified method of Weber and Osborn.¹⁴ Electrophoresis was done in cylindrical gels with a diameter of 5 mm and at a constant current of 8 mA per gel for 5 cm. Gels were stained with coomassie brilliant blue. Composition of proteins was calculated from the densitometric curves.

RESULTS

Whole muscle fracture faces. In the normal myocardial cell the SR was recognized relatively

easily in freeze-fracture replicas by its characteristic high density of IMP on the P fracture face. The free SR presented a membranous intracellular network of tubules and cisternae surrounding myofibrils, as shown in Fig. 1. Generally, the junctional and free SR consisted of tubules with widths of between 20 to 60 nm as described by Sommer.¹⁵ Fig. 2A shows the junctional SR wrapped around a T-tubule. The fracture plane sometimes exposed a large area of the SR membrane, flat enough for IMP counting. Fig. 2B shows several of the flat cisternae that are smoothly connected to tubules of the SR.

Fig.2. Freeze-fractured normal myocardial cells. A: P (SRP) and E (SRE) faces of the SR membrane and T-tubular membrane (TTP), the SR membrane has a much higher IMP density on its P face. B: Free SR shows flat cisternae smoothly connected to tubules of SR. A tubule of SR lies near mitochondria (arrow). SRP = P face of sarcoplasmic reticulum. SRE = E face of sarcoplasmic reticulum. M = mitochondria. T = T-system. Bar indicated 1 μm .

Fig.3. Ischemic myocardial cell at 30 min after coronary ligation. A: SR is slightly swollen and a reduction in the number of particles is noted on P face of SR (arrows). B,C: P face of SR. M = mitochondria. Bar indicates 1 μm .

In situ, the density of IMP on the P face was counted as $3,319/\mu\text{m}^2$ on the average.

At 30 min after coronary ligation, the SR was swollen and a slight swelling of mitochondria was

observed. The densities of IMP on the SR membrane decreased slightly, as shown in Fig. 3. Sixty to 180 min after coronary ligation, marked intracellular edema was observed and swelling of

Fig.4. A myocardial cell 60 min after coronary ligation. Sarcoplasmic reticulum appears vesiculated, and there is a decrease in particle density on P face of sarcoplasmic reticulum (SRP). SRE = E face of sarcoplasmic reticulum. M = mitochondria. T = T-system. Bar indicates 0.5 μ m.

Fig.5. A myocardial cell 120 min after coronary ligation. Severe intracellular edema is observed and sarcoplasmic reticulum is swollen and vesiculated. Particle density on P face of sarcoplasmic reticulum (SRP) is significantly reduced. M = mitochondria. T = T-system. Bar indicates 0.5 μ m.

Fig.6. A: Electron micrograph of freeze-fracture replica of isolated SR fractions from non-ischemic heart muscles showing concave (CC) and convex (CV) vesicle profiles with and without (arrow) cast shadow. Bar indicates $0.5\ \mu\text{m}$. $\times 61,500$.
B,C: Concave profiles without cast shadow reveal intramembranous particles whose density is counted by applying a test circle. $\times 150,000$.

Fig.7. Freeze-fracture replicas of isolated SR fractions from non-ischemic (A) and 60 min ischemic (B) heart muscles. A: Isolated SR from non-ischemic myocardium shows a greater particle density on the outer fracture (concave) face than on the inner fracture (convex) face. Bar indicates $0.2\ \mu\text{m}$. B: Number of particles decreases on the concave face of 60-min-ischemic microsomes. Bar indicates $0.2\ \mu\text{m}$.

TABLE I PARTICLE DENSITY OF FREEZE-FRACTURED SR MEMBRANE

	<i>P-face</i>	<i>E-face</i>
<i>SR in situ</i>		
Non-ischemic	3,319 ± 75 (100)	591 ± 55 (100)
	<i>Concave face</i>	<i>Convex face</i>
<i>Isolated SR</i>		
Non-ischemic	2,667 ± 60 (200)	570 ± 57 (200)
Ischemic		
30 min	1,989 ± 83 (200)	528 ± 55 (200)
60 min	1,550 ± 66 (200)	389 ± 40 (200)
120 min	1,506 ± 65 (200)	380 ± 48 (200)
180 min	1,269 ± 65 (200)	344 ± 45 (200)

Values are numbers of particles/ μm^2 , expressed as mean ± standard error.
Numbers in parentheses are the number of vesicles counted.

TABLE II Ca^{++} -STIMULATED ATPase ACTIVITY AND COMPOSITION OF ATPase PROTEIN

	<i>Ca⁺⁺-stimulated ATPase activity</i>	<i>Composition of major ATPase protein</i>
Non-ischemic	11.2 ± 3.3	38.7 ± 8.0
Ischemic		
30 min	5.7 ± 1.2*	22.4 ± 4.9**
60 min	6.8 ± 1.1*	19.7 ± 7.0**
120 min	3.9 ± 2.2*	13.4 ± 3.3**
180 min	4.1 ± 0.7**	13.5 ± 5.9**

ATPase activity is expressed as μ moles Pi/mg SR protein/hr at 37°C, and composition is shown as per cent. Values are mean ± standard deviation.
 $p < 0.01$; ** $p < 0.005$

the SR and T-tubules became more severe and was accompanied by mitochondrial swelling. In the swollen SR the number of IMP decreased significantly, and this decrease was moderately heterogeneous in each ischemic cell, indicating the heterogeneous ischemic changes as described previously²¹

Intramembranous particles (IMP) in the isolated SR membrane. Freeze-fracture electron micrographs of the isolated SR membrane exhibited the typical IMP of 70 to 90 Å in diameter in the same manner as seen in the SR membrane in situ, and a greater density of IMP was seen on outer fracture (concave) face than on inner fracture (convex) face of the isolated SR vesicles (Fig. 6, 7). The density of IMP of the isolated SR vesicles from the normal myocardium was 2,667/ μm^2 and was somewhat lower than that of the SR in situ. This difference was not

surprising because of (1) alterations of inside-outside membrane relationship, (2) contamination of sarcolemmal fraction, and (3) extraction of membrane-associated proteins in the isolation procedure. The particle densities in the isolated SR are summarized in Table I. The isolated SR vesicles revealed an approximately 5-fold greater concentration of IMP at the hydrophobic center of the outer (concave) versus inner (convex) fracture face. In the ischemic myocardium the average densities of IMP on concave face decreased to 1989, 1550, 1507 and 1269/ μm^2 at 30, 60, 120 and 180 min after coronary ligation, respectively. Since the protein of the SR membrane consists mainly (>38%) of one type of protein, the density of IMP should be directly proportional to the concentration of calcium pump protein of the membrane.

Biochemical studies of the sarcoplasmic

Fig.8. Electrophoresis in 10% SDS-polyacrylamide gels of isolated SR fractions from non-ischemic and ischemic myocardia. Content of the major ATPase protein band decreases in the course of ischemia.

reticulum. Ca^{++} -stimulated ATPase activity of the isolated SR was $11.2 \pm 3.3 \mu$ moles Pi/mg protein/hour at 37°C in the non-ischemic myocardium and was not significantly different from that of the normal myocardium. In the ischemic myocardium the activity decreased significantly to $5.7 \pm 1.2 \mu$ moles Pi/mg protein/hour, 50.9% of that of the non-ischemic myocardium, at 30 min after coronary ligation. After that slight further reduction was noted up to 180 min after ligation, as shown in Table II.

Electrophoretic gels of the isolated SR are shown in Fig. 8. SR proteins were composed of a major ATPase protein (100,000 dalton), several acidic proteins (50,000–70,000), and proteolipids (22,000) in the same manner as previously reported.¹ The major ATPase protein occupied about 40% of the total SR proteins in densitometry. In the ischemic myocardium a significant decrease in the band of the major ATPase was recognized, 22.4, 19.7, 13.4 and 13.5% in ischemia for 30, 60–180 min, respectively, as shown in Table II. The reduction in Ca^{++} -stimulated ATPase activity was roughly parallel with the decrease in the content of the major ATPase protein.

DISCUSSION

The SR is a highly specialized membrane which is composed mainly of Ca^{++} -pump protein.^{6,7} The presence of particles on freeze-fracture face of the SR and their possible relationship to the Ca^{++} -pump protein was first

pointed out by Deamer and Baskin.⁸ The particles observed on freeze-fracture seem to be referable to the Ca^{++} -pump protein or a complex of the Ca^{++} -pump protein with phospholipid.¹⁶ The SR membranes have a very asymmetric distribution of particles between the P and E faces. Rayns et al.⁹, in a study on guinea pig papillary muscles, found a three-to-one density difference between the P and E faces of the SR. In the present study approximately six-to-one asymmetry of particles was observed in the P to E faces. Also, a similar particle asymmetry was found in the isolated SR vesicles. This means that most of the isolated SR vesicles are right side-ont. The particle densities of the isolated SR vesicles are lower than those of the SR in situ. It is conceivable that contamination of T-system and sarcolemmal vesicles would include concave and convex faces, with a few particles corresponding to the low-density faces of these membranes.

The area of SR membrane being measured by any planimetric method will be underestimated due to the curvature of these structures. The amount of underestimation can be approximated for each area from the geometry of the organelle (vesicle, tube, flat sac, etc). And, as has been described previously,¹⁷ the number of particles in any given area of membrane fracture face also is underestimated since particles which are small in diameter and lie within the fractured membrane area tend to be lost in the background and not counted. Fortunately, in the SR membrane most of the IMP were relatively large, i.e. 70 to 90 Å in diameter, and easy to count. Therefore, there was very little particle underestimation in this study. In order to correct the underestimation of area we used Weibel's stereological methods.¹⁰ Particle densities on the P face of the normal SR were similar to those reported previously, i.e. $1030/\mu\text{m}^2$ in guinea pigs,⁹ $4938/\mu\text{m}^2$ in dogs¹⁸ and $1609/\mu\text{m}^2$ in dogs.³

In the study of conventional thin-section electron microscopy the progressive dilatation of the SR was easily recognized after 30 min of coronary ligation.⁴ Bryant and co-workers⁵ noticed that after 1 hour of ischemia enlargement of the SR was prominent, and at 4 hours the SR was remarkably swollen and some vesicles ruptured. Jennings and his co-workers^{19,20} demonstrated in the ischemic dog myocardium that the increased Ca accumulated at the intra-mitochondrial dense deposits as a form of calcium phosphate, and postulated that the

appearance of the deposits was the earliest sign of myocardial irreversibility due to ischemia. In ultrastructural histochemical studies Ozawa et al.²¹ reported a reduction in the ATPase reaction product in the SR 60 min after coronary ligation, with simultaneous swelling of the terminal cisternae of the SR and the appearance of mitochondrial dense deposits. In the ischemic myocardium, changes in the SR membrane have not been studied rigorously by freeze-fracture techniques. Imai et al.³ found that the number of IMP of the SR was reduced by up to 50% in ischemia for 60 min, and postulated that the reduced number of IMP in the ischemic zone might be an indication of their inability to transport Ca^{++} or to remove Ca^{++} from the cytoplasm.

The decrease in particle density of the SR membrane under ischemia is also conceivable. However, in the present study the SR was isolated in the same condition in both the non-ischemic and ischemic heart muscles; therefore, swelling of the SR in situ in the ischemic state should be negligible on particle density of the isolated SR vesicles.

In biochemical studies Schwartz et al.² found a defect in SR calcium release after 12–60 min of ischemia. A reduction in Ca^{++} -uptake of cardiac SR was recognized by Lee et al.²² and Hess et al.²³ within 60 min and 30 min of ischemia, respectively. Toba et al.¹ also observed reductions in Ca^{++} -stimulated ATPase activity and Ca^{++} -uptake rate in parallel with a decrease in the content of ATPase protein, and concluded that the impairment of SR activities in myocardial ischemia contributed to the degradation of ATPase protein itself. In agreement with these morphologic and biochemical studies, a decrease in the density of particles with progressive swelling of the SR and corresponding changes in the biochemical data were noted after 30–60 min of ischemia, and these indicate the degradation of ATPase protein.

Hoffstein et al.²⁴ reported that acid hydrolases were located not only in lysosomes but also in the SR with cytochemical method and attributed the latter to the degradation of the ischemic myocardium. Recently, Sasai et al.²⁵ observed the activation of hydrolases such as acid phosphatase, β -glucuronidase and cathepsin D of the SR in the early stage of myocardial ischemia. Increases in the size, number and enzymatic activity of acid phosphatase in lysosomes and SR were also observed within 15 min of coronary

ligation.²⁶ The activation of acid hydrolases in the SR should be the precipitating factor for the degradation of the SR in the acidic state in the ischemic myocardium. In this study, impaired cardiac SR was observed in ischemia for 30 to 60 min as the degradation of its intramembranous particles, which should be the ATPase protein itself; these changes of the SR are an indication of irreversible myocardial injury.

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