Correlative Studies on Sarcolemmal Ultrastructure, Permeability, and Loss of Intracellular Enzymes in the Isolated Heart Perfused With Calcium-Free Medium

Muhammad Ashraf, PhD

Effects of calcium-free perfusion and calcium-free perfusion followed by reperfusion with calcium on sarcolemmal structure, sarcolemmal permeability, and creatine phosphokinase loss were investigated in isolated perfused rat hearts. Release of creatine phosphokinase was significant ($P < 0.0002$) after 4–5 minutes of perfusion with Ca^{++}-free medium, but later releases in comparison to their immediately preceding periods became significant only after more than 20-minute perfusion. Poor correlation between enzyme loss and lanthanum permeability prior to 20 minutes of Ca^{++}-free perfusion was noted. After 20 minutes of Ca^{++}-free perfusion, the basal lamina was separated from the plasma membrane, and lanthanum was seen in the cytoplasm. The intramembranous particles began to aggregate at that time. The morphologic and enzymatic changes were dramatic following reperfusion of calcium-free perfused hearts. Morphologic changes in these hearts included separation of basal lamina, cellular separation at the intercalated disk, dissolution of actin filaments at the region of I band, contraction bands, cell swelling, and staining or filling of mitochondrial membranes with La^{+++}. Increased sarcolemmal permeability was associated with tears and aggregation of intramembranous particles in the sarcolemmal lipid bilayers. These results suggest that reperfusion of Ca^{++}-free perfused cells causes irreversible damage to the sarcolemmal lipid bilayer, and the degree of alterations induced in the cells is dependent upon the initial duration of Ca^{++}-free perfusion. (Am J Pathol 97:411–432, 1979)

IT IS WELL ACCEPTED that rapidly exchangeable Ca^{++} is involved in the generation of the contractile force of the heart,[1,2,3,4] and this Ca^{++} exchange may take place, at least in part, via calcium binding sites on the sarcolemma.[5] *In vitro* studies indicate that elimination of Ca^{++} from the medium is known to alter the myocardial cell function.[6] Consequently, the cells lose their electrical activity within a few minutes of Ca^{++} deprivation [6,7] and become highly permeable to Ca^{++} and lanthanum (La^{+++}).[5,8] Prolonged perfusion with Ca^{++}-free medium may cause permanent cellular injury [6] and loss of intracellular enzymes, including creatine phosphokinase and lactic dehydrogenase,[9,10] due to altered cell membrane permeability. All this evidence suggests that the lack of Ca^{++} induces changes in the cell membrane.

From the Department of Pathology and the Department of Pharmacology and Cell Biophysics, University of Cincinnati Medical Center, Cincinnati, Ohio 45267.

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Address reprint requests to Dr. M. Ashraf, Department of Pathology, College of Medicine, University of Cincinnati Medical Center, 231 Bethesda Avenue, Cincinnati, Ohio 45267.

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No correlative structural evidence of the loss of intracellular enzymes and altered cell membrane permeability exists. Despite the great pathophysiologic significance of calcium during ischemia and reperfusion, the role of Ca^{++} in the pathogenesis of membrane injury is not completely understood. The present study was designed to determine ultrastructural effects of Ca^{++} on the cell membrane and cell membrane permeability and the relationship of these changes to the loss of intracellular enzymes.

Materials and Methods

Sprague–Dawley rats weighing approximately 350 g were lightly anesthetized with ether, and the abdominal aorta was exposed. Heparin (0.2 ml) was injected through the abdominal aorta to prevent blood clotting. After 2 minutes, the hearts were excised and immediately placed in ice-cold Krebs–Henseleit medium to stop contraction. The aorta was cannulated and immediately attached to noncirculating Langendorff apparatus. After perfusing the hearts with oxygenated Krebs–Henseleit medium at 33 C for an equilibration period of 20 minutes, the perfusion was changed to Krebs–Henseleit medium devoid of calcium for a period of 2, 3, 4, 5, 10, 20, and 30 minutes. In the second series of experiments, the hearts perfused with calcium-free medium for 2, 4, 5, 10, 20, and 30 minutes were reperfused with calcium-containing Krebs–Henseleit medium for 20 or 30 minutes (Figure 1). Usually, 4 rats were used for each time interval. The coronary flow was recorded at 5- or 10-minute intervals.

Perfusion Medium

Krebs–Henseleit medium consisted of NaCl, 118 mM; Na HCO_3, 25 mM; KCl, 4.7 mM; KH_2 PO_4, 1.2 mM; Ca Cl_2, 2.5 mM; and glucose, 5.5 mM. The perfusion apparatus was acid washed and rinsed with distilled water. The perfusion medium was made in glass-distilled water and filtered through 0.4-μ millipore filter. In the Ca^{++}-free medium, Ca^{++} was omitted and no ethylene glycol-bis(β-aminoethylether)-N,N-tetraacetic acid (EGTA) was added. The measurements with atomic absorption spectrophotometer showed no detectable Ca^{++} in the Ca^{++}-free perfusion medium. The medium was constantly bubbled with O_2 and CO_2 mixture (95:5) and maintained at 33 C. All perfusion media were saturated with O_2 for at least 1 hour before the start of the experiment. PO_2 was measured with radiometer BMSMK2d and acid base analyzer PHM 71 (Radiometer, Copenhagen).

Determination of Creatine Phosphokinase (CPK) Activity

Three milliliters of coronary effluent was collected at the end of equilibration period and, after each 5-minute interval, placed on ice and analyzed for CPK the same day or stored at −70 C with 0.01/ml β-mercaptoethanol before analyzing in either a Gilford 2000 spectrophotometer or a Gilford micro-sample spectrophotometer according to the procedure previously described,[11] using Calbiochem single-vial reagent. The enzyme activity was expressed in milli-international units (mIU) per milliliter of coronary effluent. Reproducible data on CPK activity were obtained by analyzing the samples during the same day and without freezing the samples.

Transmission Electron Microscopy

At the termination of experiments, the hearts were fixed retrogradely through the aorta with 3% glutaraldehyde made in 0.1 M cacodylate buffer, pH 7.3. The hearts were further cut into slices, which were kept in fresh fixative for an additional 2 hours. Some hearts were perfused with 0.1 M cacodylate buffer containing 1% colloidal lanthanum for 2 min-

utes, followed immediately by 3% glutaraldehyde containing 1% colloidal lanthanum solution. Small cubes of tissue were taken from three sites of free left-ventricular wall for electron microscopy and freeze-etching.

Colloidal lanthanum was prepared according to Revel and Karnovsky [12] with modifications: 100 ml of 5% aqueous solution of lanthanum nitrate (anhydrous) was titrated with freshly prepared 0.1 N or 0.02 N sodium hydroxide over a period of 4 hours to a faint cloudiness at pH 7.5–7.6. The lanthanum solution was stirred vigorously and constantly during the entire titration while NaOH was dripped into the lanthanum solution through a 26-gauge syringe needle at a rate of 2–4 ml/hr. A slight amount of fine white precipitate was visible at the bottom of the beaker after incubation overnight. A successful titration was evidenced by the persistent cloudiness for several days. Colloidal lanthanum solution in which cloudiness disappeared after overnight incubation was discarded, since no uniform filling of T tubules or extracellular space was obtained with such preparation.

Sections 100 μ thick were cut with an Oxford vibratome and were postfixed with 1% osmium tetroxide made in 0.1 M cacodylate buffer containing 1% colloidal lanthanum solution for 1½ hours (1 part 4% osmium, 2 parts 0.2 M cacodylate buffer, and 1 part 4% lanthanum solution). The tissue was dehydrated in ethanol and embedded in Epon. Thin sections were examined in the Zeiss 10 A and Philips 300 electron microscopes. The total number of blocks examined under the light microscope from each experimental condition is given in Table 1. The number of blocks selected for thin sectioning was variable, depending on the uniformity of light-microscopic changes, which included presence of cellular swelling, clumping of nuclear material, cellular separation at the intercalated disk, and contraction bands. Representative blocks as indicated in parenthesis were thin-sectioned for electron-microscopic changes. The results of this study confirmed that the electron-microscopic changes reported here were representative of the alterations observed in 1-μ-

Table 1—Summary of Electron-Microscopic Observations on Hearts perfused with Ca^{++}-Free Medium, Followed by Reperfusion With Ca^{++}

Treatment	Percentage of blocks showing changes			Percentage of replicas showing nicks or aggregation of IP	
	Blocks examined	Altered CM permeability	Basal lamina separation	Replicas examined	With changes
Control	10(4)*	0	0	8	0
Ca^{++}-free medium (minutes)					
2	8(3)	0	0	Not	examined
4	12(4)	0	0	Not	examined
5	12(4)	0	0	Not	examined
10	8(4)	12.2	12.2	8	0
20	16(8)	87.5	68.7	8	75
30	16(6)	93.7	81.2	4	100
Reperfusion (minutes) Ca^{++}-free medium: reperfusion with Ca^{++}					
2:20	8(3)	0	0	Not	examined
4:20	16(6)	81.2	87.5	Not	examined
5:20	18(7)	94.4	94.4	Not	examined
10:20	15(5)	100	100	8	75
20–30:20	23(9)	100	100	12	100

CM = cell membrane; IP = intramembranous particles.
* Numbers in parentheses shows the blocks thin-sectioned.

thick sections. The amount of tissue in a single thick-sectioned block consisted of approximately 100 myocardial cells.

The replicas of the myocardial tissue were prepared as described previously.[13] Small pieces of glutaraldehyde-fixed tissue were soaked in 20% glycerol made in 0.1 M cacodylate buffer for an hour and were placed in gold specimen holders and immediately dipped into Freon 22 cooled in liquid nitrogen. At least four replicas consisting of six specimen grids were prepared from a single heart as indicated in Table 1. The frozen samples were stored in liquid nitrogen until use. The tissue samples were fractured in a Balzer's BA 360M freeze-etch unit at −115 C, shadowed with platinum and carbon, and reinforced with carbon. The replicas were cleaned with Chlorox, washed in glass-distilled water, and placed on uncoated 300-mesh copper grids. The direction of shadowing is indicated by an arrow in the electron micrographs. The fracture faces of the membranes were described according to the terminology adopted by Branton et al.[14] Thus, P fracture face (PF) represents the protoplasmic half of split membranes (closest to the cell cytoplasm), and E fracture face (EF) indicates the face closest to the extracellular space.

Results

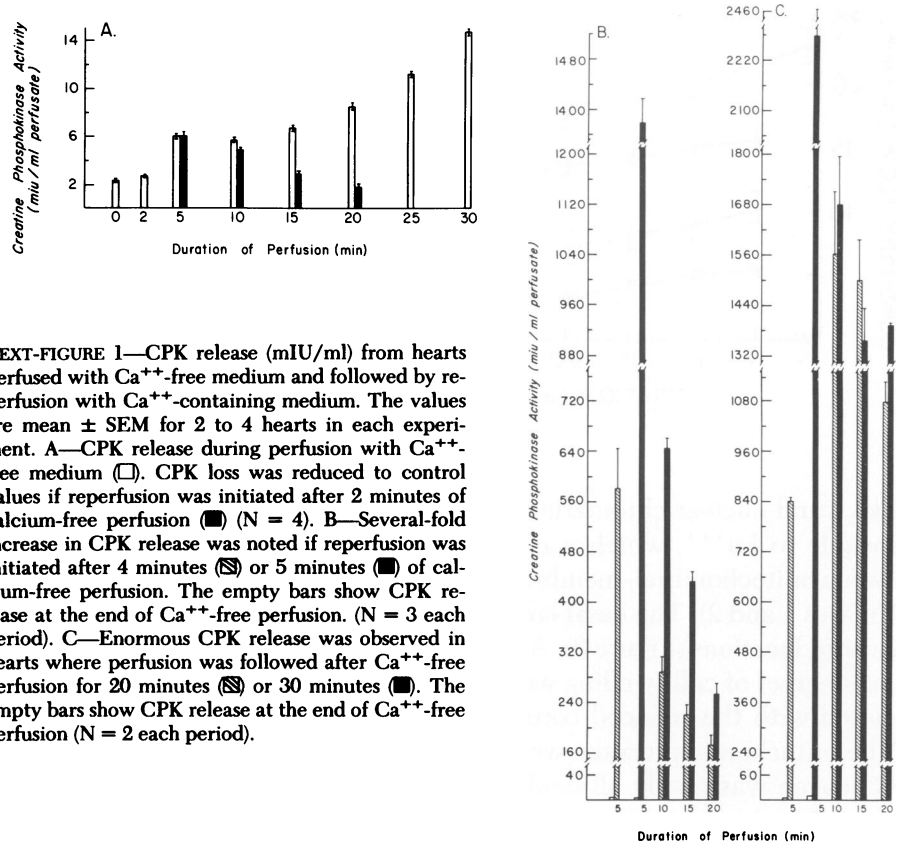
Perfusion With Calcium-Free Medium

Creatine Phosphokinase Release

During the first 2 minutes of Ca^{++}-free perfusion, the enzyme release was minimal and did not increase from the amount observed at the end of the equilibrium period. A gradual but significant increase ($P < 0.0002$) in the enzyme release was noted after 4 or 5 minutes and continued at the end of the experimental period (30 minutes) (Text-figure 1). It may be noted that CPK amount released between 5 minutes and 15 minutes of Ca^{++}-free perfusion was not significant. However, the increase was significant ($P < 0.039$) between 5 minutes and 20 minutes and longer periods of perfusion. The hearts perfused with oxygenated Krebs–Henseleit solution released only 1.15 ± 0.04 mIU/ml after 30 minutes of perfusion (Table 2). The amount of enzyme released appears to be independent of coronary flow. The flow rate was reduced approximately 20% after 30 minutes of Ca^{++}-free perfusion while the CPK release was considerably increased at that time (Text-figure 2).

Ultrastructural Changes and Cell Membrane Permeability

No major structural changes were seen in the hearts exposed to Ca^{++}-free medium for 10 minutes, compared with those hearts perfused with normal Ca^{++}-containing Krebs–Henseleit medium (Table 1). Lanthanum was confined to the extracellular space, intercalated disk, and transverse tubules (Figures 1 and 2). The mitochondria were usually elongated and intact, without any evidence of damage to the cristae. However, the basal lamina was occasionally separated from the plasma membrane, and cell membrane permeability was also occasionally altered in hearts perfused



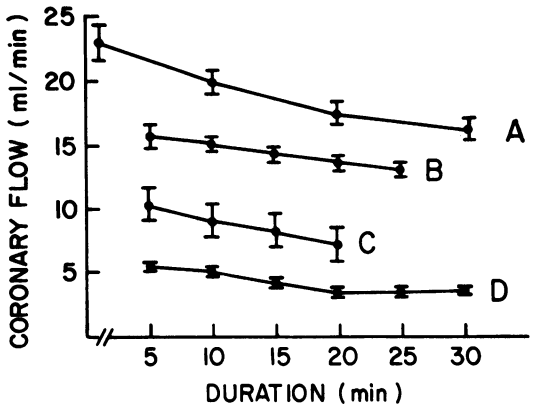
TEXT-FIGURE 1—CPK release (mIU/ml) from hearts perfused with Ca^{++}-free medium and followed by reperfusion with Ca^{++}-containing medium. The values are mean ± SEM for 2 to 4 hearts in each experiment. A—CPK release during perfusion with Ca^{++}-free medium (□). CPK loss was reduced to control values if reperfusion was initiated after 2 minutes of calcium-free perfusion (■) (N = 4). B—Several-fold increase in CPK release was noted if reperfusion was initiated after 4 minutes (▨) or 5 minutes (■) of calcium-free perfusion. The empty bars show CPK release at the end of Ca^{++}-free perfusion. (N = 3 each period). C—Enormous CPK release was observed in hearts where perfusion was followed after Ca^{++}-free perfusion for 20 minutes (▨) or 30 minutes (■). The empty bars show CPK release at the end of Ca^{++}-free perfusion (N = 2 each period).

with Ca^{++}-free medium for 10 minutes. After 20 minutes of Ca^{++}-free perfusion, the cells were mildly swollen. The cells were separated at the fascia adherens, desmosomes, and unspecialized portions of intercalated

Table 2—Release of Creatine Phosphokinase (CPK) From the Isolated Rat Hearts Perfused With Oxygenated Krebs–Henseleit Medium for 90 Minutes

Duration (minutes)	Release of CPK (mIU/ml perfusate)
0	2.63 ± 0.23
10	1.72 ± .05
20	1.88 ± .04
30	1.15 ± 0.04
40	1.27 ± .09
50	1.55 ± 0.25
60	2.02 ± 0.36
70	1.91 ± 0.17
80	1.72 ± 0.23
90	2.05 ± 0.26

Values are mean ± SEM for 4 hearts.



TEXT-FIGURE 2—The relationship between coronary flow (ml/min) and the duration of calcium depletion and calcium replenishment. A—Hearts perfused with normal medium (N = 4). B—Hearts perfused with Ca^{++}-free medium (N = 4). C—Hearts perfused with Ca^{++}-free medium for 4 minutes followed by reperfusion with normal medium (N = 3). D—Hearts perfused with Ca^{++}-free medium for 20 minutes, followed by reperfusion with normal medium (N = 3).

disks, and nuclear chromatin was evenly dispersed. The cells became permeable to La^{+++}, which was seen within the cytoplasm. The spaces between mitochondrial membranes were filled with La^{+++} (compare with Figures 1 and 2). The basal lamina was peeled off the plasma membrane at several locations (Figure 3). After 30 minutes of Ca^{++}-free perfusion, varying degrees of cell swelling were observed. The cell swelling was also associated with the reduced coronary flow from these hearts (Text-figure 2). The mitochondrial cristae were well maintained or completely disrupted. Glycogen was nearly all depleted. The basal lamina was often separated from the plasma membrane. The cells were separated from each other at intercalated disks. In all these cells, La^{+++} was observed in the cytoplasm (Figure 4).

In the freeze-fractured myocardium, which was exposed to Ca^{++}-free medium up to 10 minutes, the distribution and arrangement of intramembranous particles were similar to those of the control hearts and were randomly distributed over the sarcolemmal PF face (Figure 5). The particles were far greater in number on the PF face than on the corresponding EF face. At 20 minutes, a slight aggregation of particles was noticed, and nicks or tears became evident in the lipid bilayer (Figure 6).

Reperfusion of Calcium-Free Hearts With Normal Krebs–Henseleit Medium

The hearts ceased beating immediately after perfusing with Ca^{++}-free medium. However, normal beating of the hearts was resumed when these hearts were reperfused with normal Krebs–Henseleit medium within 3 minutes of Ca^{++}-free perfusion. Beyond 3 minutes, these hearts never resumed beating, with the exception of movement of atriums.

Release of Creatine Phosphokinase

The CPK release was greatly increased after reperfusion of hearts that were initially perfused with Ca^{++}-free medium for 2 minutes, and the enzyme release leveled off after 20 minutes of reperfusion with normal medium and was approximate to control values (Text-figure 1A). CPK was increased massively from reperfused hearts (579 mIU/ml) that were initially exposed to Ca^{++}-free medium for 4 minutes (4.0 mIU/ml) or longer (Text-figure 1B,C). The amount of enzyme released from the reperfused hearts was dependent upon the initial period of Ca^{++}-free perfusion. The rate of coronary flow was drastically reduced upon reflow, while CPK loss was very high (Text-figure 2). There was a close correlation between the amount of coronary flow and the intracellular edema.

Morphologic Changes and Cell Membrane Permeability

Lanthanum did not penetrate into the cells of the reperfused hearts after 2 minutes perfusion with calcium-free medium. The cell membrane permeability was altered in the hearts initially perfused with calcium-free medium for 4 or 5 minutes followed by reperfusion with normal Krebs–Henseleit medium, as indicated by the presence of La^{+++} in the cytoplasm. The basal lamina was separated to a varying degree from the plasma membrane of many cells (Table 1). The intercalated disks were widely separated, with the exception of nexuses, most of which remained cohesive (Figure 7). The actin filaments in the regions of I band were conspicuously damaged in the hearts which were reperfused after 5 minutes or longer of initial Ca^{++}-free perfusion (Figure 8). Z bands were also distorted. Reperfusion with normal Krebs–Henseleit medium following perfusion with Ca^{++}-free medium for 20 minutes or longer caused similar but more severe damage, as shown in Figure 9. The plasma membrane was also loosened from the swollen cells. Breaks or minute gaps were observed in the plasma membrane (Figure 10). The mitochondria were filled with La^{+++}. The myofibrils were either relaxed or contracted to form contraction bands and were stained with La^{+++}.

In the hearts which were initially perfused with Ca^{++}-free medium for 10 minutes or longer and followed by reperfusion with normal Krebs–Henseleit medium, the sarcolemmal lipid bilayer was perforated with many nicks or tears, and the intramembranous particles were aggregated (Figure 11). On the other hand in the cell membranes examined from the myocardium perfused with Ca^{++}-free medium for 10 minutes, the nicks were rarely seen and the intramembranous particles were not aggregated. After 20 minutes of Ca^{++}-free perfusion, the intramembranous particles

began to aggregate, and nicks or tears were also seen in the lipid bilayer. The reperfusion of Ca^{++}-free perfused hearts with calcium-containing normal medium accentuated the injury to the sarcolemmal lipid bilayer at a faster rate (Figure 11, Table 1).

Discussion

The rate of CPK depletion and appearance of intracellular colloidal lanthanum were employed to assess the altered permeability and structural integrity of the sarcolemma. Enzyme release was minimal but significantly different from that of the control animals during the first 5 minutes of calcium-free perfusion and became substantially higher after 20 minutes. The lack of lanthanum deposition in the cytoplasm at a time when enzyme release has occurred suggests that cell membranes were unchanged. However, the appearance of CPK in the coronary perfusate does reflect some kind of injury to the tissue, but the exact tissue source of CPK found in the perfusate could not be identified. The possibility that a few injured cells excluded from the electron-microscopic samples were involved cannot be ruled out. Moreover, it is not unreasonable to assume that conformational changes in the membrane causing enzyme leakage were so minute that they could not be detected with the methods employed in this study.

After 20 minutes of Ca^{++}-free perfusion, two structural changes became prominent. First, the basal lamina was separated from the plasma membrane. The significance of altered basal lamina was not well understood until a recent study [5] showed that zero Ca^{++} perfusion produces changes in the myocardial cell surfaces and ruptures the calcium–carbohydrate couplings located in the basal lamina. The altered cell membrane becomes highly permeable to both ionic La^{+++} [5] and colloidal La^{+++}, as shown in this and previous studies.[8] The presence of some ionic La^{+++} in our colloidal solution cannot be excluded, as determined by Moeller.[15] The overall conclusions in these two studies [5,8] are basically in agreement with each other. Secondly, the fractured membrane leaflets become slightly modified. The intramembranous particles begin to aggregate, and occasional rupturing of the lipid bilayer becomes apparent.

Both morphologic and biochemical changes were dramatic following reperfusion of Ca^{++}-depleted hearts, as shown in the previous studies.[7,16–18] The myocardial cells became severely contracted and damaged, resulting in massive enzyme release. This phenomenon has been previously called the "calcium paradox"[7] and is believed to be temperature-dependent. At 30 C or less, enzyme release is nil during "Ca^{++} paradox,"[17] and an insignificant amount of tissue injury occurs.[19] In the present study,

where the temperature of the medium was maintained at 33 C, a moderate but significant amount of CPK was released upon reperfusion of Ca^{++}-deprived hearts with normal Krebs–Henseleit medium, even for a short period, ie, 2 minutes. However, the amount of CPK released was soon reduced to control values, reflecting reversible cellular injury. On the contrary, reperfusion of hearts that were initially perfused with Ca^{++}-free medium for 4–5 minutes or for longer periods with normal medium never reduced the enzyme loss to levels observed during Ca^{++}-free perfusion. The massive enzyme leakage was also accompanied by intracellular deposits of lanthanum. All these events indicate an extreme extent of cellular injury. It also appears that the initial duration of perfusion with calcium-free medium is crucial to the ultimate cell injury if followed by reperfusion with medium containing calcium.

Reperfusion of hearts following Ca^{++}-free perfusion clearly indicates that calcium has a major role in the pathogenesis of sarcolemmal injury. This role is made evident by extensive cellular damage, which is accompanied by massive enzyme release and intracellular deposition of extracellular tracer. The precise mechanism of these changes is not known, but it is believed that increased levels of intracellular calcium [20] and the amount of ATP [16,21] play a significant role in cellular injury during "Ca^{++} paradox." The common occurrence of breaks, both in plasma membrane and basal lamina, and tears in the lipid bilayer and altered distribution of intramembranous particles soon after reperfusion or reintroduction of calcium suggest that calcium is primarily responsible for the sarcolemmal injury. The sarcolemmal alterations are immediately followed by wide cell separation, swelling, contraction bands, and dissolution of the I band.

It appears that the lack of Ca^{++} modifies the protein molecules or their arrangement within the membrane lipid bilayer. The evidence derived from this study, though indirect, points out that punctures in the lipid bilayer represent the extreme condition of injury to the membrane, and this damage is not reparable, even after reperfusion with normal Ca^{++}-containing medium. Under these conditions, the myocardial cells are severely damaged, and the sarcolemma loses its selective permeability, allowing massive enzyme leakage. The aggregation of particles in the lipid bilayer is associated with increased cell membrane permeability during "Ca^{++} paradox." Similar intramembranous particle aggregates are also believed to be related to increased cell membrane permeability after myocardial ischemia [13,22] and hormonal stimulation.[23] However, the particle clumping may be nonspecific and is known to occur under various tissue preparative conditions.[24] It is unlikely that aggregation of intramembranous particles is an artifact of tissue preparation, since no clumping of particles was no-

ticed in the control myocardium under similar procedures of tissue preparation.

This study emphasizes the need to understand the basis of myocardial cell membrane injury following Ca^{++}-free perfusion and "Ca^{++} paradox." The preservation of the myocardial-cell surface is critically important for the regulation of intracellular calcium.[25] Since "Ca^{++} paradox" has several features in common with the reperfusion of ischemic myocardium, various measures should be taken to avoid sudden "Ca^{++} paradox" during perfusion or coronary bypass of ischemic myocardium.

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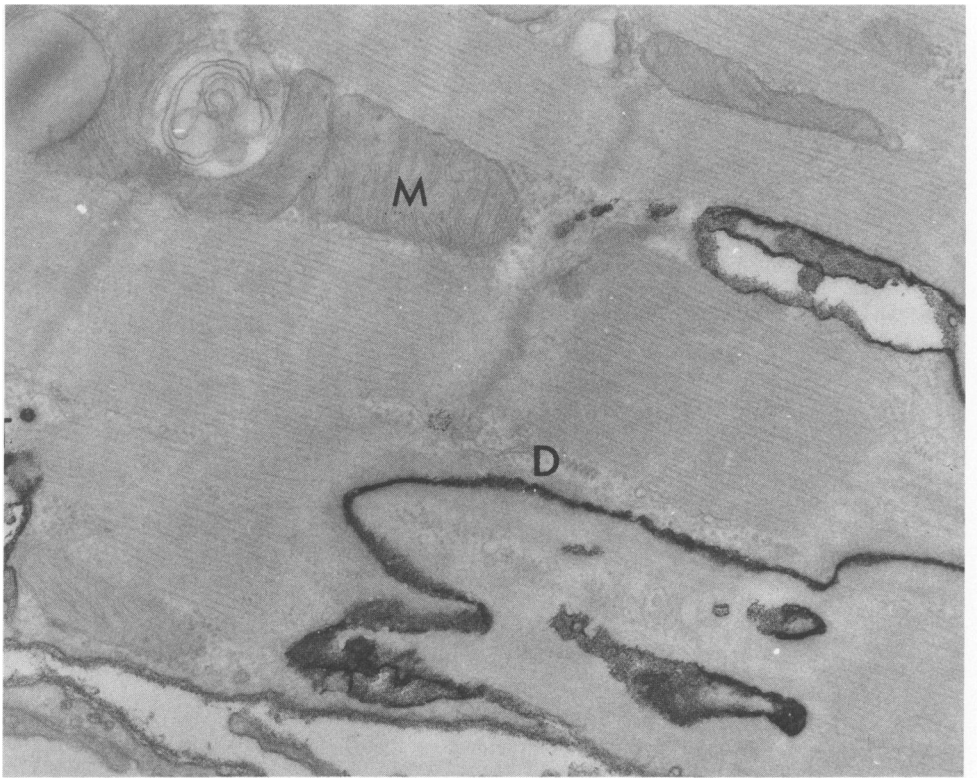
Figure 1—Electron micrograph from heart perfused with normal Krebs–Henseleit medium for 90 minutes and treated with La^{+++}. The cell membrane is well preserved, and no La^{+++} is seen in the cytoplasm except T tubules (*T*) and the intercalated disk (*D*). *M* = mitochondrion. (Unstained, ×29,166)

Figure 2—Myocardium perfused with Ca^{++}-free medium for 10 minutes. No damage is noticed to the cell ultrastructure, and La^{+++} is confined only to T tubules (*T*) and extracellular space. *V* = pinocytotic vesicles. *M* = mitochondria. (Unstained, ×35,800)



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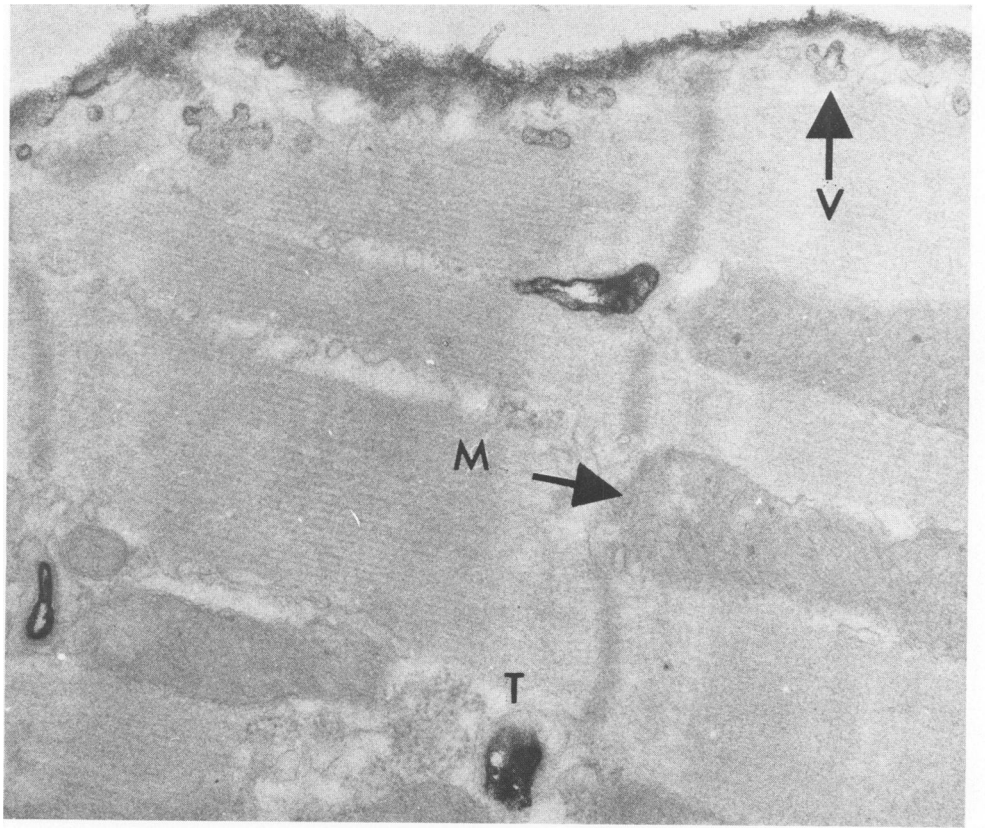

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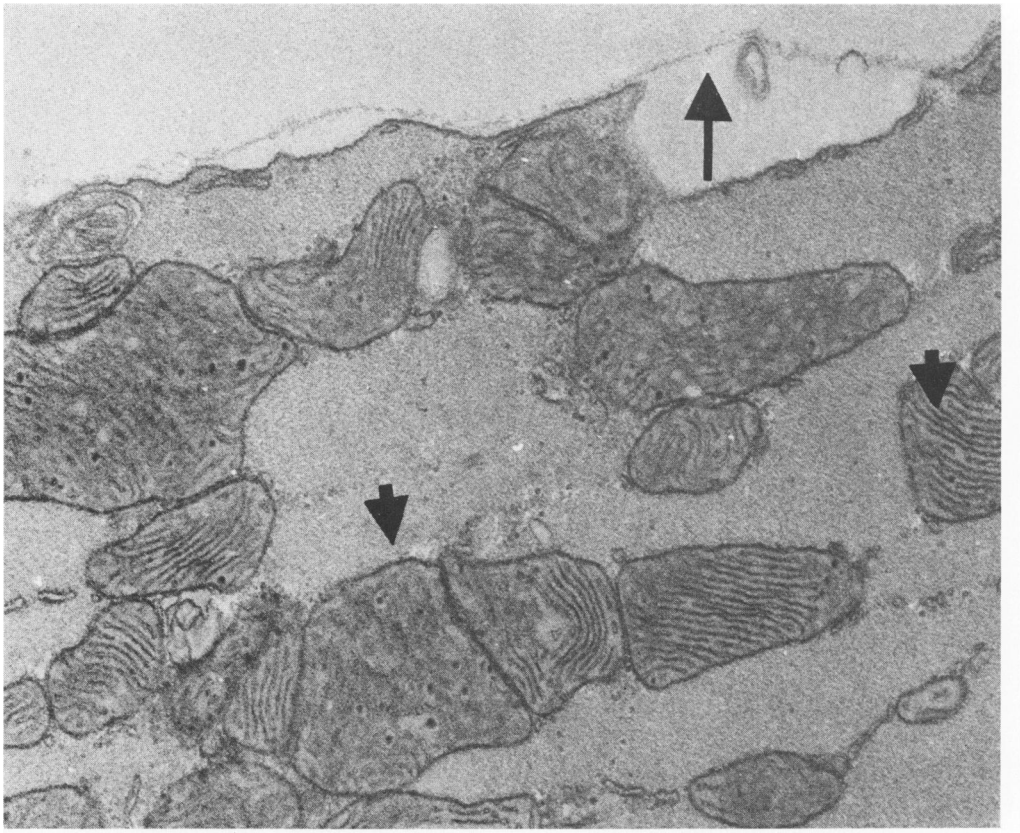
Figure 3—Myocardium exposed to Ca^{++}-free medium for 20 minutes, showing separation of basal lamina from the plasma membrane (*arrow*). La^{+++} is seen in the outer mitochondrial membranes and their cristae (*arrowhead*). (Unstained, ×44,000)

Figure 4—Myocardium exposed to Ca^{++}-free medium for 30 minutes, showing sloughing of basal lamina (*arrow*) and swollen mitochondria (*M*). La^{+++} is seen all over the cytoplasm, being more conspicuous in the mitochondrial cristae (*arrowhead*). (Unstained, ×17,250)



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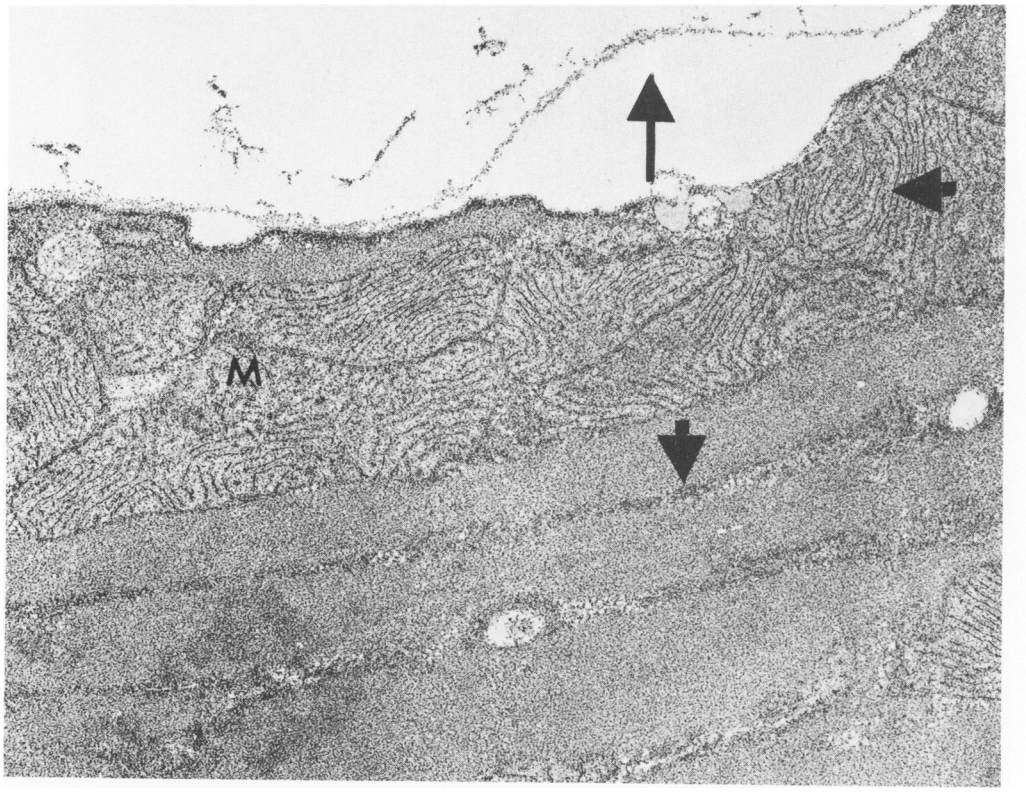

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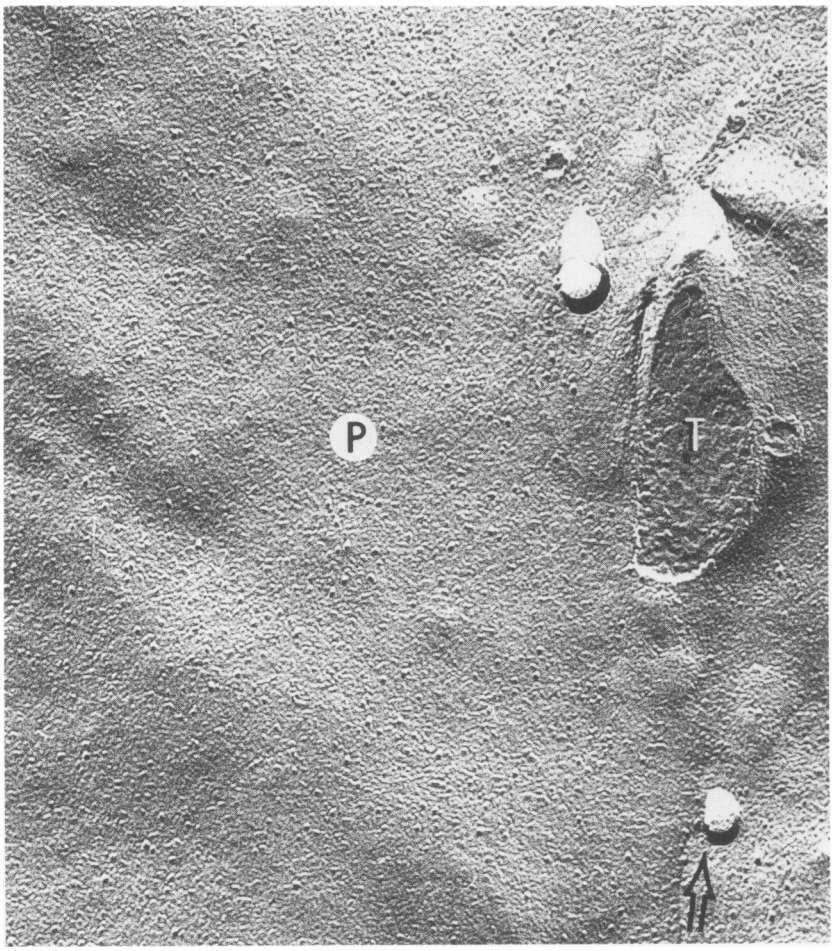

Figure 5—Replica from myocardium perfused with Ca^{++}-free medium for 20 minutes. P fractured face (*P*) of the sarcolemma shows random distribution of intramembranous particles. *T* = T tubules. An *open arrow* indicates the direction of shadowing. (×85,700)

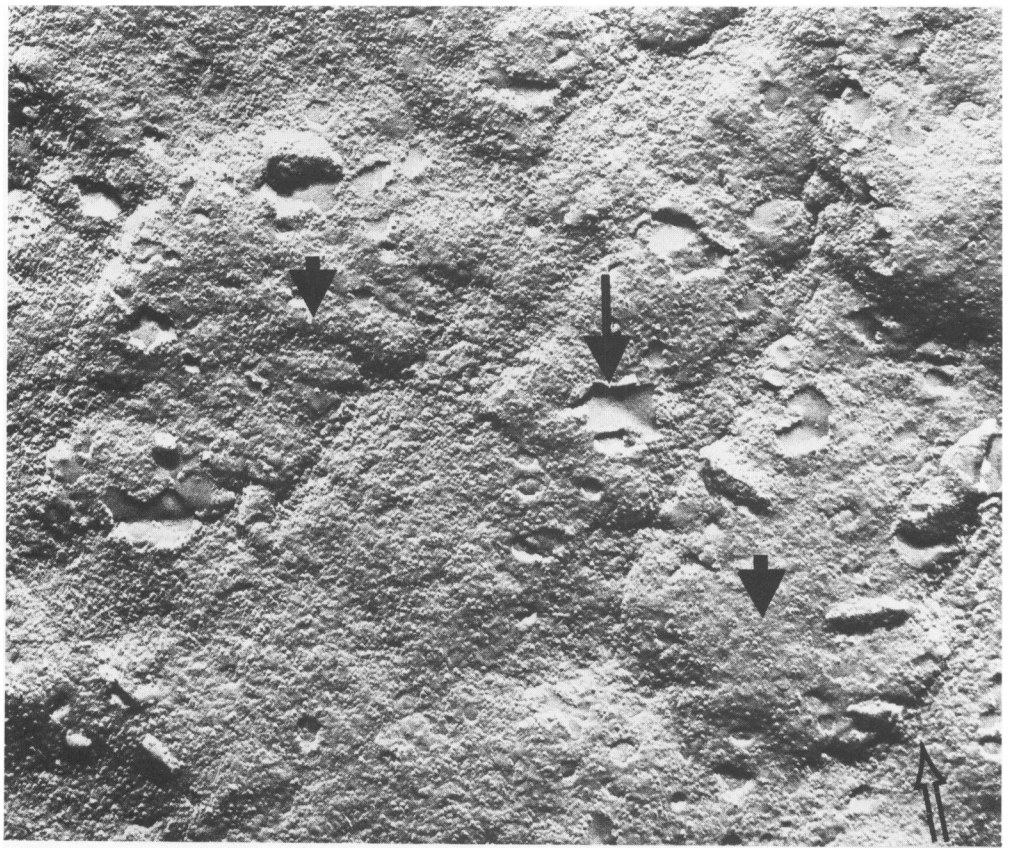

Figure 6—Replica from myocardium perfused with Ca^{++}-free medium for 20 minutes, showing tears or nicks in the sarcolemmal lipid bilayer (*arrow*). The intramembranous particles begin to aggregate over the P face of sarcolemma (*arrowhead*). An *open arrow* indicates the direction of shadowing. (×43,180)

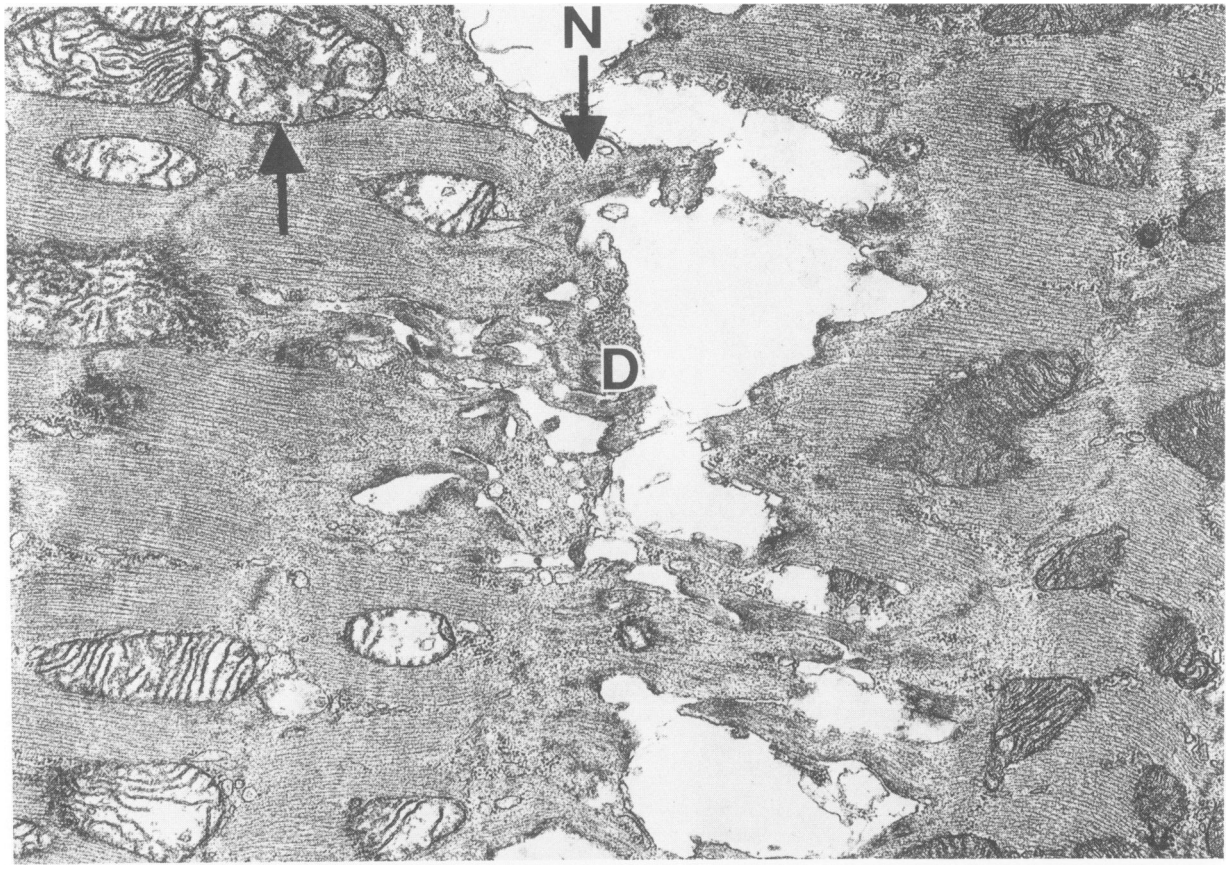

Figure 7—Myocardium exposed to Ca^{++}-free medium for 4 minutes, followed by reperfusion with normal medium for 20 minutes, showing wide separation of cells at the intercalated disk (*D*). Lanthanum has leaked into the cells, staining mitochondrial membranes (*arrow*). The nexus appears to be intact (*N*). (Unstained, ×41,600)

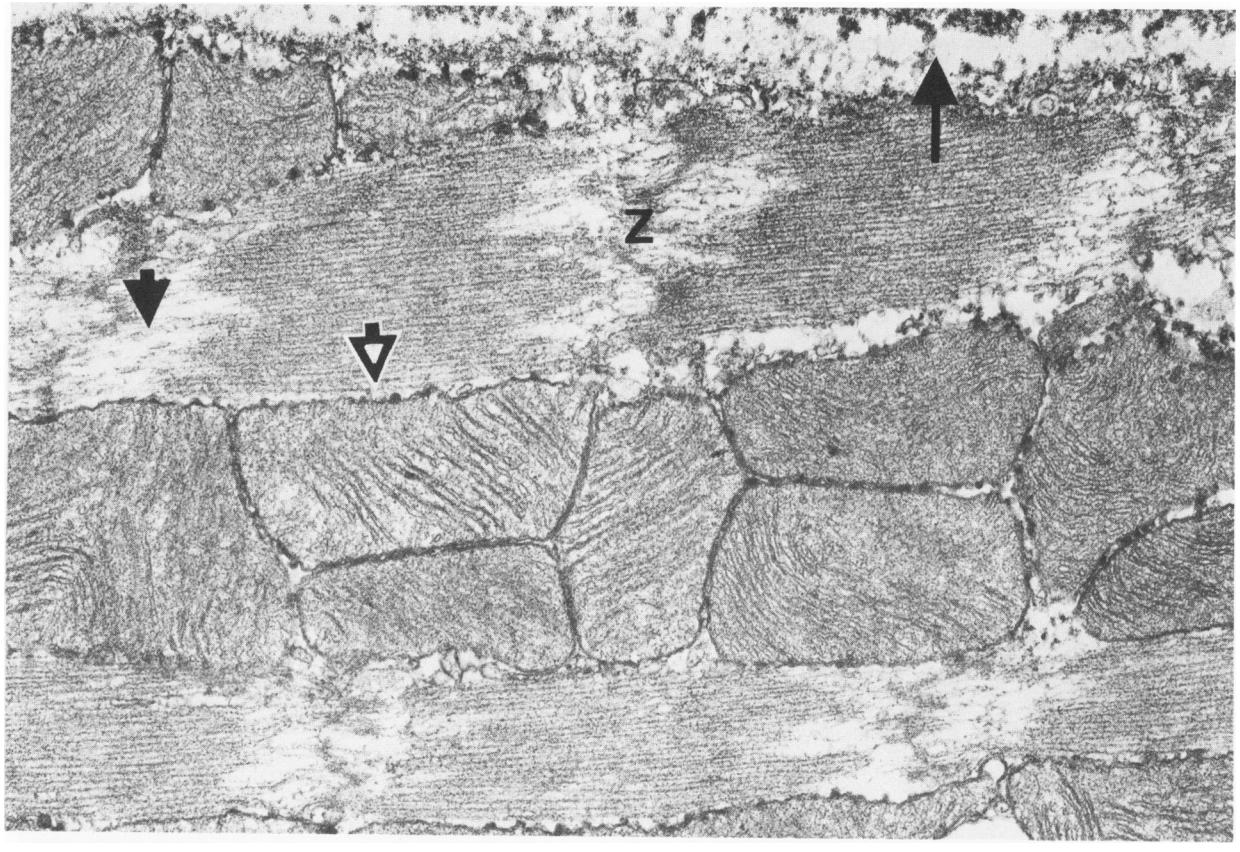

Figure 8—Myocardium treated with Ca^{++}-free medium for 5 minutes, followed by reperfusion with normal medium for 20 minutes, showing separation of basal lamina from the plasma membrane (*arrow*). Z bands (*Z*) are distorted, and actin filaments extending over the region of I band are damaged (*solid arrowhead*). Lanthanum has deeply stained the myofibrils and the mitochondrial membranes (*open arrowhead*). (Unstained, ×30,000)

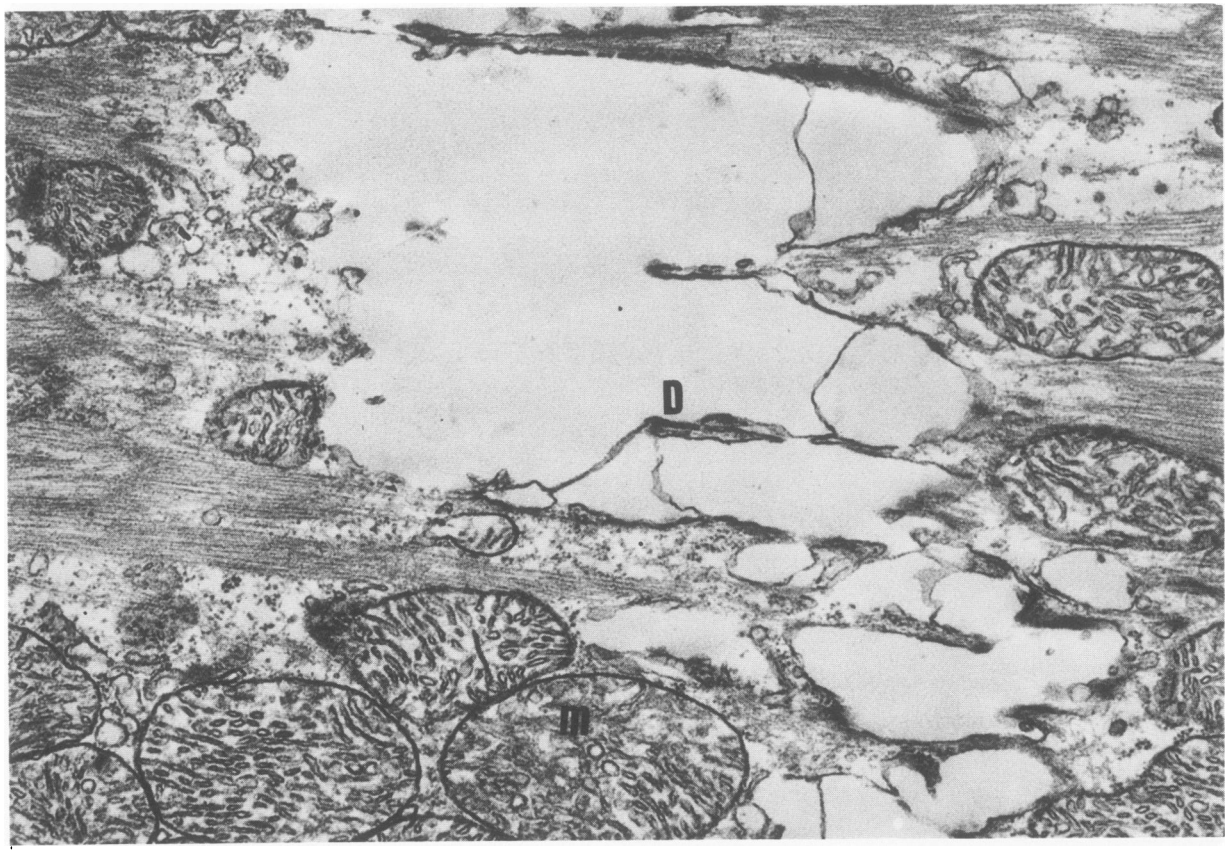

Figure 9—Myocardium exposed to Ca^{++}-free medium for 20 minutes, followed by reperfusion with normal Krebs–Henseleit medium for 20 minutes, showing severe damage to the cells with intercellular separation at the intercalated disk (*D*). Mitochondria (*M*) are swollen, and their cristae are fragmented. La^{+++} is seen in the mitochondrial cristae and between the inner and outer mitochondrial membranes (Unstained, ×30,900)

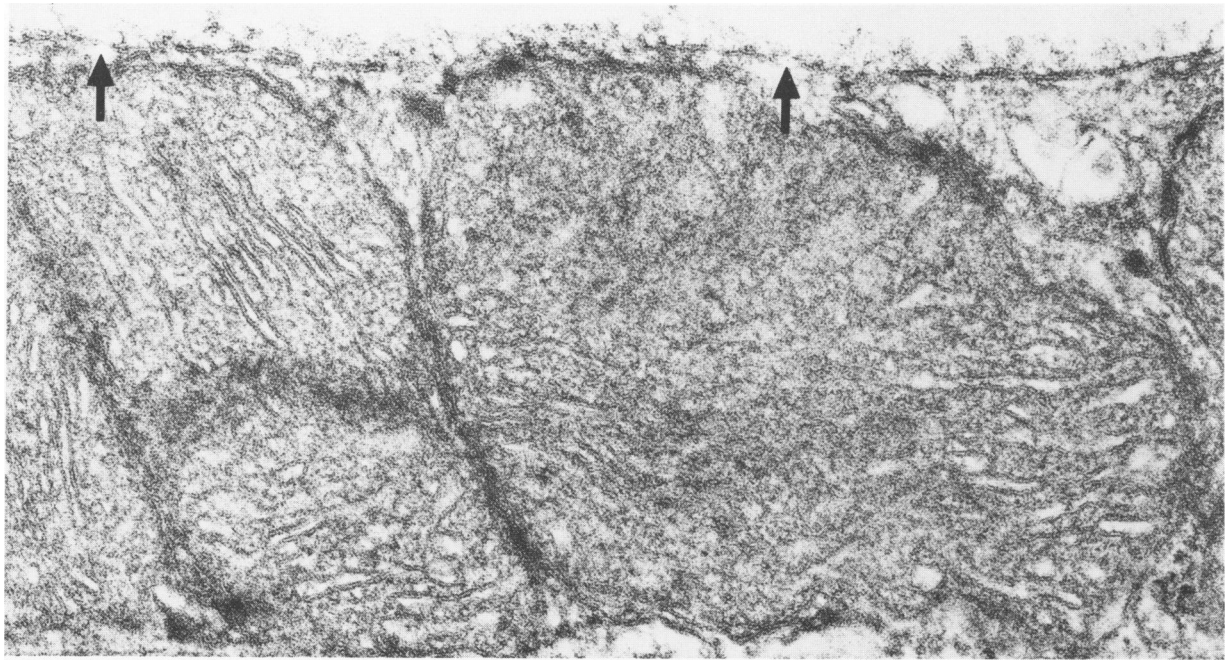

Figure 10—Myocardium exposed to Ca^{++}-free medium for 5 minutes and later reperfused with normal Krebs–Henseleit medium for 20 minutes, showing minute gaps or breaks in the plasma membrane (*arrow*) at a higher magnification. (Unstained, ×110,000)

Figure 11—Replica from myocardium exposed to Ca^{++}-free medium for 20 minutes, followed by reperfusion with normal Krebs–Henseleit medium for an additional 20 minutes, showing extensive damage to the sarcolemmal lipid bilayer in the form of nicks or tears (*arrowheads*) and aggregation of intramembranous particles (*arrow*). *Open arrow* indicates the direction of shadowing. (×60,000)