

Structural Changes in the Freeze-Fractured Sarcolemma of Ischemic Myocardium

Muhammad Ashraf, PhD, and Craig A. Halverson, BS

Ultrastructural alterations in the sarcolemma of ischemic myocardium were studied with the freeze-fracture technique. In normal myocardial sarcolemma, the P fracture face contained many intramembranous particles which were randomly distributed, while the E fracture face had few intramembranous particles; no structural abnormalities were seen within the lipid bilayer on either face. Myocardium ischemic for 45 minutes displayed no, or only slight, aggregation of intramembranous particles, but upon reperfusion for 5 to 20 minutes, the particles became significantly aggregated in the P face. Scattered nicks within the lipid bilayer were observed on both fracture faces. Intramembranous particles were similarly aggregated in myocardium ischemic for 2 hours; however, the number of nicks were greatly increased on the P and E fracture faces. These structural alterations, which were undetected in thin sections, are likely to be associated with altered function in the sarcolemma of ischemic myocardium. (*Am J Pathol* 88:583-594, 1977)

FOLLOWING ISCHEMIA, the myocardial cell membrane loses its selective permeability and, upon the return of blood flow, allows indiscriminate influx of sodium and calcium ions and water and the loss of intracellular enzymes.¹⁻² These events indicate that the membrane has undergone structural changes which are not often detected in thin sections. To view these changes, the structural and functional components of cell membranes can be exposed by the freeze-fracture technique, which splits membranes through the lipid bilayer,³⁻⁴ revealing intramembranous particles on the fractured leaflets. Some recent experimental evidence suggests that the changes in the distribution pattern of these particles are associated with an increase in cell membrane permeability in the inner cortex of ischemic kidney⁵ and in urinary bladder epithelium.⁶

We examined ischemic myocardium with the freeze-fracture method to view changes in the structural components of intact myocardial cell membranes during the early stages of ischemia. This study has shown abnormalities in the lipid bilayer and changes in the distribution of intramembranous particles.

From the Department of Pathology, University of California, San Diego, School of Medicine, La Jolla, California.

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Address reprint requests to Dr. Muhammad Ashraf, Department of Pathology, M-012, University of California, San Diego, School of Medicine, La Jolla CA 92093.

Materials and Methods

Mongrel dogs were anesthetized with sodium pentobarbital administered intravenously (25 mg/kg), and the chest was opened while respiration was maintained with a Harvard pump. The left anterior descending coronary artery was occluded by ligation for 45 minutes (2 dogs), 45 minutes with 5 minutes of reperfusion (2 dogs), 45 minutes with 20 minutes of reperfusion (2 dogs), and 2 hours (2 dogs). Details of the surgical procedure have been published previously.⁷

Preparation of Tissue for Electron Microscopy

Conventional Thin Sectioning

The hearts were flushed with 1% potassium chloride solution after the ligature was removed from the occluded artery and were fixed immediately by perfusion through the coronary arteries with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, kept at room temperature. Because the degree of structural damage in the epicardium varied widely, the tissue processed for electron microscopy was removed from the midcardial and endocardial zones of ischemic myocardium where structural alterations are uniformly present. Small pieces (1 cu mm) from midcardial and endocardial regions were immersion-fixed for an additional 2 hours at 4 C and rinsed in the buffer before postfixing with 1% buffered osmium tetroxide for 1½ hours at 4 C. The tissue blocks were dehydrated in ethanols and embedded in Araldite. Thin sections were made with a Sorvall Porter-Blum MTB2 ultramicrotome, and stained with saturated uranyl acetate, followed by Reynold's lead citrate, and picked up on 300-mesh uncoated copper grids. Sections were examined in a Zeiss 9S electron microscope.

Freeze-Fracture

Small tissue cubes were made from glutaraldehyde-fixed tissue, as described above, and infiltrated with 20% glycerol in 0.1 M cacodylate buffer for 1 hour at room temperature. Small pieces were placed in gold specimen holders and immediately dipped into Freon 22 cooled in liquid nitrogen. The frozen specimens were stored in liquid nitrogen until use. The tissue was fractured in a Balzer's BA 360 M freeze-etch unit at -115 C, shadowed with platinum and carbon, and reinforced with carbon. The replicas were cleaned with bleach (Chlorox), washed in distilled water, and placed on uncoated 300-mesh copper grids. Approximately 25 replicas from each tissue were examined. The fracture faces of the membranes were described according to the terminology adopted by Branton *et al.*⁸ Thus, *P fracture face* indicates the protoplasmic half of split plasma membranes (closest to the cell cytoplasm) and *E fracture face* indicates the faces closest to the extracellular space.

Results

Ultrastructure of Normal Sarcolemma

In this study we have investigated only the general plasma membrane that encloses the myocardial cell. That portion of the cell membrane forming intercellular attachments, i.e., the intercalated disc, has been excluded.

Normal, nonischemic myocardial cells showed intramembranous particles distributed randomly on P and E faces. P faces had a larger number of particles than corresponding E faces, which showed relatively few

particles (Figures 1 and 2). Ellipsoid and round invaginations of the membrane represented the openings of transverse tubules. Smaller circular structures, 400 Å in diameter, were the openings of pinocytotic vesicles.

Ultrastructural Alterations in Ischemic Sarcolemma

In thin sections, cell membranes appeared intact in all ischemic tissue examined, in spite of the presence of intracellular alterations (Figure 3).

Forty-five Minutes of Coronary Occlusion

General features of the fracture faces were essentially the same as for normal membranes. Slight or no aggregation of intramembranous particles was observed (Figure 4). Unusual nicks were seen infrequently in either fracture face (0.4/sq μ). We have defined nicks in this study as discontinuities resembling chips or tears in the surfaces of membranes. These nicks were seen only in ischemic cells.

Forty-five Minutes of Coronary Occlusion and Five Minutes of Reperfusion

Intramembranous particles were variably aggregated in patches or in a circular fashion, and minute nicks (0.7/sq μ) were seen in the lipid bilayer (Figure 5). T tubules invaginated into the membrane as usual, and pinocytotic vesicles were normally distributed.

Forty-five Minutes of Coronary Occlusion and Twenty Minutes of Reperfusion

Intramembranous particles were aggregated more markedly than those seen after 5 minutes of reperfusion (Figure 6). A large number of nicks (1.0/sq μ) and bare zones, which were devoid of particles, were seen in the lipid bilayer.

Two Hours of Coronary Occlusion

Intramembranous particles were scattered randomly, and no aggregation was observed over the E faces (Figure 7). P particles were aggregated in patches or in a circular fashion (Figure 8). There was an increase in the number of nicks in the membrane (2.2/sq μ).

Discussion

Involvement of cell membranes in the process of cell injury at the molecular level is poorly understood at present. However, it is believed that the alterations in membrane proteins and lipids interfere with normal functioning of the cell.⁹ Morphologically, these biochemical changes are impossible to reconcile using normal thin sectioning techniques. However, freeze-fracturing biologic membranes splits them through the lipid

bilayer and exposes the hydrophobic region for study.¹⁰ The membrane faces contain particles which are inserted in the lipid bilayer.

Intramembranous particles were randomly distributed over the P face of normal myocardial cell membranes. Within 1 hour of ischemic injury these particles began to aggregate on a limited scale, but this aggregation became greater upon reperfusion in myocardium ischemic for 45 minutes. The particle aggregation in this tissue after coronary reperfusion was greater than myocardium after 2 hours of simple coronary artery occlusion. These changes appeared at a time when the cell membrane lost its selective permeability to various electrolytes² and allowed leakage of intracellular enzymes. Thus, the alterations seen in the lipid bilayer are most likely associated with an increase in cell membrane permeability which is indirectly shown in the form of cell swelling, influx of ions, and release of intracellular enzymes.

Changes in the lipid composition of the cell membrane are likely to cause the aggregation of protein particles, a phenomenon related to the fluidity of the lipid bilayer.¹¹ However, effects of this interaction between membrane lipid and proteins on cell activities are merely speculative at present. Nevertheless, it seems likely that modified membrane areas from which the proteins have mobilized laterally are associated with structural abnormalities in the cell membrane, perhaps in altered permeability. Changes in the immediate environment of ischemic cells, such as lowering of intracellular pH due to accumulation of lactic acid,¹²⁻¹³ probably cause aggregation of particles similar to that reported to erythrocytes.¹⁴ Cell swelling may further stretch the lipid bilayer and affect the membrane-associated functions.

Intramembranous particles are also known to aggregate under different preparative procedures, such as freeze-fracture of unfixed or glycerol-treated unfixed cells,¹⁵ thus producing freeze-fracture artifacts. However, in our study the tissue was fixed with buffered glutaraldehyde before glycerol treatment. Consequently, the particles were distributed randomly over the normal membranes and were aggregated only in the ischemic tissue. Therefore, it is unlikely that the alterations described in the membrane components are due to freeze-fracture artifacts.

In addition to rearrangement of intramembranous particles, numerous nicks were seen in the lipid bilayer which were frequently observed during advanced ischemia. Because they are present in both fracture faces, these nicks obviously represent holes extending through the membrane. Swelling of ischemic cells due to abnormal retention of fluids¹⁶ and loss of cell ability to regulate its volume after reflow¹⁷ appear to follow the development of these nicks in the membrane. Thus, the role of sarco-

lemma in the pathogenesis of irreversible injury appears to be real and deserves further study.

Reflow of blood after a critical ischemic period of 40 minutes fails to prevent necrotic changes in dying myocardial cells.¹⁻² This conclusion is substantiated by the results of this study, in which irreversible ultrastructural alterations were observed, being more conspicuous in the mitochondria (Figure 3). Also, in similar cells, intramembranous particles aggregated and nicks occasionally developed in the sarcolemma, while these changes were not prominent in cell membranes of hearts occluded for 40 minutes. On the other hand, renal ischemia induced slight aggregation of intramembranous particles as early as 30 minutes and was more pronounced after 60 minutes of ischemia.⁵ This particle aggregation was reversible after resuming blood flow in the tissue ischemic up to 60 minutes. This discrepancy in degree of ultrastructural changes between the ischemic heart and kidney may be due to species variation or to different responses to ischemia by these organs themselves.

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All tissues for freeze-fracture were initially fixed with glutaraldehyde and infiltrated with glycerol.

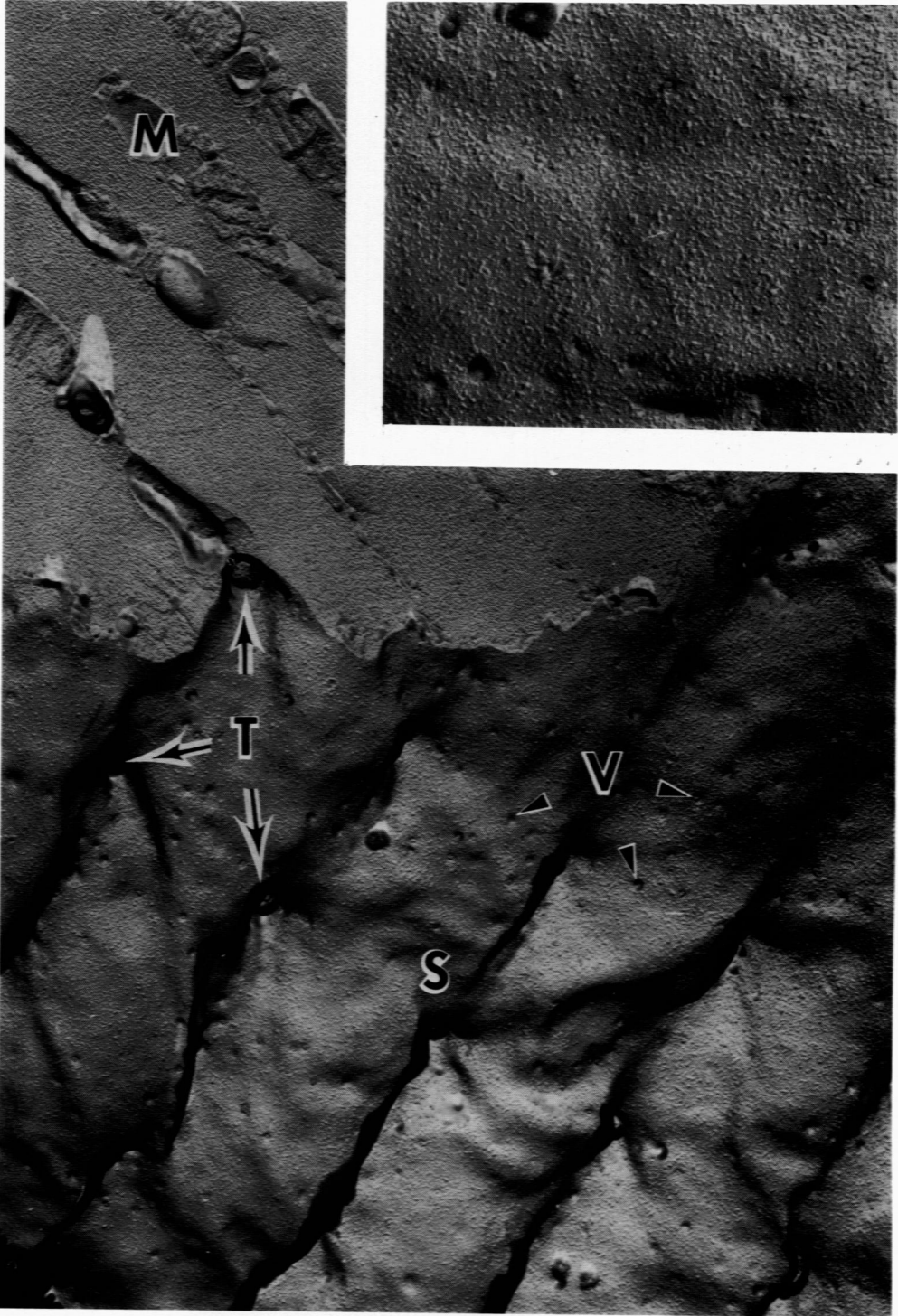


Figure 1—Longitudinal fracture of a myocardial cell showing randomly distributed particles, pinocytotic vesicles (V), and openings of T tubules (T) over the P face of the sarcolemma (S). A portion of the cell at the top is exposed, revealing myofibrils and mitochondria (M). *Inset*—Higher magnification showing particles on the sarcolemma P face. ($\times 18,400$, *inset*, $\times 38,000$)

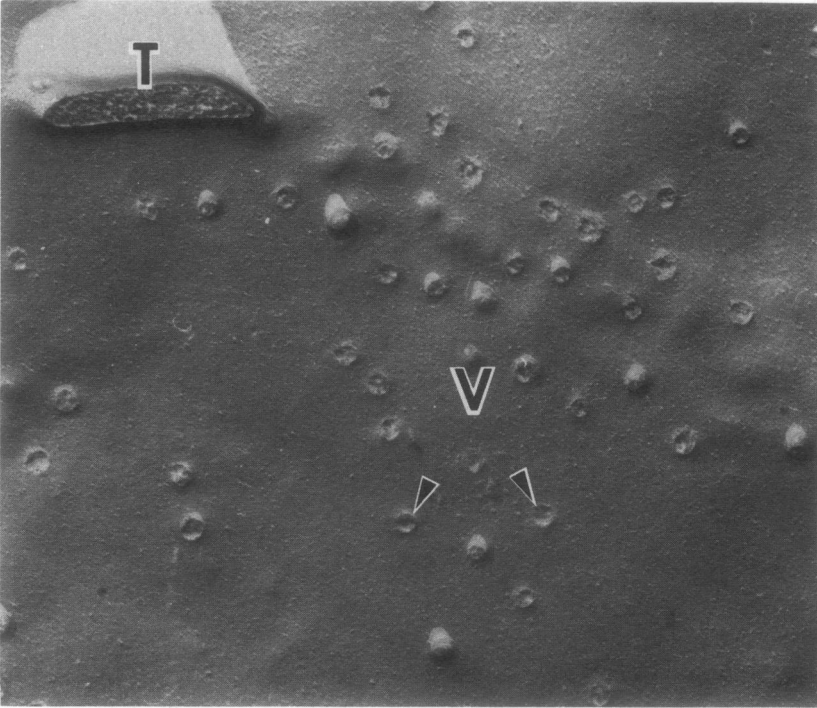


Figure 2—E fracture face of sarcolemma showing relatively few particles. V = pinocytotic vesicles, T = T tubules. ($\times 35,000$)

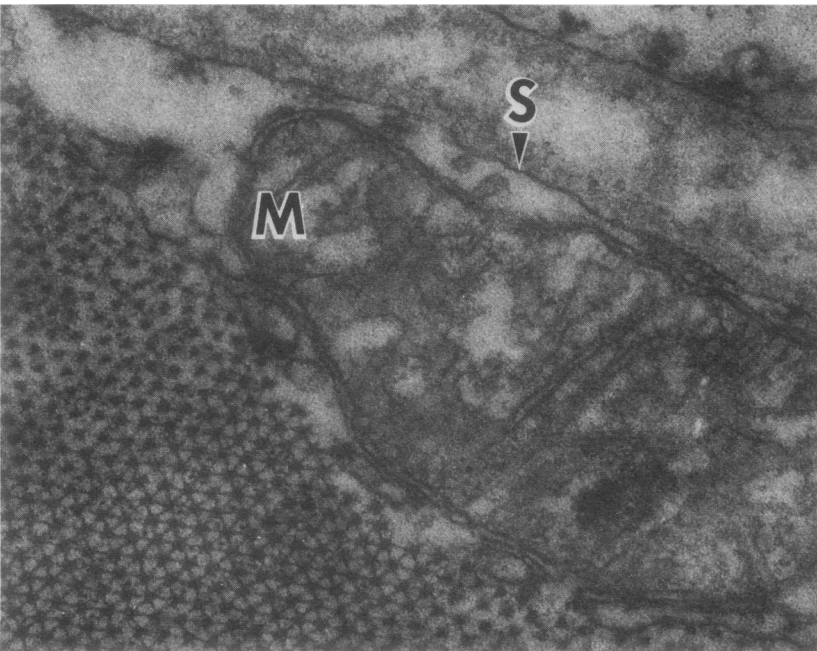


Figure 3—Thin section of myocardium following 2 hours of coronary occlusion showing intact sarcolemma (S). Electron-dense deposits are seen in the mitochondria (M). ($\times 62,000$)

Figure 4—P face of sarcolemma from myocardium following 45 minutes of coronary occlusion showing slight aggregation of particles (arrow) ($\times 35,000$).

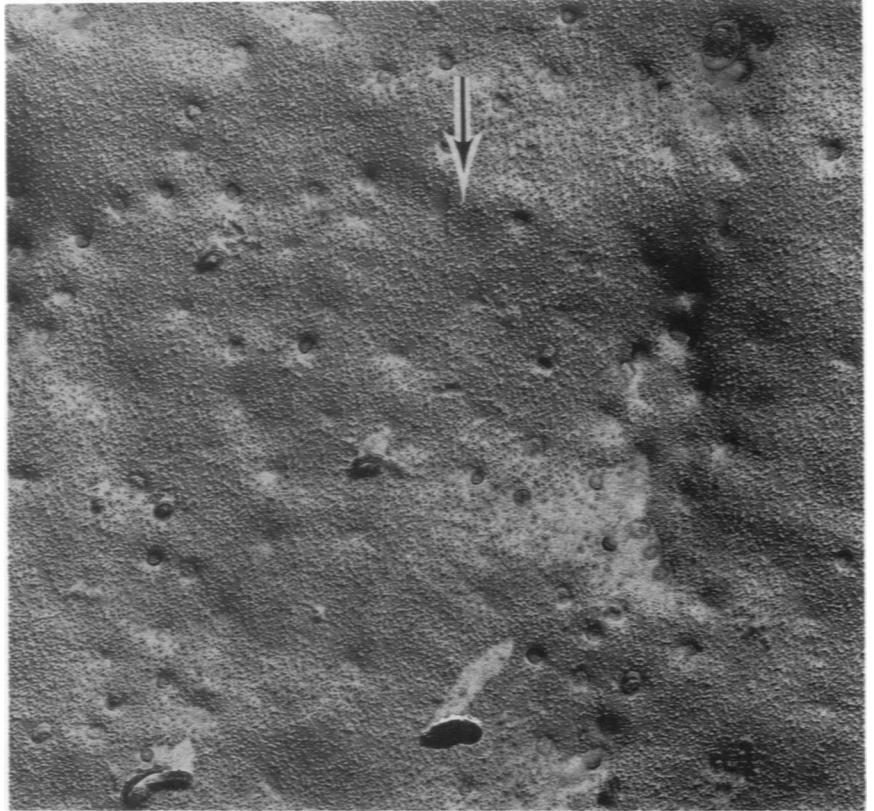
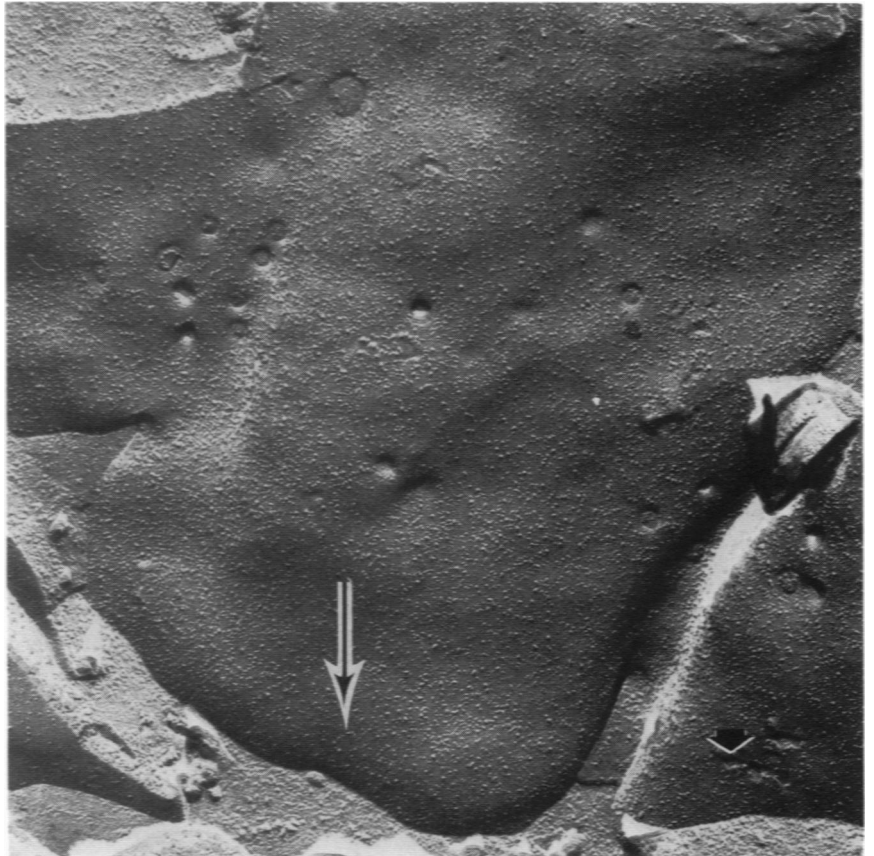


Figure 5—P face of sarcolemma from myocardium following 45 minutes of coronary occlusion and 5 minutes of reperfusion showing aggregation of particles in a circular fashion (arrow). Some nicks or breaks are also seen in the lipid bilayer (arrowhead). ($\times 43,000$)



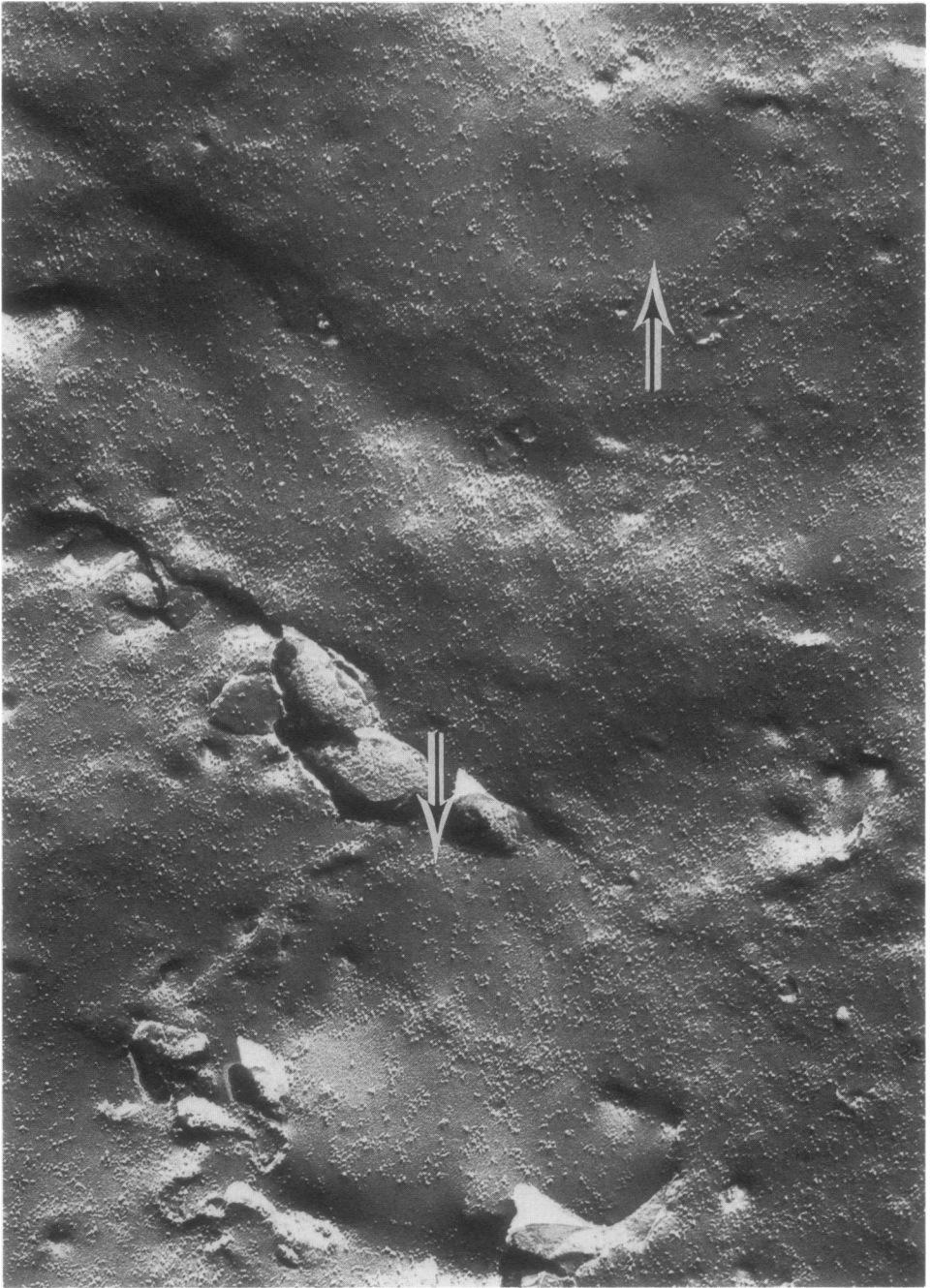


Figure 6—P face of sarcolemma from myocardium following 45 minutes of coronary artery ligation and 20 minutes of reperfusion showing marked aggregation of particles, leaving zones of lipid bilayer devoid of particles (*arrow*) ($\times 48,000$).

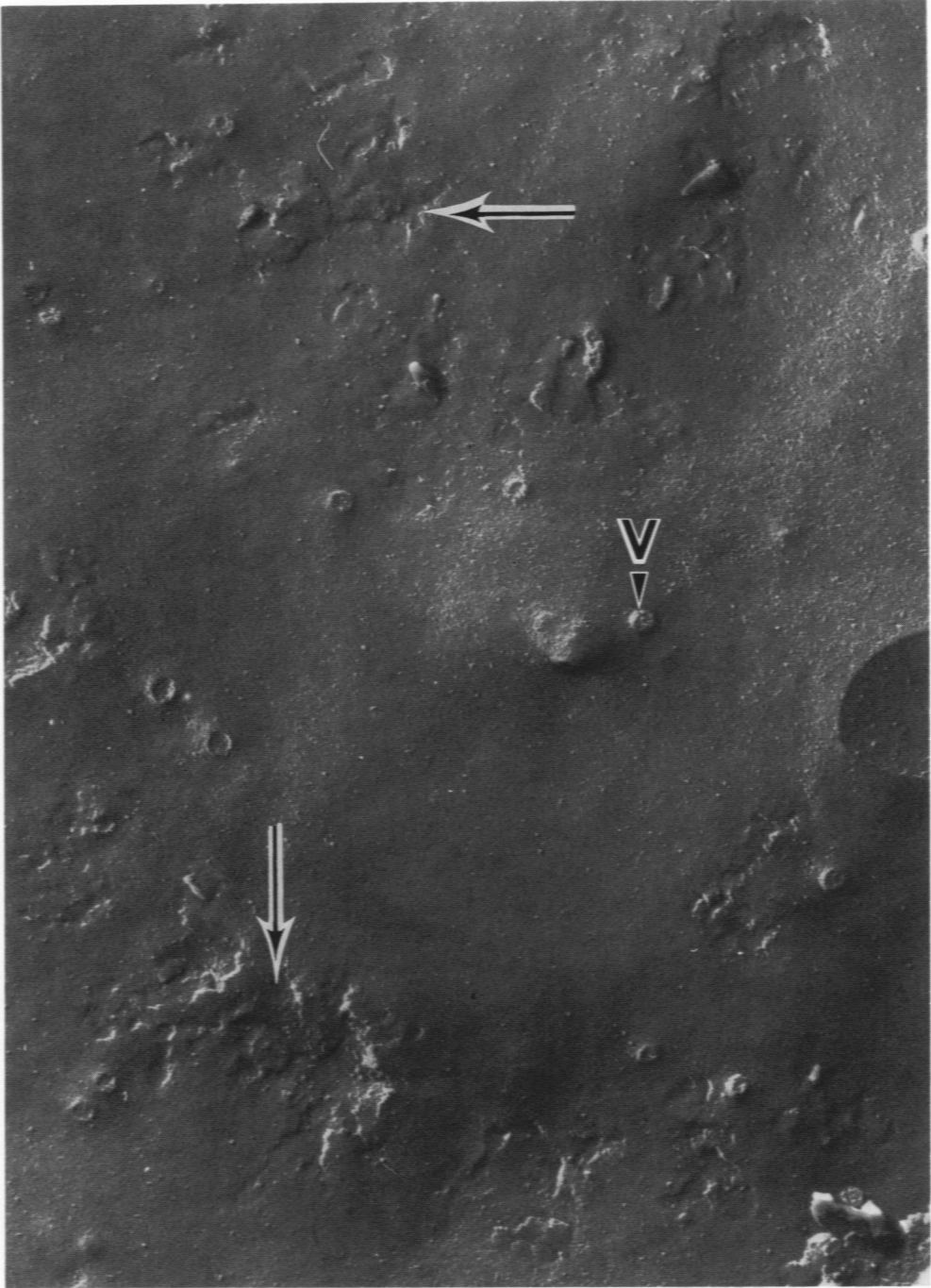


Figure 7—E face of sarcolemma from myocardium following 2 hours of coronary artery occlusion. Numerous nicks are seen in the lipid bilayer (*arrows*). The number of pinocytotic vesicles (*v*) are reduced. ($\times 45,000$)

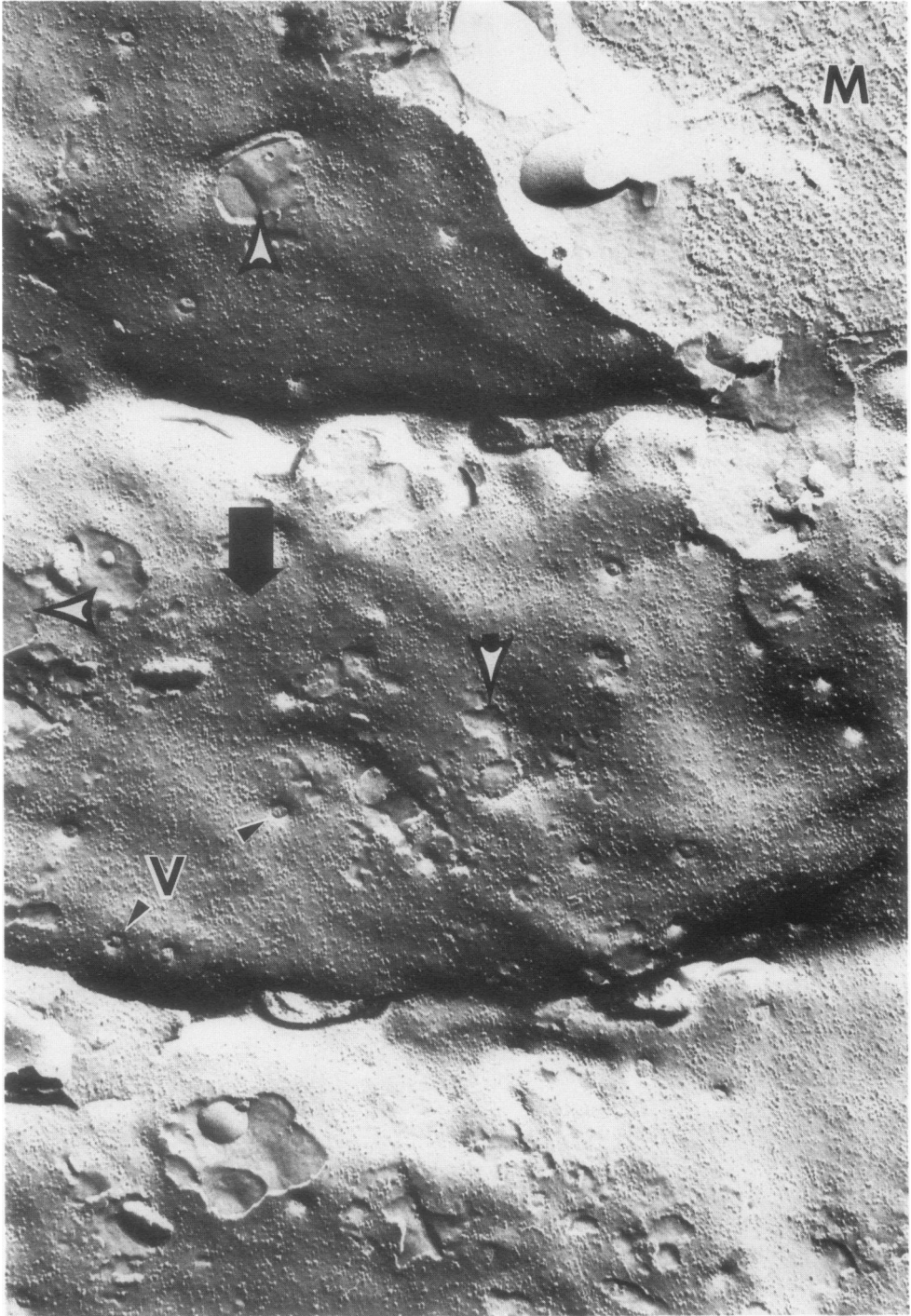


Figure 8—P face of sarcolemma from myocardium following 2 hours of coronary artery occlusion. Particles are aggregated in a circular fashion (*arrows*). Numerous nicks are observed in the lipid bilayer (*arrowhead*). *M* = mitochondrion. ($\times 40,000$)