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ALTERATIONS OF VENTRICULAR WALL MOTION AND MYOCARDIAL MEMBRANE POTENTIAL OF REGIONAL ISCHEMIC MYOCARDIUM IN THE PIG

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Chun Fu Peng, Ph. D., Marvin L. Murphy, M. D. and Karl D. Straub, M. D., Ph. D. (1985) Alterations of ventricular wall motion and myocardial membrane potential of regional ischemic myocardium in the pig. *Bull. Inst. Zool., Academia Sinica* **24**(1): 95-110. An experimental model of coronary artery occlusion was used in farm pigs to produce ischemic myocardium. Left ventricular wall motion during occlusion and reperfusion was determined by echocardiography. The membrane potential of sarcolemmal vesicles was evaluated by fluorescent sensitive probe. Ischemic insult of myocardium for 30 minutes but not for 15 minutes resulted in an irreversible alteration of systolic thickening and diastolic thinning, a decrease of the membrane potential of sarcolemmal vesicles, and an increase of calcium content in the ischemic myocardium. These results suggest that an ischemic damage of the sarcolemmal membrane allows accumulation of calcium ion in the ischemic area upon reperfusion and thus inhibits diastolic relaxation.

The membrane potential in intact cell preparations such as red blood cells (Hoffman and Laris, 1974; Sims *et al.*, 1974), squid giant axon (Cohen, 1973; Davila *et al.*, 1973; Dipolo *et al.*, 1976; Goldring and Blaustein, 1975; Tasaki *et al.*, 1972), leech segmental gangalion Salzberg *et., al.*, 1973) has been measured with the use of fluorescent potential-sensitive probes. In general, changes in the fluorescent intensity of cyanine dyes are a function of changes in the membrane potential. Based on this property, these dyes have been widely used as optical probes for measuring the membrane potential in isolated mitochondria (Laris *et al.*, 1975; Tupper and Tedeschi, 1974), sarcoplasmic reticulum (Beeler, 1980; Beeler *et al.*, 1979; Russel *et al.*, 1979; Yamamoto and Kasai, 1980), sarcolemmal vesicles (Bartschat *et al.*, 1980), as well as intact cell preparations (Hoffman and Laris, 1974; Sims *et al.*, 1974).

Calcium movements in both directions (influx and efflux) across the cell membrane are generally believed to play a crucial rôle in excitation-contraction coupling in the cardiac muscle. Calcium is thought to enter the cell upon membrane depolarization and leave the cell, during relaxation (Endo, 1977; Herzing, 1983). Mechanisms such as the Na^+-Ca^{2+} exchange process (Reeves and Sutko, 1979, 1983) and Ca^{2+}-dependent ATPase pump

(Caroni and Carofoli, 1981; Tuana and Dhalla, 1982) regulate Ca^{2+} transport, but the integrity of membrane potential of the myocardial cells is important in controlling Ca^{2+} movements (Bartschat *et al.*, 1980). Depression of myocardial contractility during ischemia has been studied (Heyndrickx *et al.*, 1975; Therox *et al.*, 1974) and loss of contraction-relaxation coupling and Ca^{2+} deposition has been observed in ischemic myocardium that has been reperfused (Bulkley and Hutchins, 1977; Murphy *et al.*, 1982; Shen and Jennings, 1972). This suggests that ischemic myocardial cells may have leaky membranes and are thus unable to extrude Ca^{2+} out of the cell. In this study, the relation between the membrane potential and "leakiness" of the membrane in the isolated sarcolemmal vesicles from non-ischemic and ischemic myocardium is investigated using fluorescent sensitive cyanine dyes.

MATERIALS AND METHODS

Experimental Protocol

The pig was chosen as the experimental model because its major coronary distribution, collateral circulation, and blood supply of the the conduction system are similar to those of man ((Kong *et al.*, 1969; Lumb and Singeltary, 1962). Sixteen mature farm pigs weighing 30 to 50 Kg were anesthetized with 6% phenobarbital sodium administered intravenously after an 18 hour fast. Tracheostomy was performed and respiration maintained with a Harvard pump respirator, using room air supplemented oxygen to maintain an arterial PaO_2 greater than 100mm Hg and a pH of 7.4. A midline sternal splitting thoractomy was made and the heart suspended in a pericardial cradle exposing the distribution of the left anterior descending coronary artery. Aortic pressure, left ventricular pressure, and lead II of the electrocardiogram were continuously monitored.

The left anterior descending coronary artery was reversibly occluded just distal to the 1st or 2nd diagonal branch using a nontraumatic rubber ligature which produced an area of ischemia which constituted approximately 25 to 30% of the total left ventricular mass. In 8 animals the ligature was applied for 15 minutes and then released with reperfusion of the ischemic zone carried out for an additional 120 minutes. A second group of eight animals had coronary occlusion for 30 minutes followed by reperfusion for 120 minutes. The coronary vein was preserved in each case to allow venous washout during reperfusion.

Ventricular arrhythmia during the experiment was treated with minimal effective doses of lidocaine or, in the case of ventricular fibrillation, with electrical countershock. At the end of reperfusion, 2.5ml of 10% alphazurine 2-G blue dye was injected in a systemic vein to identify areas of the myocardium not well perfused. This dye stains perfused and presumably viable tissue a vivid blue but does not stain ischemic or nonperfused tissue (Randoloph *et al.*, 1964) and has no effect on tissue Ca^{2+} determination or fluorescence measurement in sarcolemmal vesicles. Immediately after injection of the dye, the heart was removed and taken to a cold room where samples were excised from uninvolved and reperfused areas of the left ventricle using surface anatomy and dye staining as guidelines.

Echocardiographic methods

The left ventricular wall thickness was measured at baseline before occlusion and serially during occlusion and reperfusion by placing a 5.0 MHz nonfocused ultrasound transducer directly over the involved left ventricle. The transducer was connected to an Echoline 20A ultrasonoscope (Smith Kline and French) with data being recorded on a strip chart recorder (Honeywell). To avoid pressure artifact and distortion of the heart wall, the transducer was hand-held very lightly in contact with the epicardial surface over the center of the involved area of left ventricle, using the

coronary artery surface anatomy as a landmark. The ischemic area was easily identified by the dark discoloration and verified by S-T segment elevation on the epicardial electrode mapping over the involved area. This method resulted in reproducible echocardiographic data by assuring perpendicular orientation of the transducer with respect to the epicardial surface. Wall thickness was measured in millimeters at maximal systolic thickening and at end-diastole. Each measurement represented the mean of 5 consecutive cardiac cycles. The transducer itself caused a 3 mm layer visible as a dark stripe at the top of the echocardiograph. This thickness was subtracted from the total thickness for purposes of calculation.

Biochemical Procedures

Isolation of the sarcolemmal vesicles: After removing connective tissues and fats, portions of the nonischemic and the ischemic-reperfused myocarium were minced and washed with a medium which contained 10 mM $NaHCO_3$ and 5 mM NaN_3, pH 7.0 (medium A). The sarcolemmal vesicles were isolated with the method as previously described by Van Alstyne *et al.*, (Van Alstyne *et al.*, 1980) with slight modification. Briefly, the mince was suspended in 5 volumes of medium A and homogenized with a Polytron PT-10 homogenizer with speed setting at 6 for 3 times. The homogenate was centrifuged at 10,000×g for 20 minutes to yield supernatant 1 (which was discarded), and pellet 1. The latter was suspended again with 5 volumes of medium A and homogenized in a glass vessel with three passes of motor-driven teflon pestle, and the resulting homogenate was centrifuged at 10,000×g for 20 minutes. This yielded supernatant 2 (which was discarded), and pellet 2 which was suspended in 6 volumes of 10 mM Tris-HCl, pH 7.4 (medium B) and was subjected to four passes of a motor-driven teflon pestle as mentioned above. The resulting homogenate was centrifuged at 10,000×g for 20 minutes. This yielded pellet 3 (which was discarded), and supernatant 3. The supernatant 3 was then centrifuged at 35,000×g for 30 minutes to obtain supernatant 4 (which was discarded), and pellet 4. This pellet was suspended in 10 ml of medium B which was then layered over 15 ml of a 24% (w/v) sucrose solution containing 10 mM Tris-HCl, pH 7.4, in a type 30 Rotor tube and centrifuged at 75,000×g for 30 minutes. This yielded pellet 5 (which was discarded), and a layer of sarcolemmal vesicles between the top medium B phase and the bottom sucrose phase. This layer was carefully harvested with a Pasteur pipet and diluted with medium B to 25 ml and centrifuged at 75,000×g for 20 minutes. This yielded a final pellet which was suspended in medium B to make a final protein concentration of 1-2 mg/ml. The protein concentration was determined by the method of Lowry *et al.*, (Lowry *et al.*, 1951).

Determination of the purity of sarcolemmal preparations: The purity of sarcolemmal vesicle preparations was determined by both enzymatic assays and ultrastructural preparation. These vesicles were characterized by determination of the ouabain sensitive Na^+-K^+ ATPase, cytochrome a_3 content, oligomycin sensitive Ca^{+2}-activated ATPase and Ca^{2+} uptake in the presence of oxalate. The Na^+-K^+ ATPase is the marker enzyme of the sarcolemmal membrane (Van Alstyne *et al.*, 1980) and was determined as previously described (Straub and Carver, 1975). The cytochrome a_3 (Chance and Williams, 1955) and oligomycin sensitive Ca^{2+} activated ATPase (Peng *et al.*, 1982) are enzymes associated with mitochondrial fragment. The former was measured by the difference of changes of optical density at 445 and 455 nm under anaerobic conditions (Chance and Williams, 1955) and the latter was determined by the extent of ATP hydrolysis in the presence and absence of both oligomycin and Ca^{2+} (Peng *et al.*, 1982). Ca^{2+} uptake in the presence of oxalate is associated with sarcoplasmic reticulum vesicles and was determined as previously described (Harigaya and Schwartz, 1969). Evidence for absence of activity for the last three enzymes is an indication of the absence of contamination

from mitochondrial fragments and sarcoplasmic reticulum vesicles. The morphology of sarcolemmal vesicle preparations was determined by electron microscope. One tenth millimeter of preparations were centrifuged at 100,000×g for 30 minutes. The pellets were fixed for one hour in 2% glutaraldehyde and 0.1 M sodium cacodylate buffer, pH 7.2 and followed by postfixing in 2% $0s0_4$ and 0.1 M cacodylate buffer one hour. The samples were dehydrated with graded ethanol and embedded in Epon 812. Ultra-thin sections of each preparation were stained with uranyl acetate followed by lead citrate. The sections were examined with a Hitachi HS-8 electron microscope.

Measurement of membrane potential with fluorescence sensitive probe: For absorption and fluorescence measurements, a spectrophotometer equipped with a locally made spectrofluorometer accessory was used. A cyanine dye, 3. 3'-Diethylthiadicarbocyanine iodide (diS-C_2-(5)), was used in this study. The excitation and emission wavelengths for fluorescence measurements with the dye were 622 nm and 670 nm, respectively. The incubation medium for membrane potential measurements is indicated in the legend of the figures.

Myocardial ATP and Ca^{2+} determination: Myocardial adenosine triphosphate content was determined on duplicate tissue samples obtained by plunge biopsy using a renal biopsy needle (Travenol) just before sacrificing the animal. The needle was cooled in liquid nitrogen, plunged into the heart to obtain tissue samples, and immediately returned to a vial containing liquid nitrogen. Tissue samples for ATP

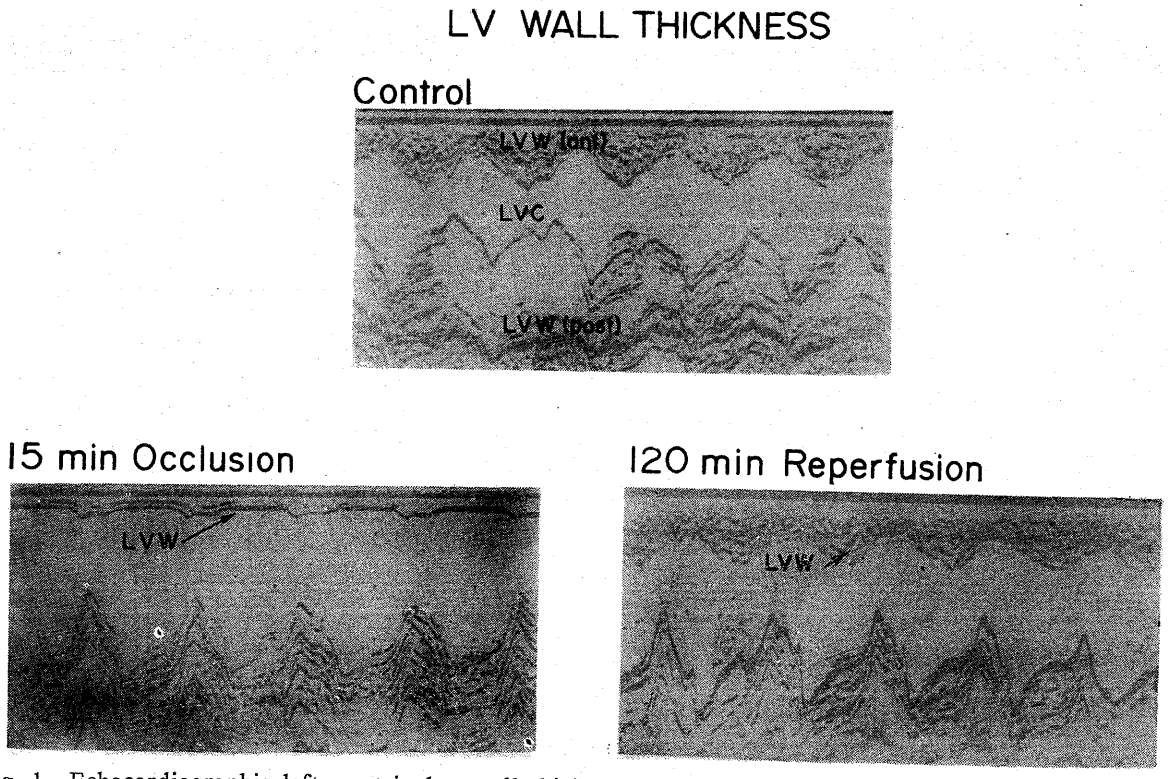

Fig. 1. Echocardiographic left ventricular wall thickness. Top panel, control echocarsdiogram recorded before coronary occlusion. The anterior (ant) left ventricular wall (LVW) thickens during systole followed by diastolic thinning. Lower left, recording performed over the same area of the left ventricle after 15 minutes of coronary occlusion. There is a loss of systolic thickening and thinning of the anterior wall. Lower right, after 120 minutes of reperfusion, nearnormal left ventricle thickening and thinning reappears. LVC=left ventricular chamber; LVW=left ventricular wall; post=posterior.

determination were thus removed and frozen within 3 to 5 seconds. These samples were extracted in boiling water and ATP content was determined by firefly luciferase reaction (Strehler, 1963).

Tissue samples for calcium content determination were extracted by a glacial acetic acid-trichloroacetic acid mixture (Sparrow and Johnston, 1964), and total calcium content was determined with an atomic absorption spectrophotometer with calcium carbonate as the standard (Willis, 1963). Data are presented as mean ± standard error and analyzed using Student's paired test.

RESULTS

Left ventricular wall motion and thickness

Immediately after coronary occlusion and ischemia the involved segment of left ventricle in both groups of animals demonstrated cyanosis, flattened veins and arteries, and paradoxic systolic bulding, which persisted throughout the entire occlusion period. Upon reperfusion, the coronary arteries and veins of the involved area became distended and coronary flow was restored.

Figs. 1 and 2 show a typical echocardiographic measurement of the left ventricular

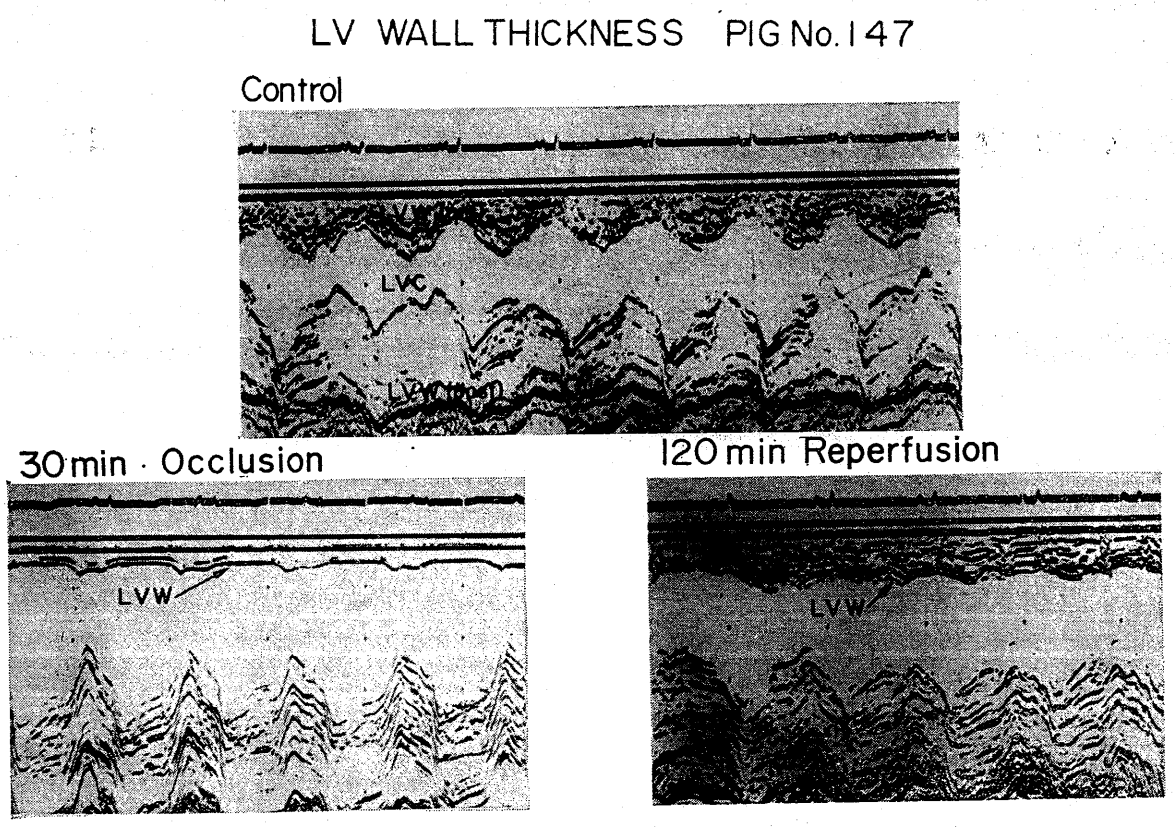

Fig. 2. The panel arrangements are identical to those in Fig. 1, but the occlusion time is 30 minutes. Note the marked diastolic thickening of the anterior left ventricular wall (LVW) with minimal systolic thickening after reperfusion.

TABLE 1
Systolic and diastolic thickness of left ventricular wall during ischemic and reperfusion periods

	Control (before occlusion)	15 minute occlusion	30 minute occlusion	2 hour reperfusion
GROUP 1 (N=8)				
Systole (mm)	13±1	4.0±1.0	—	11.0±2.0
Diastole (mm)	5±1	4.5±0.4	—	4.6±0.5 $p<0.001$
GROUP 2 (N=8)				
Systole (mm)	13±1	4.0±1.0	3.5±1.2	10.0±2.6
Diastole (mm)	5±1	4.2±1.0	3.8±1.4	9.5±1.8

Data expressed as mean±standard error of the mean.

wall motion and thickness. During the control period in each illustration (top panel), the anterior myocardial thickening illustrated normal systolic and diastolic wall motion. During occlusion of 15 and 30 minutes (bottom left panel), there was a loss of contraction (systolic thickening). After reperfusion (bottom right panel) diastolic thinning continued to occur, and systolic thickening reappearing, comparing favorably with the control in the 15 minutes ischemic group (Fig. 1). However, after reperfusion in the 30 minute ischemic group, the anterior wall was thickened and there was minimal systolic contraction. The diastolic thinning was lost (Fig. 2, bottom right panel). Table 1 summarizes the alterations of systolic and diastolic wall thickening of the ischemic left ventricles during the occlusion and reperfusion period for all animals in both groups. The most striking difference between the two groups is that diastolic thickening occurred in the 30 minute but not the 15 minute ischemic group after reperfusion, suggesting that effective systolic contraction and diastolic relaxation are lost in the former group.

Biochemical data

Table 2 shows myocardial Ca^{2+} and ATP content in both groups. The nonischemic myocardium contained 1.2 μmoles Ca^{2+}/g wet weight, the myocardium which had undergone 15 minutes of ischemia and 2 hours of reperfusion contained 1.3 μmoles Ca^{2+}/g wet weight while the myocardium which had undergone 30 minutes of ischemia and 2 hours of reperfusion contained 5.9 μmoles Ca^{2+}/g

TABLE 2
Myocardial Ca^{2+} and ATP content after 15 and 30 minutes of ischemia and 120 minutes of Reperfusion

Group	Myocardial Ca^{2+} (μmol/g wet wt)	ATP (n moles/mg protein)
15 Minute Ischemia (N=8)		
Nonischemic LV	1.2±0.2	6.8±3.6
Reperfused LV	1.3±0.2 $p<0.001$	4.8±1.4 $p<0.01$
30 Minute Ischemia (N=8)		
Nonischemic LV	1.2±0.3	6.6±3.9
Reperfused LV	5.9±1.9 $p<0.001$	3.2±1.8 $p<0.001$

Data expressed as mean±standard error of the mean.

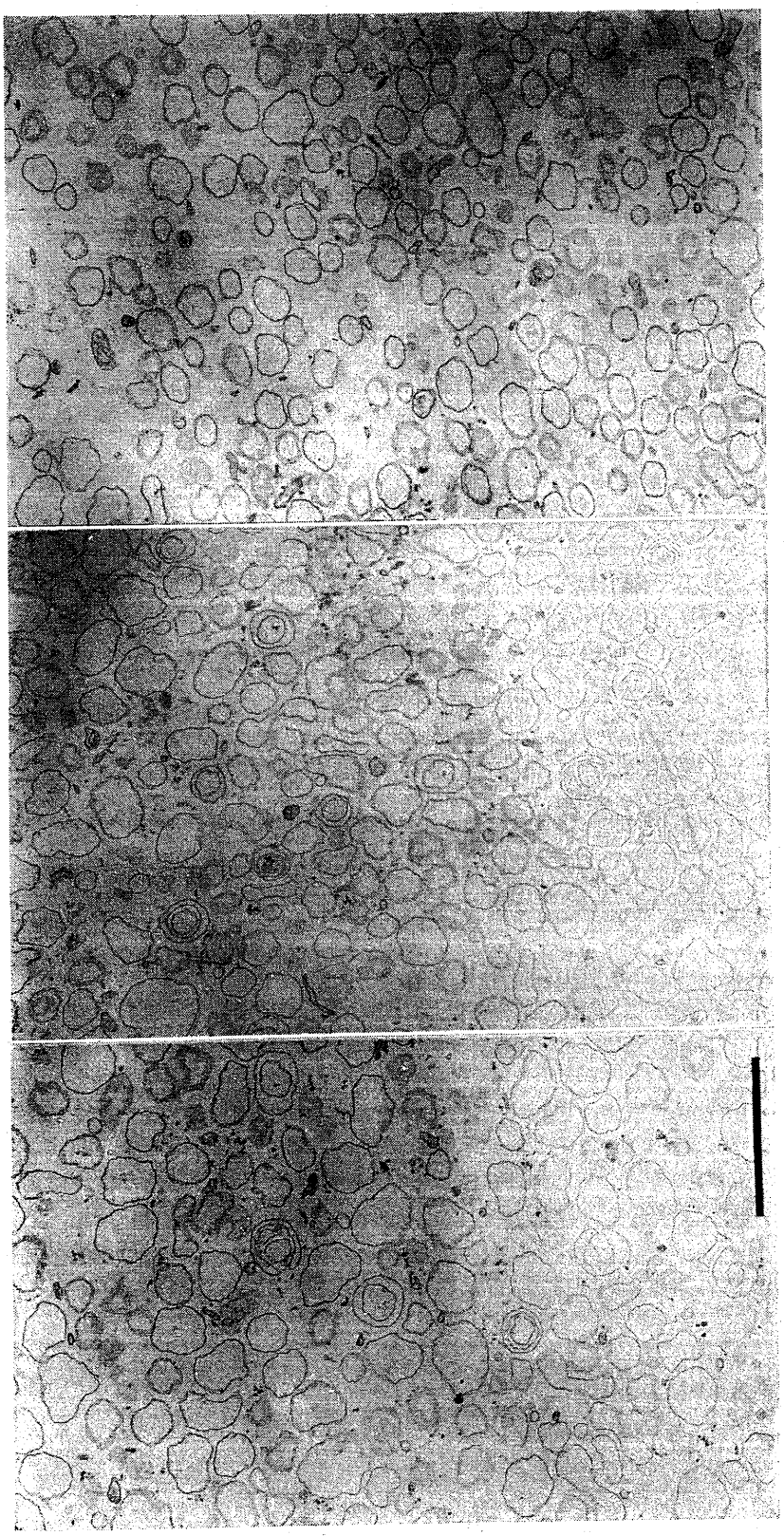

Fig. 3. Morphology of isolated sarcolemmal vesicles from pig left ventricle. Magnification 36,500×. Bar=1 μm. Sarcolemmal vesicles isolated from the nonischemic myocardium (3A), the 15 minute ischemic myocardium (3B), and the 30 minute ischemic myocardium (3C).

TABLE 3
Enzymatic activities associated with the isolated sarcolemmal vesicles from normal and ischemic myocardium

Enzymatic Assays	Normal (n=16)	15 Minute Ischemia (n=8)	30 Minute Ischemia (n=8)
(1) Ouabain sensitive Na^+—K^+ ATPase (μmol/mg/hr)[a]			
—Deoxycholate	26± 5	21± 6	23± 4
+Deoxycholate	64±10	60±10	59±15
(2) Cytochrome a_3 (pmol/mg)[b]	18± 5 (4.8%)	20± 7 (5.4%)	19± 4 (5.1%)
(3) Oligomycin sensitive Ca^{2+}—activated ATPase (nmol Pi release/mg/min)[c]	0	0	0
(4) ATP supported Ca^{2+} uptake (n mol Ca^{2+}/mg/min)[d]	15± 2 (5.4%)	12± 3 (4.3%)	8± 4 (2.9%)

Data expressed as mean±standard error of the mean.

a: Ouabain sensitive Na^+—K^+ ATPase was determined in the presence and absence of ouabain according to the method in the reference 44.

b: Pig heart mitochondria contained 369±21 pmoles cytochrome a_3/mg protein which was measured according to the method in the reference 8.

c: Oligomycin sensitive Ca^{2+}—activated ATPase was determined in the presence and absence of both oligomycin and Ca^{2+} as previously described in the reference 33.

d: Pig heart sarcoplasmic reticulum vesicles accumulated Ca^{2+} with a rate of 280±36 nmoles Ca^{2+}/mg/min which was measured according to the method described in the reference 18.

wet weight. The latter group was significantly different from the former two groups. Furthermore, ATP content in the myocardium which had undergone 15 minutes of ischemia and 2 hours of reperfusion was only slightly decreased whereas a significant decrease was observed in the myocardium of 30 minutes of ischemia and 2 hours reperfusion when compared to ATP content in the nonischemic myocardium.

Morphology and enzymatic markers of the isolated sarcolemmal vesicles

Fig. 3 shows the morphology of sarcolemmal membrane vesicles isolated from the nonischemic and ischemic left ventricles. The membrane fragments demonstrated a vesicular configuration with diameters ranging from 0.15 to about 0.40 μm. There was no marked difference of vesicular configuration and size between 15 minute and 30 minute ischemic groups. There was almost an absence of mitochondrial fragments and sarcoplasmic reticulum vesicles in these sarcolemmal vesicle preparations, as shown in Fig. 3.

Table 3 shows the results of the enzyme marker studies. These vesicles showed high Na^+-K^+-ATPase activity. This ouabain sensitive Na^+-K^+-ATPase increased three fold by the addition of deoxycholate in the incubation medium. Similarily, the same magnitude of stimulation of this enzyme was observed when the vesicle preparations were subjected to 15 consecutive freeze-thaw cycles. Together, these results suggest that two thirds of vesicles were in the right-side out configuration. Table 3 also demonstrates that there was no marked difference in Na^+-K^+ATPase activity between the nonischemic and ischemic groups suggesting this enzyme was not damaged in a brief period of coronary arterial occlusion. Sarcolemmal vesicles isolated in this study contained small amounts of cytochrome a_3 and accounted for approximately 4.8 to 5.4% of contamination in our preparations. Oligomycin sensitive

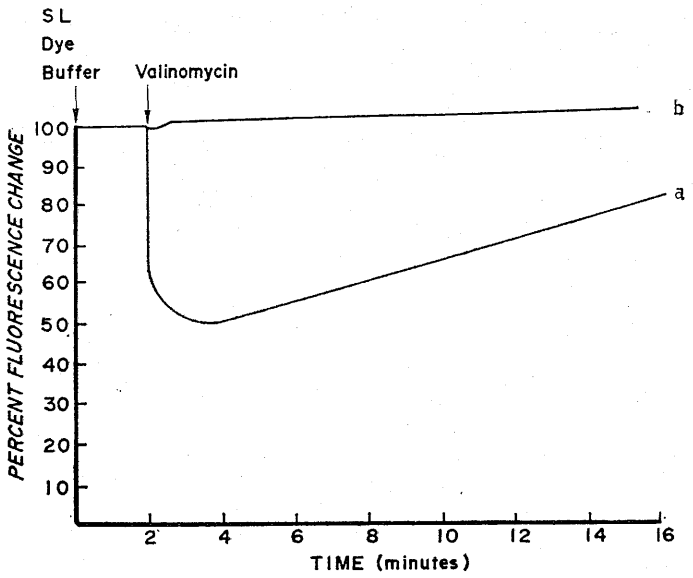

Fig. 4. Effect of types of salt-loaded vesicles on membrane potential development. (a) 150 mM KCl-loaded sarcolemmal vesicles (30 μg/10 μl) were diluted to 3 ml of medium containing 150 mM NaCl (or LiCl), 10 mM Tris-HCl, pH 7.0 and 5μM diS-C_2(5), (b) 150 mM NaCl-loaded vesicles (30 μg/10 μl) were diluted to 3 ml of medium containing 150 mM KCl (or LiCl), 10 mM Tris-HCl, pH 7.0 and 5 μM diS-C_2(5). Valinomycin (2 μM) was added to the reaction system to initiate the development of membrane potential.

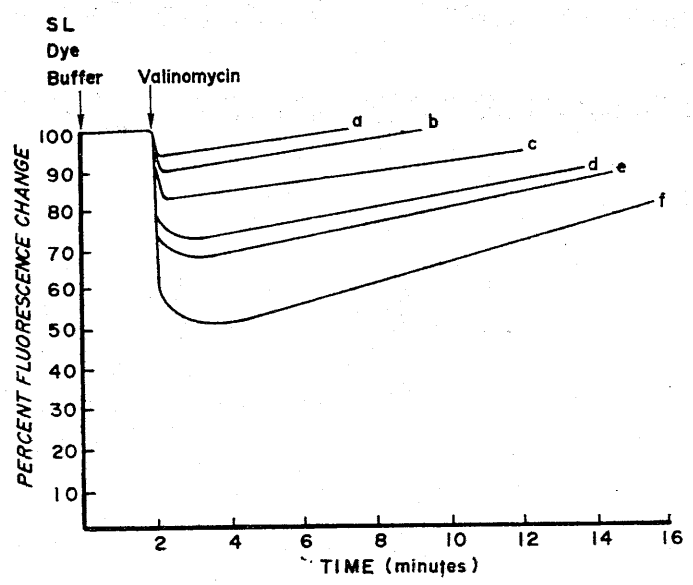

Fig. 5. A correlation between membrane potential and fluorescence alterations: Incubation medium contained 10 mM Tris-HCl, pH 7.0 and 100 mM NaCl plus 50 mM KCl (a); 125 mM NaCl plus 25 mM KCl (b); 140 mM NaCl plus 10 mM KCl (c); 145 mM NaCl plus 5 mM KCl (d); 149 mM NaCl plus 1mM KCl (e); and 150 mM NaCl (f). diS-C_2(5) (5 μM) and sarcolemmal vesicles (30 μg/10 μl) were added to 3 ml of incubation medium as indicated. Valinomycin (2μM) was added one minute after sarcolemmal vesicles were added

Ca^{2+}-activated ATPase demonstrated that there was little contamination from mitochondrial fragments. The ability of the sarcolemmal vesicle preparations to take up Ca^{2+} in the presence of oxalate was also determinated. As noted in Table 3, a very small amount of Ca^{2+} was taken up by these vesicles, suggesting that contamination from sarcoplasmic reticulum was minimal (2.9–5.4%).

Membrane potential of the isolated sarcolemmal vesicles

Fig. 4 shows a relationship between the fluorescent intensity of diS-C_2-(5) and the membrane potential of sarcolemmal vesicles. Fluorescent intensity decreased with the addition of valinomycin (hyperpolarization) to K^+-loaded sarcolemmal vesicles which were incubated in 150 mM NaCl or LiCl medium (4a). However, valinomycin addition did not decrease the fluorescent intensity of Na^+-loaded vesicles which were incubated in KCl or LiCl medium (4b). Instead, an increase in fluorescent intensity was observed. Since diS-C_2-(5) is a positively charged molecule, the results in Fig. 4 suggest that this fluorescent sensitive dye is accumulated inside the sarcolemmal vesicles in an aggregated form when electrical potential inside is negative relative to the outside. Thus, the vesicles loaded with K^+ and then incubated in a K^+ free medium developed a membrane potential.

Fig. 5 demonstrates a decrease in membrane potential caused by increasing K^+ concentration in the external medium (depolarization). The higher the K^+ concentration used, the less the fluorescent intensity changed. When decreases in fluorescent intensity reached a minimum after valinomycin addition, an increase in fluorescent intensity ensued, which is believed to be caused by a loss of the membrane potential as a result of the diffusion.

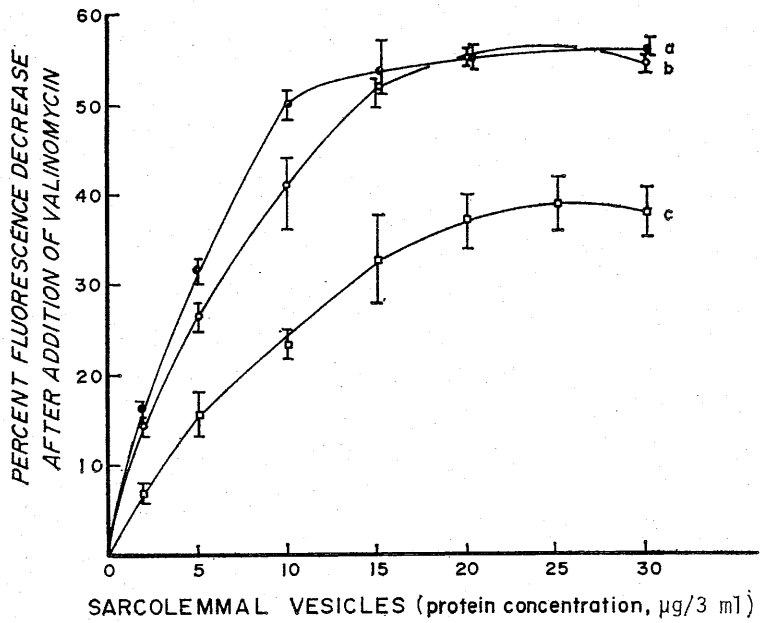

Fig. 6. Effect of ischemia on sarcolemmal vesicles membrane potential: Incubation medium contained 150 mM NaCl, 10 mM Tris-HCl, pH 7.0 and 5 μM diS-C_2(5). Sarcolemmal vesicles (150 mM KCl-loaded) at various concentrations were added to the incubation medium in a final volume of 3 ml as indicated in the horizontal axis. Valinomycin (2 μM) was added one minute after vesicles were added. Percent fluorescence decrease is shown in the vertical axis. Normal (a, ●---●), 15-minute ischemia (b, ○---○), 30-minute ischemia (c, □---□).

of K^+ from vesicles to the external medium. The greater the membrane potential sarcolemmal vesicles possess, the longer the time required for the membrane potential to collapse.

Fig. 6 shows the magnitude of membrane potential determined in sarcolemmal vesicles isolated from the nonischemic, 15 minute ischemic-2 hour reperfused, and 30 minute ischemic-2 hour reperfused left ventricle, respectively. The magnitude of the membrane potential in vesicle preparations was determined at various protein concentrations of vesicles. As noted in Fig. 6, the magnitude of membrane potential increased from 1 μg to approximately 70μg and reached a plateau between 20 to 30μg of protein which were added to the incubation medium in a final volume of 3 ml. Under such conditions, the membrane potential developed in sarcolemmal vesicles that were isolated from myocardium which had undergone 15 minutes of ischemia and 2 hours of reperfusion was not markedly different from the nonischemic group. However, the membrane potential developed in vesicles that were isolated from myocardium which had undergone 30 minutes of ischemia and 2 hours of reperfusion showed a significant difference from the nonischemic group. This result suggests that a leaky membrane of vesicles may result from 30 minute but not 15 minute ischemic insult.

Fig. 7 shows a reciprocal relationship between the magnitude of the membrane potential, the extent of Ca^{2+} deposition and the degree of diastolic thickening. The membrane potential decreased while the Ca^{2+} deposition and diastolic thickening occured, suggesting

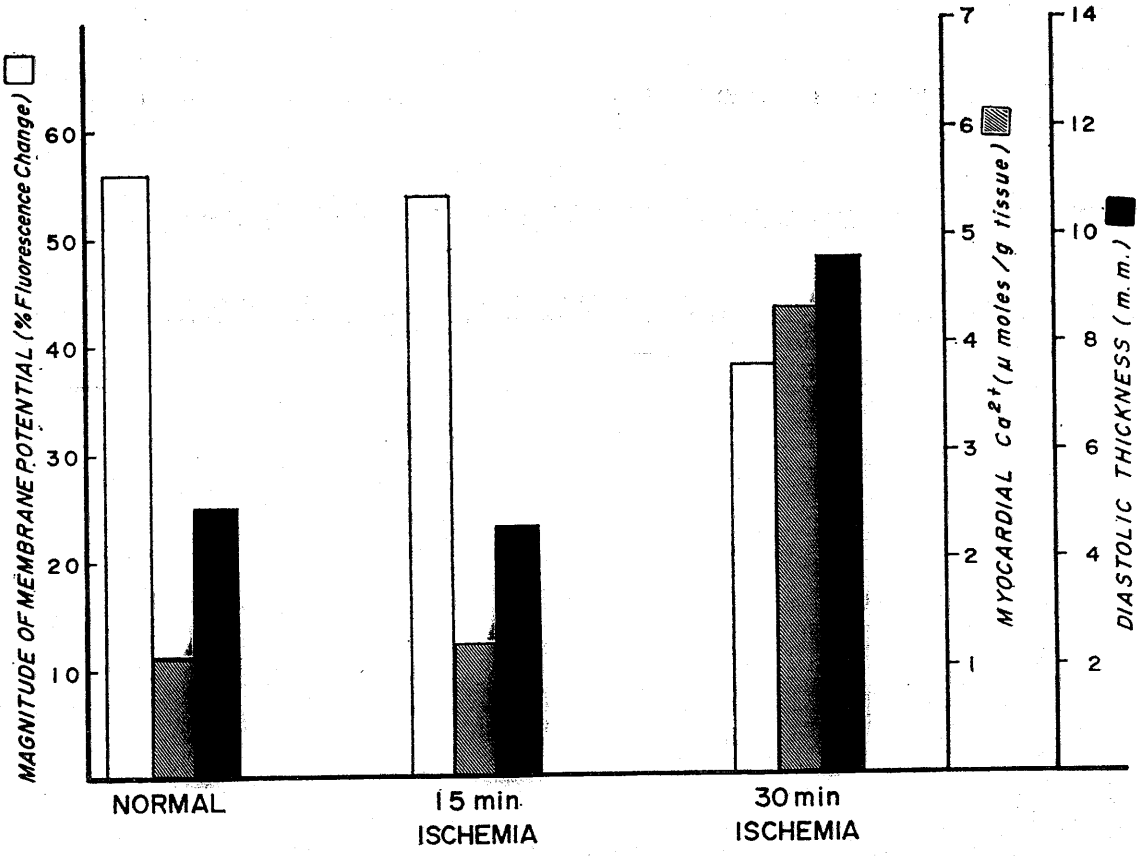

Fig. 7. A reciprocal relationship between the magnitude of the membrane potential, the diastolic thickening, and the extent of Ca^{2+} deposition in the irreversibly damaged ischemic myocardium. Plain bar, membrane potential; solid bar, diastolic thickening; hatched bar, myocardial Ca^{2+} content.

that the leakiness of the membrane is responsible for the irreversible damage after 30 minutes of the ischemic insult.

DISCUSSION

This study demonstrates that acute coronary occlusion resulted in the absence of systolic thickening in the ischemic left ventricle. This thinning persisted throughout the length of occlusion in both groups of animals. The absence of contractile activity and wall motion with ischemia have previously been reported by others (Gaasch and Bernard 1977; Heyndrickx *et al.*, 1975; Murphy *et al.*, 1982; Therox *et., al.*, 1974). Reperfusion is generally thought to be beneficial for the ischemic myocardium by providing essential vital substances to repair injury or regenerate cellular components. However, in this study, reperfusion of myocardium after 30 minutes of ischemia resulted in the left ventricular wall thickening, ionic Ca^{2+} accumulation and decreasing membrane potential of the sarcolemmal vesicles. On the contrary, these were not true for the 15 minute ischemic group. These data suggest that irreversible myocardial damage occurred between 15 and 30 minutes of the ischemic insult.

Myocardial injury related to the coronary reperfusion in experimental animals and human subjects have been well described (Bulkley and Hutchins, 1977; Gaasch and Bernard, 1977; Jenning and Ganote, 1976; Katz and Tada, 1972; Murphy *et al.*, 1982; Shen and Jennings, 1972). Gaasch and Bernard (Gaasch and Bernard, 1977) indicated that increases in the left ventricular wall thickness on reperfusion after relatively short periods of ischemia were related to the acute increase in coronary flow (reactive hyperemia) that occurred upon ligature release. As reactive hyperemia is a transient phenomenon, it would appear unlikely that the prolonged increase in the left ventricular wall thickness observed in this study resulted merely from an increase in blood volume of involved myocardium. Intramyocardial hemorrhage during reperfusion after a prolonged ischemia (one hour or longer) has been described (Bresnahan *et al.*, 1974; Capone and Most, 1978). However, absence of or slight hemorrhage in the 30 minute ischemia (Murphy *et al.*, 1982) indicated that this mechanism could not account for the thickened left ventricular wall after reperfusion. It has been suggested that the severe depletion of cellular ATP that is associated with prolonged ischemia may be responsible for the onset of the ischemic contracture (Jennings and Ganote, 1976; Katz and Tada, 1972). Mitochondria isolated from myocardium with ischemia lasting 30 minutes or longer were unable to efficiently generate ATP (Jennings and Ganote, 1976; Murphy *et al.*, 1982). There was a positive correlation between the abnormality of left ventricular wall thickening and the alteration of mitochondrial functions (Murphy *et al.*, 1982). However, mitochondrial energy production was known to be inhibited by ionic calcium (Peng *et al.*, 1978). Upon reperfusion, one of the most striking abnormalities was the several fold increase in calcium content in the ischemic myocardium (Murphy *et al.*, 1982; Shen and Jennings, 1972). These high levels of calcium in the ischemic region may have resulted from the inability to pump calcium ions out of the cell because of a defect in Ca^{2+}-dependent ATPase. This, in turn, inhibits ATP production by mitochondria. It is conceivable that these high level of Ca^{2+} ion in the tissue and low level of ATP produced by mitochondria may result in the delay or failure of myocardial relaxation, a thickened left ventricular wall, and ischemic contracture with little or no return of function.

Recently, a correlation of phospholipid depletion, techneticum pyrophosphate uptake, and Ca^{2+} depositions in an irreversibly ischemic myocardium has been reported (Chien *et al.*, 1981). The activation of lysosomal enzymes such as phospholipase was suggested to be responsible for the depletion of phospholipids of the sarcolemmal membrane and thus the alteration of the permeability of this membrane

to calcium ions. It appears that the intactness of cell membrane is essential for myocardial cells to maintain both normal membrane potential and ionic composition. Bartschat *et al.*, (Bartschat *et al.*, 1980) have shown that Ca^{2+} uptake by isolated sarcolemmal vesicles was moderately affected by polarization of the vesicles but was markedly and rapidly enhanced by depolarization of the vesicles. These data suggest that decrease in membrane potential (depolarization) of myocardial cells may result in increasing Ca^{2+} accumulation. In this study, we have shown that decreases in membrane potential of isolated sarcolemmal vesicles and increases in myocardial Ca^{2+} occurred only in 30 minute but not 15 minute ischemic insult. This is in agreement with the above mentioned observations that depolarization enhanced Ca^{2+} accumulation. Furthermore, a reciprocal relationship between the magnitude of membrane potential, the extent of Ca^{2+} deposit and the degree of diastolic thickening observed in this study suggest that the leakiness of membrane is responsible for the irreversible damage observed after 30 minutes of ischemia.

The method of the fluorescent sensitive probe for the membrane potential study deserves some comment. Many techniques have been employed to determine cell membrane potential, such as impaling cells with single conventional microelectrodes and measuring the membrane potential and membrane conductance in the course of repeated injection of current pulses (Morad *et al.*, 1982), by following the inside/outside distribution ratio of the radioactive compound, i.e., ^{3}H-triphenylmethylphosphonium (Goldinger *et al.*, 1983), and by using fluorescent probes (Goldring and Blaustein, 1975; Hoffman and Laris, 1974; Sims *et al.*, 1974). The latter two methods are noninvasive probes. Each technique has its own advantage and weakness (Goldinger *et al.*, 1983; Goldring and Blaustein, 1975; Laris *et al.*, 1975; Morad *et al.*, 1982). Complemental study by using two or all the three techniques usually provides more reliable data. In this study, however, only the result of fluorescent study was reported because the fluorescent probe permits the detection of early changes in the membrane potential.

The techniques used to isolate sarcolemmal vesicles are important also. Several techniques have been developed by various laboratories for the sarcolemmal vesicle isolation. Reeves and Sutko (Reeves and Sutko, 1979, 1983) have isolated cardiac vesicles with Na^+-Ca^{2+} exchange activity. However, their vesicle preparations were contaminated with sarcoplasmic reticulum and mitochondrial fragments (Reeves and Sutko, 1979). Another method which was developed by Philipson and Nishimoto (Philipson *et al.*, 1980; Philipson and Nishimoto, 1980) produced a relatively pure preparation. However, the procedure is tedious and time consuming. The method used in this study was first developed by Van Alstyne *et al.*, (Van Alstyne *et al.*, 1980). We have found that vesicles isolated by this method are relatively free of sarcoplasmic reticulum and mitochondrial fragments (see Table 3). In addition, it takes less than three hours to complete the entire isolation procedure.

In conclusion, this study, by using fluorescent sensitive probe, shows that ischemia of myocardium for 30 minutes results in damaged sarcolemmal membrane, which is not present at 15 minutes. This damage allows accumulation of calcium ion in the ischemic area upon reperfusion. Ca^{2+} deposition apparently interferes with mitochondrial ATP production and inhibits diastolic relaxation that is related in part to high calcium ion and low ATP levels.

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心臟肌局部缺血 (Myocardial ischemia) 對左心室心肌伸縮能力與心肌細胞膜電勢改變的影響

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本報告以四十～五十公斤重之豬爲材料，以外科手術之方法，紮結左下降冠狀動脈 (left anterior descending coronary artery) 十五分鐘或三十分鐘，隨後去除紮結使血液回流 (reperfusion) 至缺血之心肌，以便觀測局部缺血後所造成之損傷程度。在紮結與回流過程中，以心音圖觀察該缺血之心肌在心臟收縮與疏張間的變化情形。血液回流二小時後，心臟取出，正常與缺血之心肌分別以生化方法，分離其細胞膜囊，測定其電勢，並分析心肌細胞鈣離子之分佈。再將心音圖所得之結果與生化變化的結果相互比較，以便尋求可能之關聯。實驗結果顯示，紮結冠狀動脈三十分鐘後心肌細胞產生了不能逆轉的損傷。反之，十五分鐘之紮結，心肌細胞大部份之生理與生化之變化均能逆轉至正常狀態。此結果可供臨床研究之參考。