FINE-STRUCTURAL CHANGES IN RAT LIVER MICROSOMES TREATED WITH PHOSPHOLIPASE C

JOAN A. HIGGINS
Department of Human Biology and Anatomy, University of Sheffield, Sheffield S10 2TN, U.K.

SUMMARY
Phospholipase C hydrolyses 50 % of the phospholipids of rat liver microsomes. Our previous observations suggest that the hydrolysis is of the outer leaflet of the membrane bilayer. In the present investigation the products of hydrolysis and the effect of phospholipase C treatment on the morphology of total, rough and smooth microsomes were investigated. The only product of phospholipase C treatment of microsomes was diglyceride. After hydrolysis of half the phospholipid, the microsomes retained their vesicular structure; however, the average diameter of total and smooth microsomes was reduced by approximately half, and that of rough microsomes by a third. Profiles were observed that suggested that small vesicles may form by pinching off from large vesicles. About 10-15 % of the phospholipase-treated microsomes had associated with them an amorphous droplet yielding a 'signet-ring'-like structure. The rest of the vesicle membrane retained a bilayer structure. About 10-15 % of the treated vesicles adhered in groups of two or three. In these groups the shared membrane was trilaminar. Freeze-fracture replicas of phospholipase C-treated microsomes exhibited a similar morphology to untreated microsomes having both concave and convex surfaces, the former exhibiting a greater density of intramembranous particles. These observations suggest that microsomal vesicles remain closed after treatment with phospholipase C and that the bilayer structure is retained. However, there are morphological changes that are possibly related to maintaining the stability of the membranes in which the outer leaflet consists essentially of diglyceride.

INTRODUCTION
There is considerable evidence that there is an asymmetric distribution of phospholipids about the bilayer of the red blood cell membrane (Van Deenen, 1981; Op den Kamp, 1979). This has been demonstrated by the use of phospholipases as probes (Colley, Zwaal, Roelofsen & Van Deenen, 1973; Verkleij et al. 1973; Zwaal, Roelofsen & Colley, 1973; Kahlenberg, Walker & Rohrlich, 1974), by chemical labelling of the membrane phospholipids (Bretscher, 1972; Gordesky & Marinetti, 1973; Gordesky, Marinetti & Love, 1975; Marinetti & Love, 1976), and by investigation of the availability of phospholipids for exchange by means of phospholipid-exchange proteins (Bloj & Zilversmit, 1977; Kramer & Branton, 1979; Van Meer et al. 1980; Crain & Zilversmit, 1980). Investigations of intracellular membranes, especially microsomes derived from the endoplasmic reticulum of rat liver, have been more limited, Phospholipid-exchange proteins catalyse complete exchange of phospholipids of microsomes and, therefore, have not been used successfully in studies of these membranes (Zilversmit & Hughes, 1977;
We have used phospholipase C from Clostridium perfringens and phospholipase D as probes of the distribution of phospholipids in open and closed microsomal vesicles (Higgins & Dawson, 1977; Higgins, 1979; Bollen & Higgins, 1980; Higgins, 1981). These results suggest that these membranes are asymmetric with phosphatidylethanolamine and phosphatidylserine in the inner leaflet. However, work by other investigators using phospholipase A has yielded conflicting results (Nilsson & Dallner, 1977a, b; Sundler, Sarcione, Alberts & Vagllos, 1977). For studies of membrane asymmetry it is essential that the probe used does not perturb the structure of the membrane. In view of the conflicting results between studies of the distribution of phospholipids in microsomal membranes, we have now examined the effect of phospholipase C on the fine structure of these membranes using both sections and freeze-fractured specimens. These observations suggest that, although there are morphological changes, microsomes retain the bilayer structure after treatment with phospholipase C.

**MATERIALS AND METHODS**

**Treatment of microsomes with phospholipase C**

Rat liver microsomes, and rough and smooth subfractions of these, were prepared as described previously (Higgins & Barnett, 1972; Higgins, 1976; Higgins & Dawson, 1977) and incubated with and without phospholipase C (C. perfringens, Sigma type X, chromatographically purified, or Sigma type 1) 10 units/ml in 0.25 M-sucrose, 0.005 M-Tris buffer (pH 7.4), 1 mM-CaCl₂ (1 ml) for 10 min. Both the products of hydrolysis and the morphology of the microsomes were the same after treatment with either preparation of phospholipase C.

**Preparation of microsomes for electron microscopy**

After incubation of microsomes in buffer alone, buffer without CaCl₂, or buffer plus phospholipase C, 10 vol. of ice-cold buffer were added and the microsomes isolated by centrifugation at 105000 g for 30 min. The pellets were fixed in 2% osmium tetroxide, 0.05 M-cacodylate buffer (pH 7.4) containing 4.5% dextrose for 15 h, dehydrated, and embedded in Araldite. Thin sections were cut, stained with uranyl acetate and lead citrate in a Philips EM301 electron microscope.

For stereological analysis, the experiment was repeated 3 times and 3 random blocks selected from each group of sectioning. Several fields were taken from each block for measurement of profile diameters. Diameters were measured on micrographs at a magnification of 36000 using a x10 lens fitted with a scale, classified in groups within 0.5 mm steps (equivalent to 14 nm).

For preparation of freeze-fracture replicas microsomal pellets were fixed for 3 h with 2% glutaraldehyde in 0.1 M-phosphate buffer (pH 7.4), rinsed with the same buffer and left overnight in 40% glycerol in the same buffer. Freeze-fracture replicas of small pieces of the pellets were made using a Leybold Heraeus BioEtch 2005.

**Analysis of the products of phospholipase C treatment**

After treatment with phospholipase C, microsomes were extracted with chloroform/methanol (2:1, v/v) as described previously (Higgins & Dawson, 1977) except that 10 mM-EDTA was added to the incubation medium immediately prior to extraction in order to prevent further action of phospholipase C. In some experiments 10 vol. of ice-cold buffer were added to the incubation medium, and the microsomes were pelleted by centrifugation at 105000 g for 30 min. The pellets were resuspended in a small volume of water and extracted...
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with chloroform/methanol (2:1). The lipids were separated by chromatography on thin layers of silica gel (Merck 60 F 254) using petroleum ether/ether/glacial acetic acid (80:20:2, by vol.). Spots were detected with iodine vapour, scraped and extracted with chloroform/methanol (2:1). The solvent was removed and free fatty acids were determined by the method of Duncombe (1963), and diglyceride by the method of Snyder & Stephens (1959). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) and phospholipid phosphorus by that of Bartlett (1959).

RESULTS

Products of phospholipase C treatment of microsomes

In the preparations of microsomes examined, between 45 and 50% of the microsomal phospholipids were hydrolysed as described previously (Higgins & Dawson, 1977; Higgins, 1979, 1981; Bollen & Higgins, 1980). For each μmol of phospholipid lost an equivalent amount of diglyceride was produced (Table 1). No detectable free fatty acids were produced, indicating that no significant further hydrolysis of diglyceride took place. On centrifugation all of the microsomal phospholipid and diglyceride were pelleted. The microsomal protein pelleted by centrifugation was the same in untreated microsomes and those treated with phospholipase C. In the preparations of microsomes examined in the electron microscope, therefore, there is no loss of membrane protein, phospholipid or diglyceride into the supernatant.

It has been demonstrated by other investigators (Michell, Coleman & Finean, 1973; Cater & Hallinan, 1971) that there is an acylhydrolase in microsomal membranes, which acts on diglyceride, the product of phospholipase C treatment, to produce free fatty acids. We did not detect any free fatty acids under our experimental conditions. However, on examination of the results of Cater & Hallinan (1971) it is apparent that treatment of microsomes for 60 min at 5 °C with phospholipase C yielded mainly diglyceride and that free fatty acid production was only 22% of that produced when the microsomes were post-incubated at 37 °C for 60 min. The difference between our results and those reported by others, therefore, probably lies in the experimental conditions used. In our experiments microsomes were treated with phospholipase C for 10 min, and were either extracted immediately or

Table 1. Products of phospholipase C treatment of microsomes

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>Hydrolysis of phospholipid (%)</th>
<th>Phospholipid phosphorus lost (μmol)</th>
<th>Diglyceride produced (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracted without centrifugation</td>
<td>46</td>
<td>1.89</td>
<td>1.94</td>
</tr>
<tr>
<td>With centrifugation</td>
<td>48</td>
<td>1.84</td>
<td>1.90</td>
</tr>
</tbody>
</table>

Microsomes (10 mg protein) were incubated with and without phospholipase C as described in Materials and Methods. One group of samples was extracted immediately; a second group was centrifuged (105 000 g, 30 min) and the pellet extracted. Lipids were extracted and analysed as described in Materials and Methods. Results given are means of 3 determinations.
Fig. 1. Effect of phospholipase C treatment on the size distribution of microsomal vesicles. Microsomes were treated with phospholipase C and prepared for electron microscopy as described in Materials and Methods. The size distribution of microsome diameters was determined as described in Materials and Methods. All vesicles were measured, those having a signet-ring structure (see text) were measured across the membrane portion and adhering vesicles were measured individually. Between 800 and 900 vesicles were counted in each experimental group. The number of vesicles within each size range as a % of the total counted, is plotted against diameter. Microsomes incubated without phospholipase C (---); microsomes incubated with phospholipase C (- - - - -).
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cooled rapidly and extracted after a 30 min centrifugation spin at 0 °C. Under these conditions the hydrolysis of diglyceride appears to be minimal.

Morphology of phospholipase C-treated microsomes

Sections. Microsomes incubated in a medium with calcium but lacking phospholipase C were morphologically similar to unincubated microsomes. After incubation with phospholipase C, however, microsomes exhibited several morphological changes. The most uniform change was a reduction in the average diameter of the microsomal vesicles (Figs. 1, 2–5). The mean diameter of vesicles incubated in the absence of phospholipase C was 84–98 nm, and that of those incubated in the presence of phospholipase C was 42–56 nm. Similarly, the mean diameter of rough microsomes was reduced from 140–154 nm to 98–112 nm by phospholipase C treatment, while the mean diameter of smooth microsomal vesicles was reduced from 84–98 nm to 42–56 nm. In total rough and smooth vesicles a number of profiles were observed, which were consistent with small vesicles forming by pinching off from the large vesicle membrane (Fig. 6A, B). These profiles were relatively infrequent, however, suggesting that if vesiculation occurs it is rapid.

After phospholipase C treatment the unit membrane structure of the membrane was retained; however, two morphological changes were observed, each involving approximately 10–15% of the vesicles measured in several preparations. Vesicles adhering to each other in groups of two or more were observed (Fig. 6C, D). Usually the shared membrane between these was a trilaminar structure rather than a pentalaminar structure, which would be expected if adherence of only the outer leaflet occurred. It is difficult to interpret these changes in molecular terms. However, if diglyceride accumulation in the outer leaflet results in a contraction of this leaflet, one mechanism that would stabilize such vesicles would be fusion so that the shared bilayer is derived from the inner leaflet of each vesicular membrane. To determine whether such adhering vesicles are intermediates in a process of fusion, microsomes were treated with phospholipase C followed by incubation at 37 °C for 60 min to allow any fusion process to be completed. However, after such treatment a fall in the diameter of the vesicles and approximately 10–15% adhering vesicles were still observed.

The second morphological change observed was the presence of an amorphous electron-dense structure associated with some vesicles. The membrane portion retained a bilayer structure (Fig. 6E, F). Similar signet-ring structures were observed by Meldolosi, Jamieson & Palade (1971) in rat liver microsomes incubated with the post-mitochondrial supernatant of rat pancreas. These droplets were believed to be pools of free fatty acid produced by hydrolysis of phospholipid by pancreatic lipase. Finean & Martonosi (1965) also observed small droplets associated with muscle microsomes after treatment with phospholipase C. Coleman, Finean, Knutton & Limbrick (1970) and Limbrick & Knutton (1975) observed amorphous droplets of diglyceride, much larger than those seen in our preparations, in phospholipase C-treated red blood cells. In our preparations diglyceride is the only detectable
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product of phospholipase C treatment and it is probable that the droplets observed, are composed, at least in part, of this lipid.

A further morphological change observed in the phospholipase C-treated microsomes was an apparent reduction in the number of bound ribosomes. Other investigators have reported that treatment of stripped microsomes with phospholipase C prevents rebinding of ribosomes, suggesting that the outer membrane phospholipids play a role in the association of the ribosomes with the membranes (Kishore & Carubelli, 1979).

Freeze-fractured preparations. It is generally considered that during freeze-fracture of membranes the fracture plane splits the membrane bilayer (Branton, 1966; Pinto de Silva & Branton, 1970; Tillack & Marchesi, 1970). Rat liver microsomes exhibit either a concave replica, in which the inner face of the cytoplasmic leaflet of the membrane bilayer is replicated (P face), or a convex replica, in which the inner face of the cisternal leaflet is replicated (E face) (Losa, Weibel & Bollander 1978; Dippier & Dallner, 1975). On average the P face of the membrane contains more particles than the E face. As the particles probably represent proteins these observations are consistent with an asymmetric structure of microsomal membranes. Untreated microsomes and those incubated without phospholipase C showed similar morphology to that reported by others (Fig. 7). Phospholipase C-treated microsomes also showed a similar morphology, exhibiting both P and E faces; the former exhibiting a greater particle density than the latter (Fig. 8). These observations suggest that the phospholipase C-treated microsomes retain the bilayer structure, and hence that the basic organization of the membrane is retained. Occasional vesicles were observed in which an area occurred with a different fracture plane from the rest of the membrane and lacking particles. These structures may be of the droplets observed in vesicles in sectioned material. Limbrick & Knutton (1975) observed regions devoid of particles in phospholipase C-treated red blood cell membranes. They attributed these to diglyceride droplets, which would not fracture in the same way as the rest of the membrane. These areas were much larger than those observed in our preparations, as were the droplets seen associated with the membranes in their sectioned material.

DISCUSSION

Phospholipase C hydrolyses approximately 50% of the phospholipids of rat liver microsomes without causing loss of labelled vesicular contents or increasing mannose-6-phosphate activity (Higgins & Dawson, 1977; Higgins, 1979). If the microsomes

Figs. 2-6. Morphology of phospholipase C-treated microsomes in sections. Microsomes were incubated with or without phospholipase C as described in the text.

Fig. 2. Microsomes incubated without phospholipase C. Smooth profiles and profiles with bound ribosomes are observed in these preparations. × 36000.

Fig. 3. Phospholipase C-treated total microsomes. Vesicles remain closed. Occasional vesicles exhibit signet-ring structure (arrows) and others adhere in groups of two or three (arrowheads). × 36000.
Phospholipase C-treated microsomes are opened, either mechanically using the French pressure cell, or chemically using detergents or high pH, hydrolysis of the phospholipids is increased (Higgins & Dawson, 1977; Bollen & Higgins, 1980). These results suggest that microsomal vesicles remain intact during phospholipase C-treatment and that only phospholipids at the outer leaflet of the membrane bilayer are hydrolysed. The present observations also indicate that the microsomal membranes remain closed and that the bilayer is retained in vesicles viewed in conventional section and in freeze-fractured specimens. The organization of the membrane is not destroyed by phospholipase C treatment using fine structure as a criterion. After treatment with phospholipase C the mean diameter of microsomes fell by approximately 50%. Limbrick & Knutton (1975) observed that after hydrolysis of 70% of the phospholipid there was a 27% reduction in the diameter of red blood cells. They observed treated red blood cells under the phase-contrast microscope and did not see vesiculation of the cells, and concluded, therefore, that reduction in diameter is a consequence of pooling of diglyceride, which is seen in droplets associated with the membranes. A similar explanation might be suggested for our observations. However, signet-ring structures occurred in a limited number of vesicles and these showed a size distribution similar to the whole population. It can be calculated that the average microsomal vesicle would give rise to 4 smaller vesicles having half the diameter. If the fall in vesicle diameter observed after phospholipase treatment were simply due to the loss of three-quarters of the membrane area, hydrolysis of phospholipid would be greater and accumulation of diglyceride drops more extensive. An alternative explanation of the reduction in diameter is that vesiculation of large vesicles gives rise to smaller vesicles. This is consistent with the morphological observations. Profiles intermediate in the formation of small vesicles were seen in sections. In addition, although measurements were not made because of problems of possible particle aggregation in freeze-fracture preparations, the particle density observed in concave replicas of phospholipase C-treated microsomes was not noticeably different from that of untreated microsomes. As these particles represent membrane proteins, removal of three-quarters of the lipid should result in a large and obvious increase in particle density.

It has been demonstrated that asymmetric modifications of the red blood cell membrane, using phospholipase C (Allan, Low, Finean & Michell, 1975), calcium ionophores (Allan & Mitchell, 1975; Allan, Billah, Finean & Michell, 1976) or amphipathic drugs (Sheetz & Singer, 1974), induce shape changes in the membrane. Accumulation of drugs or enzyme-produced diglyceride in the outer leaflet induced inward vesiculation, while accumulation of drugs or diglyceride in the inner leaflet induced outward vesiculation. Thus, the 2 halves of the bilayer behave as separate monolayers that are in contact and have been described as a 'bilayer couple' by

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Fig. 4. Phospholipase C-treated smooth microsomes. This field has a higher than average percentage of signet-ring-like profiles and was selected to illustrate these. Adhering vesicles (arrows) are also present. × 36000.

Fig. 5. Phospholipase C-treated rough microsomes. Both adhering vesicles and signet-ring profiles are present. × 36000.
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Sheetz & Singer (1974). Poste & Allison (1973) suggested that membranes having a high degree of curvature do not repel each other to the same degree as flat membranes and that, if membranes overcome long-range repulsive forces and come close together (1 nm), fusion might occur without involvement of other factors. In phospholipase C-treated microsomes, invaginations of the membrane caused by accumulation of diglyceride in the outer leaflet would have small radii of curvature and vesiculation could occur by this mechanism. Further stabilization of the smaller vesicles in which the outer leaflet is largely diglyceride might be achieved by fusion of vesicles, as suggested earlier.

Liver endoplasmic reticulum is not a static structure, but is continually turned over, increasing and decreasing in response to the physiological state of the hepatocyte. In addition, the endoplasmic reticulum is involved in synthesis and secretion of lipoproteins and plasma proteins. Small vesicles form from this membrane and transport the secretory product to the Golgi membranes. There are many cellular events, therefore, that involve changes in the membrane shape and the formation and fusion of vesicles, both in the endoplasmic reticulum and the Golgi membranes. The present observations suggest that asymmetric modification of the phospholipid bilayer can cause such morphological changes. This presents a simple mechanism, which is readily controlled by either the location of phospholipases in the membrane, or the action of these enzymes on specific phospholipids. As only a small fraction of the total endoplasmic reticulum is involved in vesicle formation at one time, hydrolysis need only be of a small proportion of the phospholipid, restricted to special sites of the membrane, e.g. the ends of the cisternae. Thus, observations of red blood cells, which do not form vesicles under physiological conditions, may be extended to the endoplasmic reticulum, many of the diverse functions of which are dependent on its ability to change shape.

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Fig. 6. Phospholipase C-treated microsomes. A, B. Profiles consistent with 'pinching off' of small vesicles from large vesicles. Membranes exhibit shape changes (double arrows), close opposition (single arrows) and profiles consistent with membrane fusion and separation of small vesicles (arrowheads). A, × 96,000; B, × 218,000. C, D. Profiles of adhering vesicles. The bilayer structure is retained and the shared membranes also have a bilayer structure (arrows). × 140,000. E, F. Signet-ring profiles. The membrane portion of the vesicles retains a bilayer structure. × 140,000.
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REFERENCES


Figs. 7-8. Freeze-fracture replicas of phospholipase C-treated and untreated microsomes.

Fig. 7. Untreated microsomes. Concave faces (arrows) and convex faces (arrowheads) are present, the former having a higher density of intramembranous particles. 
\[ x \times 72,000 \]

Fig. 8. Phospholipase C-treated microsomes. Convex faces (arrowheads) and concave faces (arrows) are similar to those in Fig. 7. Some profiles exhibit an area (large arrowheads) in which the fracture plane differs from the rest of the membrane. These may represent the droplets seen in signet-ring profiles in sectional material. 
\[ x \times 72,000 \]


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