Transverse Organization of Phospholipids across the Bilayer of Plasma-Membrane Subfractions of Rat Hepatocytes

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Phospholipase C treatment of vesicular subfractions of plasma membranes derived from the three functional domains of rat liver indicated that there is an asymmetric distribution of phospholipids across the bilayer of these membranes. The bile-canalicular and sinusoidal membranes were similar and different from the contiguous membrane.

Studies, mainly with erythrocyte membranes, have shown that some proteins and lipids are arranged asymmetrically relative to the cytoplasmic and external milieux. To determine whether phospholipids are also arranged asymmetrically in the plasma membranes of nucleated cells and to investigate the constancy of phospholipid organization in a physically continuous plasma membrane differentiated to carry out physiologically diverse functions, the effects of phospholipase treatment on membranes derived from regions of the hepatocyte surface were investigated. Plasma-membrane subfractions originating from the three major functional domains of the hepatocyte surface, i.e. blood sinusoidal, contiguous and bile canalicular, have been prepared and characterized (Kremmer *et al.*, 1976; Wisher & Evans, 1975, 1977). Their phospholipid organization is now examined with a technique used successfully to demonstrate the asymmetric distribution of phospholipids in a liver microsomal fraction (Higgins & Dawson, 1977). Since it is often proposed that there is transient functional continuity between intracellular membranes and the blood sinusoidal plasma membrane in hepatocytes, the present results also allow discussion of the mechanism of synthesis of the plasma membrane.

Methods

Protein was determined by the method of Lowry *et al.* (1951), phospholipid phosphorus by the method of Bartlett (1959) after digestion of samples with perchloric acid, and lactate dehydrogenase was assayed with a kit supplied by Sigma (London) Chemical Co., Kingston upon Thames KT27 BH, U.K.

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Preparation of liver plasma-membrane subfractions

Bile-canalicular (zonal-light subfraction) contiguous (zonal-heavy subfraction) and blood sinusoidal (microsomal-light subfraction) plasma membranes were prepared as described previously (Wisher & Evans, 1977).

Treatment of plasma-membrane fractions with phospholipase C

Samples of plasma membrane fractions were treated with phospholipase C of *Clostridium welchii* (Sigma Chemical Co.) and the extent of hydrolysis of the phospholipids determined as previously for liver microsomal fraction (Higgins & Dawson, 1977).

Results

Orientation of plasma-membrane vesicles

To ascertain whether vesicles present in the three plasma membrane fractions were predominantly of the right-side-out configuration, an antiserum that inhibited 5'-nucleotidase, a membrane glycoprotein (Evans & Gurd, 1973) with its active site facing the external medium (Farquhar *et al.*, 1974; Trams & Lauter, 1974) was used. The inhibition of enzyme activity before and after treatment designed to open up vesicles was investigated. Fig. 1 shows that more than 90% of enzyme activity was inhibited in bile-canalicular and contiguous plasma-membrane fractions; the lower inhibition (about 80%) recorded in the blood sinusoidal plasma-membrane fraction probably reflects the presence in this fraction of vesicles derived from the Golgi apparatus (Wisher & Evans, 1975). Furthermore, very little (less than 15%) increase in inhibitable activity was elicited in the presence of 0.5% deoxycholate in contrast with the latency shown by Golgi membranes (Little &

Fig. 1. Availability of 5'-nucleotidase of plasma-membrane subfractions to antiserum

Plasma membranes from the bile-canalicular (□) (16 μg of protein), contiguous (○) (60 μg of protein) and blood sinusoidal (●) (80 μg of protein) surfaces were preincubated for 30 min at 30°C with the indicated amount of a rabbit antiserum raised against a membrane-bound 5'-nucleotidase antigen (Evans & Gurd, 1972) and 5'-nucleotidase activity then determined by the method of Avruch & Wallach (1971).

Widnell, 1975). Non-immune antisera did not affect enzyme activity and the antiserum did not affect alkaline phosphodiesterase. The results suggest that the majority of the vesicles in the three fractions now investigated had maintained the same orientation as in the intact cell.

Hydrolysis of phospholipids of plasma-membrane subfractions by phospholipase C

Hydrolysis of the phospholipids of each plasma-membrane subfraction was rapid and reached a plateau within 15 min of incubation. The final extent of hydrolysis varied between subfractions and was 55% of the total phospholipid in the case of the bile-

canalicular membranes, 56% in that of the sinusoidal membranes and 40% in that of the contiguous fraction (Fig. 2). Under identical conditions, all phospholipid components of these membranes dispersed by sonication were hydrolysed with the exception of phosphatidylinositol (Higgins & Dawson, 1977). The lack of complete hydrolysis of the plasma membrane phospholipids is, therefore, not a consequence of enzyme specificity.

The phospholipid composition of the three fractions and the phospholipid/protein ratios were similar to previous reports (Kremmer *et al.*, 1976). The individual phospholipids available for hydrolysis by exogenous phospholipase C were similar for both the bile-canalicular membranes and the sinusoidal membranes and were respectively 53 and 59% of the phosphatidylethanolamine, 85 and 86% of the phosphatidylcholine, 67 and 63% of the sphingomyelin, and no phosphatidylserine (Fig. 2). Phosphatidylinositol is not a substrate for the phospholipase C used in these studies. Therefore no conclusion can be reached concerning its distribution. In contrast, no phosphatidylethanolamine, 80% of the phosphatidylcholine, 7% of the sphingomyelin, 6% of the phosphatidylserine and no phosphatidylinositol of the contiguous surface membrane were hydrolysed (Fig. 2). There is therefore a striking difference in the available phospholipid between the plasma-membrane subfractions. Assuming that the hydrolysed phospholipid is in the outer leaflet of the bilayer, it appears that in the contiguous membrane this consists almost completely of phosphatidylcholine, whereas in the bile-canalicular membrane and the sinusoidal membrane this consists of approximately 19% phosphatidylethanolamine, 23% sphingomyelin and 59% phosphatidylcholine.

Effect of phospholipase C or deoxycholate on the lactate dehydrogenase released from plasma-membrane vesicles

There is no well-established marker for the contents of plasma-membrane vesicles. However, it is possible that cytosol markers such as lactate dehydrogenase may be trapped in the plasma-membrane vesicles during tissue homogenization. The effect of phospholipase C on the release of this enzyme from plasma-membrane subfractions was therefore investigated. Detectable lactate dehydrogenase was released from all plasma-membrane subfractions by incubation at 37°C (Table 1). This was not increased by phospholipase C treatment, but was increased 50–100% by treatment of the vesicles with deoxycholate at concentrations that should make these more permeable.

Treatment of plasma-membrane subfractions

Fig. 2. Hydrolysis of phospholipases of plasma-membrane subfractions by phospholipase C. Plasma-membrane subfractions (2–3mg) were incubated with and without phospholipase C for 30min as described in the Methods section. The lipids were extracted at the end of the incubation period and the hydrolysis of total and individual phospholipid determined as described in the Methods section. The results plotted are averages of four determinations on two membrane preparations. The distribution of each phospholipid available (upper part of Figure) and unavailable (lower part of Figure) for hydrolysis by phospholipase C is plotted as a fraction of its contribution to the whole membrane. The standard deviations are indicated by bars.

Table 1. Effect of phospholipase C and deoxycholate treatment on lactate dehydrogenase activity of plasma-membrane subfractions

Subfractions of plasma membranes (bile-canalicular membrane, 0.136 mg of protein; sinusoidal membrane, 0.256 mg of protein and contiguous membrane, 0.39 mg of protein) were incubated with and without phospholipase C as described in the Methods section. Similar samples were preincubated with deoxycholate (0.05% in the case of the bile-canalicular membranes and 0.1% in that of the sinusoidal and contiguous membranes) for 30 min before addition of phospholipase C. At the end of the incubation period, the membranes were isolated by centrifugation. The pellets were extracted with chloroform/methanol (2:1, v/v) and the hydrolysis of the phospholipids determined as described in the Methods section. Samples (10μl) were used to assay lactate dehydrogenase. Results are averages of four determinations on two membrane preparations, with ranges in parentheses.

	Lactate dehydrogenase activity (μmol of pyruvate reduced)		
	Untreated	Phospholipase C-treated	Deoxycholate-treated
Bile canaliculi	0.10 (0.06–0.11)	0.09 (0.07–0.13)	0.19 (0.15–0.23)
Contiguous	0.30 (0.25–0.37)	0.25 (0.21–0.30)	0.52 (0.49–0.55)
Sinusoidal	0.60 (0.56–0.63)	0.57 (0.52–0.60)	0.80 (0.74–0.82)
	Hydrolysis of total phospholipids by phospholipase C (%)		
	No pretreatment	Deoxycholate treatment	
Bile canaliculi	52 (45–55)	71 (67–73)	
Contiguous	40 (39–42)	65 (62–67)	
Sinusoidal	56 (53–59)	80 (77–84)	

with deoxycholate also results in an increased hydrolysis of the phospholipid consistent with more of the vesicles being opened to phospholipase C in the

incubation medium (Table 1). As in our previous studies with microsomal membranes (Higgins & Dawson, 1977), hydrolysis of the plasma-membrane

phospholipids is not complete, suggesting that not all the vesicles are opened by deoxycholate.

Discussion

To investigate transverse asymmetry, it is necessary to prepare membranes in the form of closed and impermeable vesicles of the same orientation, so that the outer leaflet of the phospholipid bilayer may be probed without the inner leaflet being accessible to the probing reagent. The present work using an antiserum to inhibit a plasma-membrane ectoenzyme, when taken in conjunction with morphological results (Wisher & Evans, 1975, 1977) and especially the density of intramembranous particles in membrane leaflets produced by freeze-fracture of vesicles (Losa, 1976; Schafer *et al.*, 1978; Trams & Lauter, 1974), indicates that on tissue homogenization and subcellular fractionation, the plasma membrane forms vesicles that are predominantly of a right-side-out orientation. It is difficult to demonstrate impermeability of these vesicles to the exogenous enzyme as there is no unambiguous marker for the content of the vesicles, and the yields of membrane are too small to perform isotope-dilution experiments. However, we found a detectable lactate dehydrogenase activity in the plasma membrane subfractions that was increased up to 2-fold by treatment of the vesicles with low concentrations of deoxycholate, suggesting that some of this enzyme, a cytoplasmic marker, is trapped in the vesicles during homogenization of the liver. The low lactate dehydrogenase activity released from plasma membranes during incubation at 37°C was not increased by treatment of the vesicles with phospholipase C. This observation, together with the plateau of hydrolysis of the phospholipid, which was increased by treatment of the vesicles with deoxycholate, is strongly indicative that the vesicles are impermeable to phospholipase C.

We have previously demonstrated, using phospholipase C, that there is an asymmetric distribution of phospholipids on either side of the bilayer of liver microsomal fraction (Higgins & Dawson, 1977). Recently, however, Sundler *et al.* (1977) have reported that there is no difference in the hydrolysis by phospholipase A₂ of phospholipids of microsomal fraction compared with dispersed extracted microsomal lipids. They interpret their results as indicating a lack of asymmetry in the phospholipid bilayer of these membranes. The reasons for the discrepancy between our results and theirs is not clear. However, in our hands phospholipase A₂ from several sources caused immediate and rapid leakage of microsomal contents, indicating that the vesicles were no longer intact, and complete hydrolysis of all phospholipids (Higgins & Dawson, 1977; J. A. Higgins & W. H. Evans, unpublished work). As Sundler *et al.* (1977)

did not demonstrate that vesicles remain intact during their procedure, their results may be due to opening of vesicles even at early times of incubation with phospholipase A₂ and consequently hydrolysis of phospholipids of both sides of the membrane.

The results reported here clearly demonstrate a difference in the action of phospholipase C on three functionally defined plasma-membrane subfractions of rat liver. The membranes of the free surfaces, the bile canaliculi and the sinusoidal surface, are similar and differ from the membranes derived from the contiguous surfaces, both in the amount of phospholipid available for hydrolysis and the composition of the phospholipids hydrolysed. As the free surfaces have microvilli, whereas the contiguous surface is planar, these observations suggest that there is a relationship between the morphological structure and the molecular architecture of the phospholipids of the membrane.

The sinusoidal surface is the only domain of the plasma membrane having transient morphological continuity with the intracellular membranes. In the living cell, the plasma-membrane domains are continuous. It appears probable, therefore, that the contiguous membranes and the bile-canicular membranes may be formed by flow of membrane laterally from the sinusoidal membrane. Our results, however, indicate that the distribution of phospholipids across these membranes differs. There must therefore be constraints against the free lateral movement of molecules between the plasma-membrane domains, for example, at the tight junctions that surround the bile canaliculi, or alternatively, a rapid mechanism for the reorganization of phospholipids in the different membrane domains must exist.

There is a functional continuity during secretion between the endoplasmic reticulum, the Golgi membranes, and the sinusoidal membrane of the hepatocyte (Claude, 1970; Ehrenreich *et al.*, 1973; Glaumann *et al.*, 1975; Hamilton *et al.*, 1967). It has been suggested, therefore, that the plasma membrane is derived from the endoplasmic reticulum. Consistent with this, the composition of the Golgi membranes is intermediate between that of the endoplasmic reticulum and the plasma membrane (Keenan & Morré, 1970; Yungmans *et al.*, 1970). However, our previous investigations of the distribution of phospholipids across the endoplasmic-reticulum membrane indicate that the outer leaflet of the membrane bilayer consists of 84% phosphatidylcholine, 8% phosphatidylethanolamine, 9% sphingomyelin, and 4% phosphatidylserine, whereas the inner leaflet consists of approximately equal amounts of phosphatidylcholine, phosphatidylethanolamine with small amounts of phosphatidylserine and sphingomyelin (Higgins & Dawson, 1977). If membrane components synthesized in the endoplasmic reticulum are transported as vesicles to the

plasma membrane via the Golgi membranes during secretion, then the inner surface of the endoplasmic reticulum would become the outer surface of the plasma membrane. The transverse distribution of phospholipids in the endoplasmic reticulum and the plasma membrane are not consistent, therefore, with a simple movement of membrane between those two compartments. Thus, if the endoplasmic reticulum contributes directly to the plasma membrane, re-arrangement of the transverse distribution of phospholipids must occur, as well as a change in the membrane composition, owing possibly to the selective insertion of membrane components into the plasma membrane (Bergeron *et al.*, 1973). Little & Widnell (1975) have demonstrated, with cytochemical techniques, that 5'-nucleotidase is located on the cytoplasmic side of the microsomal membrane vesicles and the Golgi cisternal membranes. However, this enzyme is located on the outer surface of the plasma membrane and the inner surface of the secretory vesicle membrane. There appears, therefore, to be a transverse shift of the enzyme across the membrane at some point during the formation of the secretory vesicle. Our observations suggest that a similar re-arrangement of the phospholipids in the vesicles may occur en route from the endoplasmic reticulum to the plasma membrane. The biogenetic relationship between the sinusoidal plasma membrane and the intracellular membranes is thus complex.

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