Irreversible Ischemic Cell Injury

Prevention by Chlorpromazine of the Aggregation of the Intramembranous Particles of Rat Liver Plasma Membranes

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Ischemic rat liver tissue has been shown previously to exhibit a markedly accelerated rate of phospholipid degradation, producing a loss of almost one half the total cellular phospholipid with 3 hours of ischemia. Pretreatment of the rats with chlorpromazine completely prevented the disturbed phospholipid metabolism at the same time that it prevented the cell death associated with as much as 3 hours of ischemia. Lipid-depleted microsomal membranes were shown previously to manifest alterations in their structure and function. The present report documents that similar structural alterations are evident in ischemic liver cell plasma membranes. The technique of freeze-fracture electron microscopy was used to examine the morphology of ischemic liver cell plasma membranes. Freeze-fracture replicas of whole tissue fragments exhibited a diffuse aggregation of the intramembranous particles in the P face of the plasma membranes. The incidence of this change correlated with the duration of ischemia. Pretreatment of the rats with chlorpromazine (20 mg/kg) for 30 minutes before inducing ischemia prevented the aggregation of the membrane-associated particles. These findings establish the existence of plasma membrane alterations in ischemic liver cells. The time course of these changes, their prevention by chlorpromazine, and their similarity to the previously described structural alterations in the microsomal membranes suggest that they are related to the loss of liver cell phospholipid. The data in the present report support the hypothesis that an accelerated phospholipid degradation and its resultant membrane dysfunction are the critical alterations that produce irreversible liver cell injury and, ultimately, cell death in ischemia. (Am J Pathol 92:713–732, 1978)

RECENT STUDIES in our laboratory using a rat liver model have shown that ischemia induces a marked disturbance in phospholipid metabolism.[1,2] Whole homogenates and post-mitochondrial supernatants from livers ischemic for 3 hours showed a 40% and 55% decrease in total phospholipids, respectively. Pretreatment of the animals with chlorpromazine prevents the liver cell death produced by as much as 3 hours of ischemia[3] and prevented the loss of phospholipids from both the whole homogenates and post-mitochondrial supernatants.[1,2] This loss of phospholipid is entirely due to an accelerated rate of degradation, with a half-life of 2 to 4 hours for ischemic compared with 24 hours for control microsomal membrane phospholipids.[1,2]

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Phospholipid loss is associated with manifestations of membrane dysfunction.[1,2,4] Microsomes prepared from the lipid-depleted post-mitochondrial supernatants exhibited considerable alterations in their structure and function with inhibition of glucose-6-phosphatase and calcium pump activities and a 25 to 50-fold increase in their passive permeability to Ca^{2+} ions. Electron micrographs of freeze-fractured ischemic microsomes showed fewer intramembranous particles, bare membrane regions devoid of particles, and areas containing aggregations of intramembranous particles.[2]

A number of reasons lead us to believe that this disturbance in phospholipid metabolism may be the critical alteration that produces irreversible cell injury in ischemia. The time course closely parallels that of the loss of reversibility. Chlorpromazine prevents the changes in phospholipid in parallel with the prevention of cell death. Finally, the functional consequences, particularly as reflected in an altered permeability of isolated microsomal membranes, are relevant to the increasing evidence that disturbed membrane function participates in the production of irreversible ischemic cell damage.[5]

The critical link in any such sequence connecting an accelerated phospholipid degradation with the onset of irreversible injury is the plasma membrane, for the changes in cell physiology that characterize irreversibly injured ischemic cells are indicative of plasma membrane dysfunction specifically.[5] It became important, therefore, to establish that the alterations in phospholipid metabolism in ischemic liver cells are reflected in changes in plasma membrane phospholipids. However, attempts to isolate plasma membranes from ischemic liver cells by the conventional methods based on membrane buoyant density [6] have been unsuccessful.[7] In the present study we have used the technique of freeze-fracture electron microscopy to look for morphologic alterations in ischemic rat liver plasma membranes and have observed an extensive aggregation of the membrane-associated particles dependent on the duration of ischemia and prevented by pretreatment with chlorpromazine. These findings suggest that alterations in the structure of the plasma membrane occur in parallel with the previously described changes in microsomal membrane structure.[2] Similar changes have also been reported in ischemic kidney and heart muscle cell plasma membranes.[8,9]

Materials and Methods

Female Wistar rats (Charles River Farms) weighing 140 to 150 g and fasted for 18 hours overnight were used in all experiments. With the rats under light ether anesthesia, an abdominal midline incision was made and liver ischemia was induced by clamping the portal venous and hepatic arterial blood supply (Thomas, 27 mM, No. 3873–S60) to the

left lateral and median lobes. After the desired duration of ischemia, the animals were again placed under light ether anesthesia and the abdomen was reopened through the original wound. The liver was excised and samples were taken from the left lateral lobe. Fixation was at room temperature in a 0.1 M phosphate buffer, pH 7.2, containing 2.5% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.). After fixation, the small blocks of tissue (1.5 cu mm) were washed in two 15-minute changes of the same buffer and infiltrated with 25% glycerol in the 0.1 M phosphate buffer for 1.5 hours at 4 C. Small pieces of liver were mounted on cardboard disks, rapidly frozen in liquid Freon 12 (Allied Chemical Corp., Morristown, N.J.), cooled with liquid N_2, and stored in liquid N_2 until used. Freeze-fracturing was performed with a Balzers device (model BA 360 Balzers High Vacuum Corp., Santa Ana, Calif.). The specimen table temperature was maintained at −103 C at the time of fracture and replication. The platinum–carbon replicas were cleaned with commercial bleach and, after washing in distilled H_2O, were picked up on formvar-coated grids. All replicas were examined in a Philips EM 300 operating at 80 kV. Images were recorded on Kodak Electron Image Plates.

Results

Normal Liver Plasma Membranes

Liver cell plasma membranes are readily identified in freeze-fracture replicas of whole tissue fragments by the presence of characteristic structures representing intercellular contacts within these membranes. In Figure 1, long branching and anastomosing "chains" of intramembranous particles may be seen on the protoplasmic (P) face[10] of the plasma membrane immediately adjacent to a bile canaliculus. In liver cells, such a tight junction [11-13] is always found as a ribbon or band on either side of a bile canaliculus, forming a seal between liver intercellular space and the lumen of the bile canal. In contrast, gap junctions are found on the lateral surfaces of liver cells between the bile canaliculus and the space of Disse. Figure 1 illustrates that in our freeze-fracture replicas, gap junctions are readily identifiable as a dense particular lattice coplanar with the P face of the membrane.[11-13] The nonjunctional areas of liver cell plasma membranes exposed by freeze-fracture display either one of two appearances representing the different fracture faces of the membrane. One membrane surface (*EF* in Figures 1, 3, 4, 6, and 7) is sparsely studded with particles and corresponds to the half of the membrane closest to the extracellular space. The other membrane surface (*PF* in Figures 1 through 7) has a dense population of particles and corresponds to the half closest to the cytoplasm. Figure 1 shows that these particles are randomly and uniformly distributed in the plane of the membrane. Figure 2 shows the pattern of distribution of these particles under higher magnification. There are very few aggregates: most of the particles are single, with an occasional group of two particles seen together. Except for areas immedi-

ately adjacent to a tight junction, there are no significant areas in the P surface devoid of particles.

Effect of Ischemia on Plasma Membrane Morphology

Figures 3, 4, and 5 illustrate the effect of periods of ischemia associated with irreversible cell injury on the morphology of rat liver plasma membranes. Readily apparent is the diffuse aggregation of the membrane-associated particles in the exposed P faces. This aggregation affects the entire area exposed by the freeze-fracture technique, leaving no areas with a normal particle distribution. In Figure 5 the aggregates can be seen to consist of from 10 to 30 individual particles, leaving relatively large bare areas devoid of particles or containing one or two particles that are widely spaced. While there is no aggregation of the particles in the E face, there is clustering of the pits in the E face as shown in the portion of this face visible in the lower portion of Figure 3.

The incidence of intramembranous particle aggregation is dependent on the duration of ischemia. The frequency with which aggregation of the membranous particles was observed in random replicas of freeze-fractured whole liver fragments increased with the duration of ischemia. It was seen in only 1 of 40 replicas examined from liver tissue ischemic for only one-half hour. With 1 hour of ischemia, approximately one quarter to one third of the replicas examined showed aggregation of the membrane particles. Over one half the replicas showed this change after 90 minutes of ischemia. It was very difficult to study the plasma membrane changes associated with ischemic periods much longer than 90 minutes, since such durations were associated with considerable swelling of the cells, rendering their analysis by freeze-fracture difficult. In addition, prolonged periods of ischemia rendered the plasma membranes relatively resistant to fracturing through their interior as opposed to along either their inside or outside surface.

Although it was apparent that there was a correlation between the duration of ischemia and the incidence of the aggregation of intramembranous particles, it was more difficult to appreciate variations in the extent of aggregation. Comparison of Figures 3 and 4 suggests that there is greater aggregation of the particles in Figure 3. At both 1 and 1.5 hours of ischemia there seemed to be this kind of variation in the extent of aggregation in different replicas in contrast to a more uniform aggregation of the particles in any one replica. It is, of course, not known if this variation between replicas represents different stages in the reaction of the plasma membranes to ischemia or simply similar stages of a variable response pattern.

Effect of the Pretreatment with Chlorpromazine

Pretreatment of the rats with chlorpromazine prevents the aggregation of the intramembranous particles seen in the untreated ischemic cells. This is illustrated by the essentially normal-appearing plasma membranes in Figures 6 and 7. In all of the replicas from the chlorpromazine-pretreated animals, nothing resembling the appearance of the membranes that results from ischemia alone was observed. In the chlorpromazine-treated case, the intramembranous particles are distributed randomly and homogeneously throughout the P faces of the exposed plasma membranes (Figure 6). With higher magnification (Figure 7), the particles can be seen to be distributed randomly, with no tendency to form aggregates as in Figures 3, 4, and 5.

Discussion

We have shown that there is an extensive reorganization of the distribution of the membranous particles visible in the P face of freeze-fractured rat liver plasma membranes in association with periods of ischemia that produce irreversible cell injury. Protection of the cells from such injury by pretreating with chlorpromazine prevents this aggregation. These findings establish that structural alterations in plasma membranes develop in parallel with the disturbances in phospholipid metabolism produced by ischemia and prevented by chlorpromazine. The main questions raised by these observations are a) whether the alterations in the plasma membranes are related to changes in the phospholipids and b) whether the protective effect of chlorpromazine is a consequence of its ability to prevent such changes or is related to some other effect of this drug on cellular membranes.

It is becoming increasingly clear that modulation of the distribution of intramembranous particles is a relatively common reaction of a variety of membranes to a number of different kinds of physiologic, pharmacologic, and pathologic stimuli. In all these cases one usually observes some degree of aggregation of the intramembranous particles in the lateral plane of the membrane as revealed by the freeze-fracture technique. In most cases the mechanism responsible for this aggregation and the functional consequences with respect to membrane physiology are not well understood.

Probably the most readily definable mechanism of particle aggregation is that related to a decreased fluidity of the membrane accompanying a reduction in temperature. Changes in the distribution of membrane-associated particles at low temperature have been attributed to phase transitions (liquid crystal to gel state) in the membrane phospholipid bilayer.[14-22] Lateral motion and consequent aggregation of intra-

membranous particles occur with low temperatures and have been attributed to the growth of protein-excluding regions of liquid crystal to gel state phase transitions in the bilayer lipid that are observed as smooth, particle-free patches. In natural membrane systems as well as in reconstituted membrane systems [14-24] exposure to low temperatures causes particle-rich regions in which the phospholipids are still in the liquid crystal state to separate from these particle-poor regions (gel state lipid).

Changes in the phospholipid content of liver cell plasma membranes could produce a similar reduction in membrane fluidity and play a role in the particle aggregation we have observed. In this case reduced fluidity would obviously not be brought about by reduced temperature. We would suggest that a decreased phospholipid content produces essentially the same end result as the temperature-induced partial freezing-out of phospholipid molecules during phase transitions with simply less total fluid lipid in which the proteins can disperse.

There are several features of the plasma membrane alteration induced by ischemia that would suggest it is directly related to the disordered phospholipid metabolism previously described.[1,2] The structural alterations in the plasma membranes develop with the same time course as the changes in liver cell phospholipid content. Chlorpromazine prevents the plasma membrane alterations in parallel to its protection against the lipid losses. Finally, the structural alterations in the plasma membrane are very similar to those observed in the ischemic lipid-depleted microsomal membranes.[2]

We have suggested that the accelerated phospholipid degradation in ischemic liver cells results from the activation of endogenous, membrane-bound phospholipases.[1,2] Virtually all membrane systems that have been isolated from liver cells have been shown to contain phospholipase activity.[25] Rat liver microsomes [26,27] and plasma membranes [26,28] contain an alkaline phospholipase A_1 activity. Plasma membranes also seem to contain an additional phospholipase A_2 activity.[29,30] Both enzymes require Ca^{2+} with optimal activity from 0.5 to 2.0 mM. We have recently shown that the *in vitro* incubation of normal liver microsomes with excess Ca^{2+} produces a progressive loss of phospholipid with a time course in hours similar to that with ischemia and associated with inhibition of glucose-6-phosphatase and calcium pump activities and an increased permeability to Ca^{2+}.[31] Incubation of the microsomes with Ca^{2+} in the presence of chlorpromazine prevented the loss of phospholipids, consistent with the reported inhibition of phospholipases from a variety of sources by chlorpromazine.[32,33] We would suggest that a similar activation of the analogous phospholipase activity (or activities) in liver cell plasma membranes

with consequent loss of membrane lipid and a decreased fluidity could readily account for the structural alterations described here.

An alternate explanation for particle aggregation is the action of Ca^{2+} ions themselves on cellular membranes. Exposure of whole cells or isolated cellular organelles to Ca^{2+} ions (in some cases with an ionophore to promote uptake of the Ca^{2+} ions) can result in aggregation of membrane-associated particles.[34-38] Such calcium-induced changes in membrane conformation are believed to promote membrane fusion and to be intimately associated with the known role of Ca^{2+} ions in the stimulus–secretion coupling in a wide variety of cell types.[36-41] There are several possible bases for this action of Ca^{2+} ions. They may act by an interaction with spectrin or "spectrin-like" proteins, leading to aggregation of integral membrane proteins, somewhat analogous to the action of Ca^{2+} in muscle contraction.[34] It is difficult to invoke this mechanism to account for the aggregation of particles described here, since it is not known if there are proteins analogous to spectrin in liver cell membranes. On the other hand, Ca^{2+} ions may be interacting directly with the lipids of the membranes to produce particle aggregation. The binding of Ca^{2+} to phosphatidylserine in lipid bilayers yields solid aggregates of phosphatidylserine and allows the other phospholipids to form a separate fluid phase.[42,43] This is somewhat analogous to the effect of temperature on the membrane lipids. As noted above, Ca^{2+} ions activate endogenous, membrane-bound phospholipases, with resultant changes in the lipid composition of the membrane. Ca^{2+} has been shown to fuse chicken erythrocytes at an alkaline pH in the absence of any added promoting agent like Sendai virus.[44] Under these conditions, a low percentage of the membrane phosphatidylethanolamine and lecithin are hydrolyzed.

Either of these mechanisms may actually be responsible for Ca^{2+}-induced particle aggregation; this phenomenon could play a role in the plasma membrane changes in ischemic liver cells. There is a progressive increase in the liver cell calcium content during the period of ischemia that is distinct from the much more marked increases in cell Ca^{2+} associated with reperfusion.[3] This increased calcium content is not sequestered in the mitochondria[3] in the absence of oxygen nor is it likely to be pumped into the cisternae of the endoplasmic reticulum in the face of the severe loss of ATP. We realize that this increased Ca^{2+} content could simply be an effect of the membrane changes we have described. It is also possible, however, that they are causally related to them.

It is important to point out that if either of the mechanisms we have discussed to account for the aggregation of membranous particles, ie, loss of phospholipids or a direct effect of Ca^{2+} ions, is operating in the

ischemic liver cells, the ability of chlorpromazine to prevent this reaction is readily explainable. We have reported previously that pretreatment with chlorpromazine prevents both the loss of phospholipid [1,2] and the increased liver cell Ca^{2+} content during the ischemic period and following reflow.[3]

Chlorpromazine is also known to have a variety of effects on the physical properties of membranes [45]; it is possible, therefore, that the protective effect of chlorpromazine that we have observed may not be related to a specific and direct antagonism of the causal mechanism itself, eg, prevention of phospholipid depletion. Interaction of chlorpromazine with dipalmitoyl phosphatidylcholine model membranes produces a higher fluidity in the fatty acid chain region and a reduced mobility of the polar headgroups of the phospholipids.[46] A model for the incorporation in the lipid bilayer was proposed in which the dialkylaminoalkyl chains are located near the polar headgroups and the ring system penetrates the hydrocarbon phase slightly beyond the glycerol backbone.[46] As a result of such interaction, the fluidity of the membrane is increased,[46] the volume of bilayer is expanded,[45] and calcium ions bound to the phosphate groups of the lipids are displaced.[45] Such effects on phospholipid bilayers presumably relate to the ability of chlorpromazine to modulate a variety of membrane-dependent effects in a variety of cell types. Chlorpromazine provides protection against osmotic lysis of erythrocytes [47] and inhibits several kinds of cell-to-cell interactions (platelet adhesiveness,[48,59] cell fusion,[50] and leukocyte adherence to endothelium [51]). Chlorpromazine inhibits the secretion of plasma proteins from liver cell slices [52] and prevents and disrupts immunoglobulin caps in lymphocytes.[53] The latter is prevented and reversed by raising the extracellular calcium concentration.[53] All of these effects of chlorpromazine imply that it can affect many diverse membrane-associated reactions. This would suggest that the prevention of intramembranous particle aggregation in liver cell plasma membranes reported here could be a nonspecific consequence of the interaction of chlorpromazine with cellular membranes and, therefore, not necessarily directly related to prevention of either the calcium increases or the accelerated phospholipid degradation.

It is interesting to speculate on the role, if any, of plasma membrane particle aggregation in the altered Ca^{2+} homeostasis that characterizes the loss of reversibility of ischemic liver cell injury. The reperfusion of irreversibly injured ischemic liver cells is accompanied by a marked increase in the cell calcium content.[3] We have shown that microsomal membranes isolated from liver cells that have been ischemic for 2 hours are at least 25 times more permeable to Ca^{2+} ions at 1 mM total calcium

than are control membranes.[1,2] Wade et al [54] suggested a number of mechanisms whereby aggregations of intramembranous particles might produce increased permeability, perhaps simply by increasing the number of small aqueous pores in the membrane. Or, by bringing integral membrane proteins into close apposition, transmembrane pathways might be produced in the membrane between the now adjacent proteins. Another possibility is that aggregation alters the position of proteins in the membrane such that they span the membrane and thus serve as transmembrane pathways. Or it could be that by being in close apposition the proteins in some way alter the lipid moieties at sites of aggregation. These speculations mainly serve to indicate that the aggregated particles could represent sites of altered membrane calcium permeability.

In conclusion, the demonstration in this report of the development of plasma membrane alterations in ischemic rat liver supports our current working hypothesis that changes in membrane structure and function as a result of a markedly increase rate of phospholipid degradations underly the development of irreversible ischemic cell injury. The present report also focuses on the necessity for further study of the effects of the activation of the endogenous, plasma membrane-bound phospholipases on the structure and function of this membrane.

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[*Illustrations follow*]

Figure 1—Freeze-frature replica of a normal rat liver cell. A bile canaliculus (*BC*) is separated from a lateral surface plasma membrane by a tight junction (*arrow*). A gap junction (*arrowhead*) is seen at the *right middle* of the field. *PF* = P face according to Branton et al [10]; *EF* = E face. (× 38,000)

Figure 2—Freeze-fracture replica of a normal rat liver cell. The protoplasmic face (*PF*) of a lateral surface plasma membrane is seen with a tight junction (*arrow*) in the *upper right corner* and a gap junction (*arrowhead*) in the *lower right corner*. The intramembranous particles can be seen to be uniformly and randomly distributed in the P face. (× 84,000)

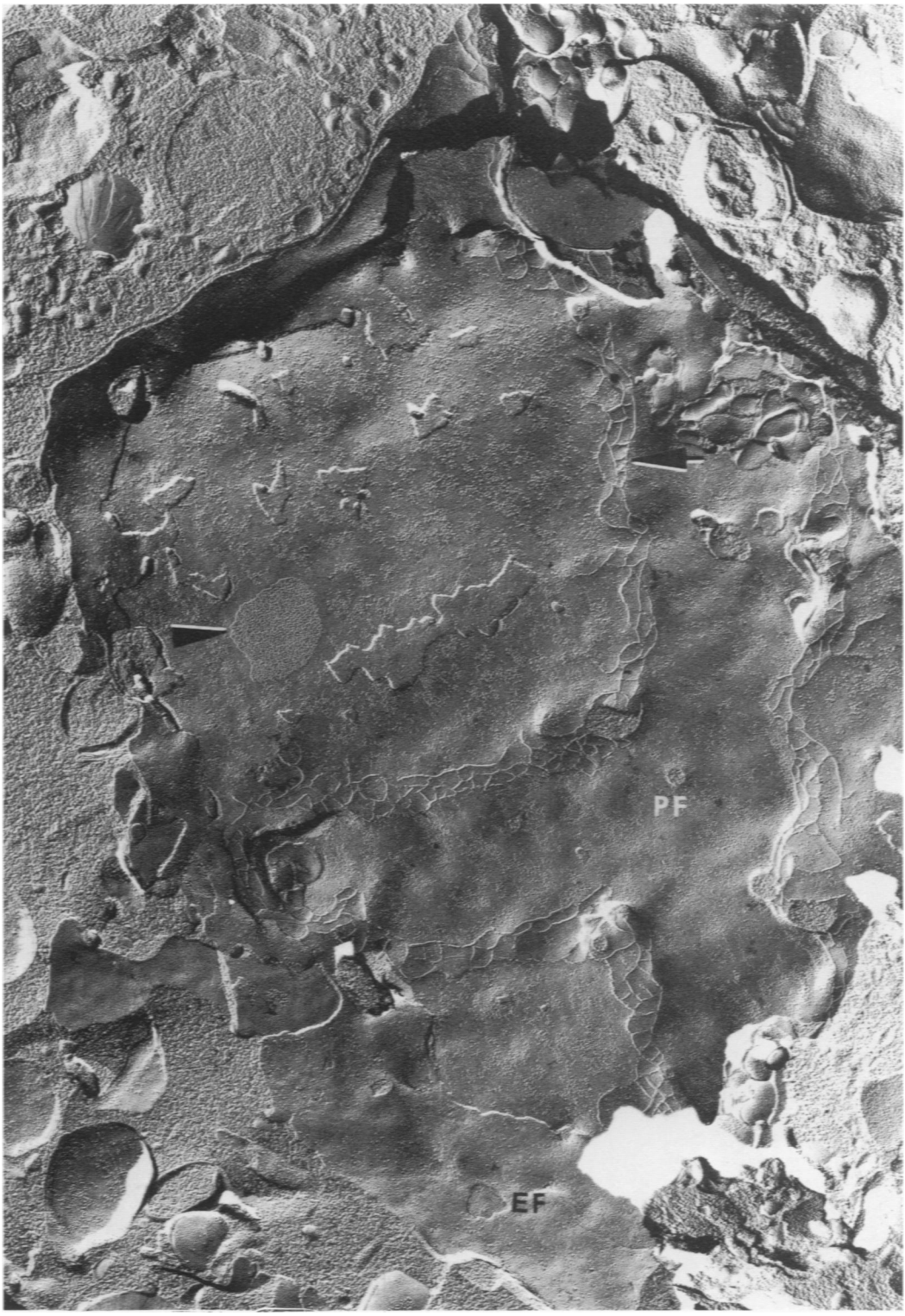

Figure 3—Freeze-fracture replica of a rat liver cell made ischemic for 1 hour by clamping the portal venous and hepatic arterial blood supply to the left lateral and median lobes. An extensive tight junction (*arrow*) runs through the center of an exposed lateral surface plasma membrane. A small portion of the bile canaliculus can be seen immediately above the arrow in the *upper right portion* of the field. A gap junction (*arrowhead*) is seen in the *left center*. The intramembranous particles in the exposed P face are extensively aggregated with virtually no areas containing a normal distribution of particles. *PF* = P face; *EF* = E face. (× 27,500)

Figure 4—Freeze-fracture replica of a rat liver cell made ischemic for 1 hour. A lateral surface plasma membrane is seen, with the E face on the *left* and the P face on the *right*. A portion of a tight junction (*arrow*) is visible in the E face at the *lower left*. The intramembranous particles visible in the P face are aggregated with many clumps consisting of 10 to 30 individual particles. Bare areas devoid of particles are seen between the aggregates. *PF* = face; *EF* = E face. (× 53,125)

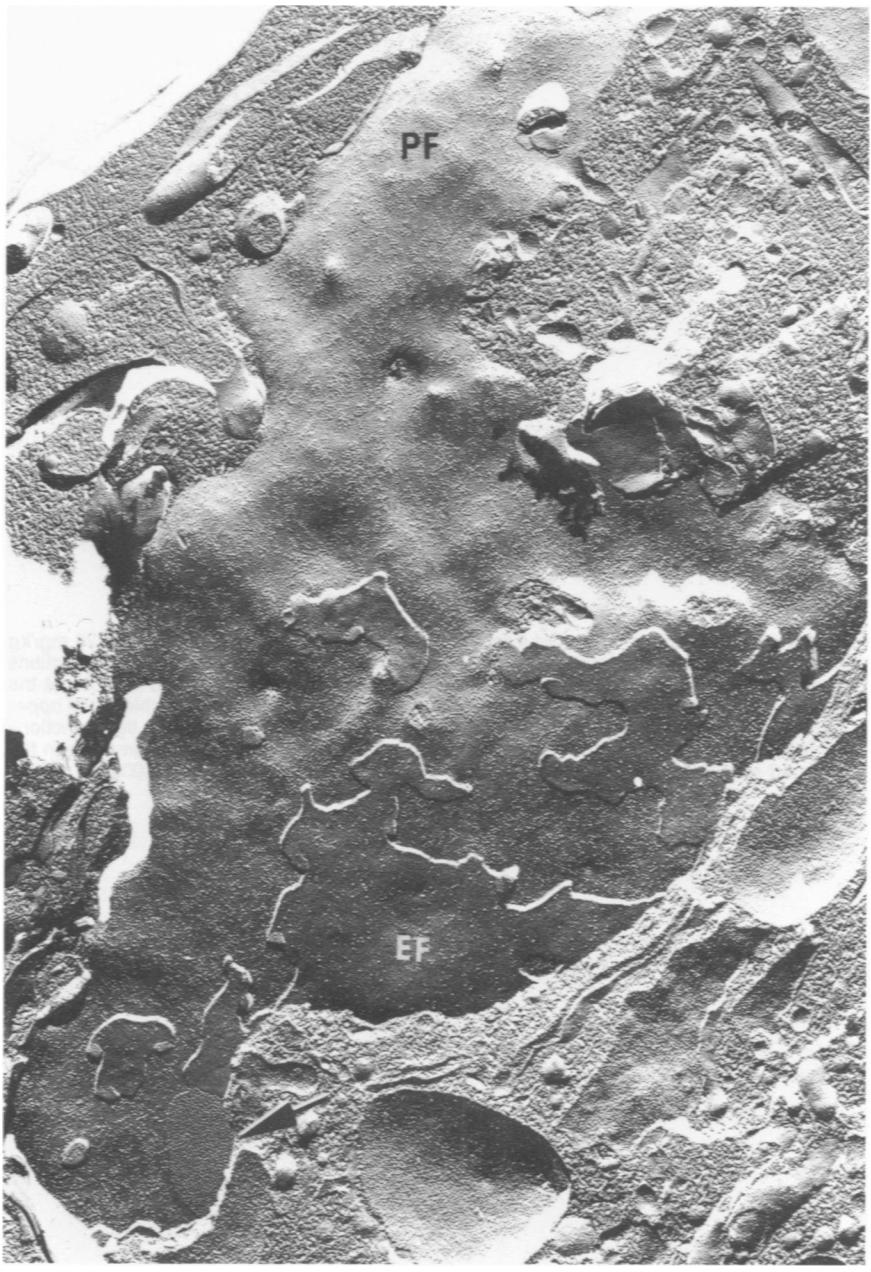

Figure 5—Freeze-fracture replica of a rat liver cell made ischemic for 1 hour. A lateral surface plasma membrane has been exposed. A large area of the P face is visible, with smaller and interrupted areas of the E face. A gap junction is visible in the *lower left corner*. The intramembranous particles in the P face of the plasma membrane are diffusely aggregated; no normal areas are visible. *PF* = P face; *EF* = F face. (× 27,500)

Figure 6—Freeze-fracture replica of a rat liver cell pretreated with chlorpromazine (20 mg/kg body weight) 30 minutes before induction of ischemia for 1 hour. Two extensive tight junctions (*arrow*) run through the center of the field and are seen in both the P and E faces of the exposed lateral surface plasma membrane. A gap junction (*arrowhead*) is visible in the *upper left corner*. The intramembranous particles in the P face above and below the tight junctions are distributed in a diffuse and random manner, with no tendency to form clumps as in the untreated cells ischemic for the same time. *PF* = P face; *EF* = E face. (× 30,000)


PF
EF

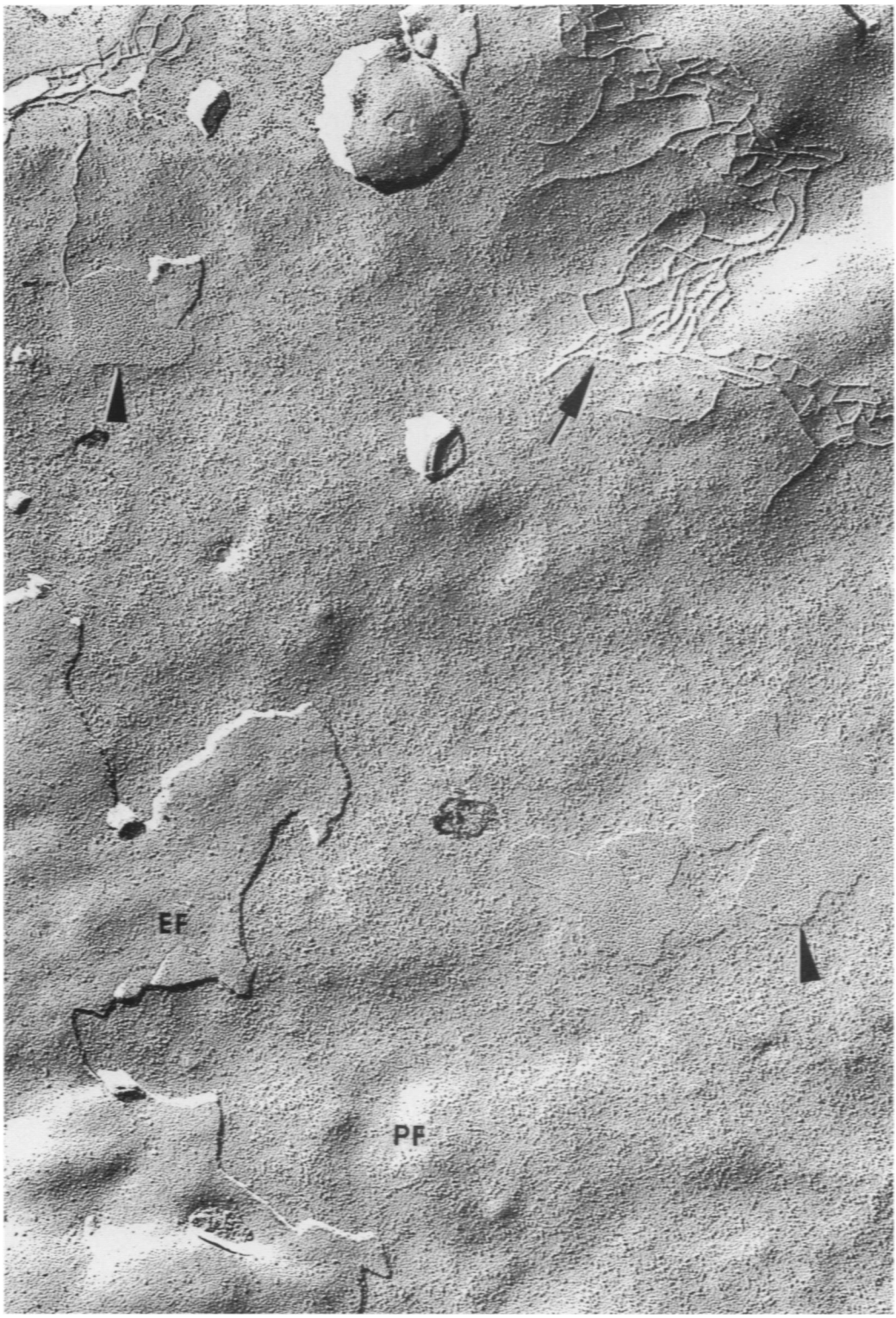

Figure 7—Freeze-fracture replica of a rat liver cell pretreated with chlorpromazine (20 mg/kg body weight) 30 minutes before induction of ischemia for 1 hour. A tight junction (*arrow*) runs across the upper portion of the field delimiting a lateral surface plasma membrane below. A gap junction (*arrowhead*) is visible in the *lower right*. The intramembranous particles visible in the exposed P face are randomly distributed with no tendency to form clumps. *PF* = P face; *EF* = E face. (× 38,000)