Phospholipid asymmetry in rough- and smooth-endoplasmic-reticulum membranes of untreated and phenobarbital-treated rat liver

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Phospholipase C was used as a probe for the distribution of phospholipids about the membrane of rough and smooth microsomal fractions from normal and phenobarbital-treated rat liver. All membranes exhibited an asymmetric distribution, with phosphatidylethanolamine and phosphatidylserine concentrated in the inner leaflet of the bilayer and phosphatidylcholine and sphingomyelin concentrated in the outer leaflet. The only phospholipid showing a significant difference in distribution between fractions was phosphatidylcholine, which was shifted towards the outer leaflet in the smooth microsomal fraction compared with the rough microsomal fraction, and towards the outer leaflet in both rough and smooth microsomal fractions from phenobarbital-treated liver compared with the same preparations from untreated rat liver. Apart from this small change, the asymmetric distribution of phospholipids was conserved in microsomal fractions which had proliferated in response to phenobarbital and in which the protein composition had changed.

The endoplasmic reticulum of rat liver consists of two forms, the rough endoplasmic reticulum and the smooth endoplasmic reticulum. The rough membranes are concerned primarily with protein synthesis, and the smooth membranes with a variety of functions, including oxidative demethylation of drugs. We have previously demonstrated that there is an asymmetric distribution of phospholipids on either side of the bilayer of the total microsomal fraction, which is derived from the endoplasmic reticulum, of rat liver (Higgins & Dawson, 1977). In view of their functional differences, it is important also to determine whether there is a similar asymmetric distribution of phospholipids in separated rough and smooth membranes.

In response to intraperitoneal injections of phenobarbital, the smooth endoplasmic reticulum of rat hepatocytes proliferates up to 4-fold after 4 days of treatment, with no significant change in the rough membranes (Remmer & Merkel, 1963; Orrenius & Ericsson, 1966; Staubel *et al.*, 1969). This is accompanied by a selective increase in the smooth membranes of the specific activity of the enzymes involved in drug detoxification, including the cytochrome *P*-450 system (Conney *et al.*, 1960; Brazda & Baucum, 1961; Orrenius *et al.*, 1965; Orrenius, 1965a,b; Orrenius & Ericsson, 1966). The smooth endoplasmic reticulum thus differs in its enzymic composition and function from that of untreated rat

liver. The phenobarbital-treated rat liver therefore provides an excellent model system in which to investigate the effects of modulation of the function of the endoplasmic reticulum on the molecular architecture of the membrane bilayer.

Methods

Materials

Phospholipase C of *Clostridium welchii* (EC 3.1.4.3) (specific activity 7 units/mg) was purchased from Sigma Chemical Co. Phenobarbital was purchased from Sigma and prepared as a solution for injection in 0.14 M-NaCl by addition of small amounts of NaOH to convert the free acid into the sodium salt.

General methods

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Phospholipid phosphorus was determined by the method of Bartlett (1959) after digestion of the lipid with 70% (v/v) HClO₄.

Preparation of microsomal fractions

Rough, smooth and total microsomal fractions were prepared as described previously (Higgins &

Barnett, 1972) from livers of rats (male, Sprague-Dawley, 150–250 g body wt.), which were either untreated, or injected intraperitoneally with phenobarbital (10 mg/100 g body wt.) at daily intervals for 4 days before they were killed on day 5.

Incubation of microsomal fractions with phospholipase C

Microsomal fractions were incubated with phospholipase C, as described previously (Higgins & Dawson, 1977), except that the final enzyme concentration was increased 10-fold to 10 units/ml (Higgins, 1979). The extent of hydrolysis was the same with either enzyme concentration, but reached a plateau after 5–10 min incubation with the higher concentration. In all experiments microsomal fractions were incubated with phospholipase C for 15 min to ensure complete hydrolysis of available phospholipid.

After incubation, microsomal lipids were extracted with chloroform/methanol (2:1, v/v) as previously described (Higgins & Dawson, 1977) and the phospholipids separated on thin layers of silica gel (Merck 60 F 254) in a one-dimensional system, chloroform/methanol/acetic acid/water (60:50:1:4, by vol). Lipid spots were detected with iodine vapour, and then scraped into test tubes and digested with HClO_4 (1.5 ml) for 60 min at 180°C. Water (7.6 ml) was added and the tubes centrifuged (500 rev./min, 10 min, MSE Minor instrument) to remove the silica gel. Phosphate was determined in the supernatant (Bartlett, 1959).

In some experiments microsomal fractions were treated with NH_3 to open the vesicles (Dallner *et al.*, 1978). These were suspended in 0.1 M- NH_3 for 15 min at 37°C, isolated by centrifugation (40 000 rev./min, Spinco 40 rotor, 45 min) and resuspended in buffer. Mannose 6-phosphatase latency (Arion *et al.*, 1976) in the untreated microsomal fraction was 80% compared with that of fractions treated with NH_3 .

Results

Phospholipid composition of microsomal fractions prepared from livers of untreated and phenobarbital-treated rats

The composition of the phospholipids of microsomal fractions was similar for preparations from both normal and phenobarbital-treated rat liver (Table 1). The results for microsomal fractions from untreated rat liver were similar to those reported previously (Higgins & Dawson, 1977) and by other investigators (DePierre & Dallner, 1975; Glaumann & Dallner, 1968; Dallner & Ernster, 1968). However, Young *et al.* (1971) have reported that in livers of phenobarbital-treated rats the newly formed phospholipid is predominantly phosphatidylcholine, resulting in a relative increase in this phospholipid in the microsomal fraction. This has been questioned by Davison & Wills (1973, 1974), who found no difference in the phospholipid composition between the microsomal fractions from untreated and phenobarbital-treated rat liver. Our results support those of the latter group. Thus, although there is a significant change in the enzymic composition of the endoplasmic reticulum and an increased synthesis of phospholipid (Orrenius *et al.*, 1965; Davison & Wills, 1973, 1974; Higgins, 1974), this is not accompanied by a change in the total pattern of the phospholipid components of either rough or smooth endoplasmic reticulum.

Hydrolysis of phospholipids of microsomal fractions by phospholipase C

Hydrolysis of total phospholipids by phospholipase C reached a plateau after approx. 5 min incubation, and no further hydrolysis occurred on continued incubation up to 30 min. The extent of hydrolysis of phospholipids of the total microsomal fraction was approx. 50%, as reported previously (Higgins & Dawson, 1977). However, hydrolysis of phospholipids of the smooth microsomal fraction was statistically greater (65–67%) than that of the

Table 1. *Phospholipid composition of microsomal fractions from livers of phenobarbital-treated and untreated rats*
Microsomal fractions were prepared from livers of untreated and phenobarbital-treated rats and the lipids were extracted and separated by t.l.c. as described in the Methods section. Results are means $\pm$ S.D. for four determinations.

Rats	Microsomal fractions	Composition (%)				
		Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-serine	Phosphatidyl-inositol	Sphingomyelin
Untreated	Total	50.7 $\pm$ 9.9	23.0 $\pm$ 2.3	7.0 $\pm$ 1.7	12.5 $\pm$ 0.5	6.0 $\pm$ 2.6
	Rough	52.4 $\pm$ 3.3	25.4 $\pm$ 3.6	4.4 $\pm$ 1.1	12.3 $\pm$ 0.7	4.0 $\pm$ 1.1
	Smooth	55.1 $\pm$ 6.7	24.0 $\pm$ 2.0	5.1 $\pm$ 6.9	11.1 $\pm$ 2.9	3.8 $\pm$ 3.2
Phenobarbital-treated	Total	51.9 $\pm$ 6.1	25.1 $\pm$ 1.4	5.7 $\pm$ 2.6	13.9 $\pm$ 1.2	2.7 $\pm$ 5.9
	Rough	51.2 $\pm$ 4.2	23.8 $\pm$ 8.8	5.5 $\pm$ 4.2	14.3 $\pm$ 1.1	7.5 $\pm$ 3.8
	Smooth	51.9 $\pm$ 4.2	27.5 $\pm$ 2.4	4.3 $\pm$ 1.9	12.0 $\pm$ 2.6	4.3 $\pm$ 0.5

Table 2. *Hydrolysis of phospholipids of microsomal fractions from livers of normal and phenobarbital-treated rats by phospholipase C*

Microsomal fractions were incubated with phospholipase C, the lipids extracted and the percentage hydrolysis determined as described in the Methods section. Results expressed are means $\pm$ S.D. of at least four determinations.

Rats	Microsomal fractions	Hydrolysis (%)
Untreated	Total	52.9 $\pm$ 5.4
	Rough	50.9 $\pm$ 6.3
	Smooth	65.4 $\pm$ 4.3
Phenobarbital-treated	Total	53.5 $\pm$ 9.3
	Rough	53.6 $\pm$ 3.1
	Smooth	66.7 $\pm$ 3.2

Table 3. *Loss of labelled contents of microsomal vesicles on treatment with phospholipase C*

Microsomal protein contents were labelled by injection of [3 H]leucine (5 μ Ci/100 g) intraperitoneally into rats 30 min before they were killed for preparation of the total liver microsomal fraction (Kreibich *et al.*, 1973). The membranes were suspended in the buffer for phospholipase-C treatment. One group of samples were centrifuged immediately, a second group incubated for 20 min at 37°C and a third group incubated with phospholipase C for 20 min at 37°C. All samples were centrifuged at 40000 rev./min for 45 min, and samples of the supernatant were taken and counted for radioactivity in a Beckman c.p.m. 100 liquid-scintillation counter. Loss of contents is expressed as radioactivity appearing in the medium as a percentage of radioactivity in the original sample. Results are averages of two determinations.

	Loss of label into supernatant (%)		
	No treatment	20 min incubation	20 min incubation + phospholipase C
Smooth membranes	12.3	13.8	16.0
Rough membranes	8.0	7.4	12.1

rough fraction (51–52%) from both normal and phenobarbital-treated rats (Table 2).

When microsomal vesicles were opened by treatment with NH_3 , hydrolysis was increased to 71% of the phospholipids of the rough fraction and 80% of that of the smooth fraction. Complete hydrolysis could not be achieved, possibly because the vesicles reseal or are not all opened. As in our previous observations (Higgins & Dawson, 1977), increased hydrolysis of open-vesicle phospholipid was due to increased hydrolysis of phosphatidylethanolamine and phosphatidylserine. Hydrolysis of phosphatidylcholine and sphingomyelin was not increased.

The secretory proteins contained in both rough and smooth microsomal fractions were labelled by injection of [3 H]leucine into rats 30 min before they were killed (Kreibich *et al.*, 1973). Loss of these labelled contents during treatment with phospholipase C was not increased compared with microsomal fractions incubated without enzyme, or washed with cold buffer without incubation (Table 3). Loss of contents from the rough fraction was slightly increased by phospholipase-C treatment. However, this was a small fraction of the loss of label when the vesicles were opened by NH_3 . Treatment of either rough or smooth fractions with phospholipase C does not therefore open the

vesicles, and presumably only phospholipids of the outer bilayer leaflet are hydrolysed (Higgins & Dawson, 1977).

Hydrolysis of individual phospholipids of microsomal fractions by phospholipase C

The hydrolysis of individual phospholipids of the total microsomal fraction was similar to that reported previously, suggesting an asymmetric distribution of phospholipid, with phosphatidylcholine and sphingomyelin predominantly in the outer leaflet (cytoplasmic surface) and phosphatidylethanolamine and phosphatidylserine predominantly in the inner leaflet (cisternal surface). A similar asymmetric distribution of phospholipids was found in both rough and smooth fractions (Table 4), with no detectable difference in the distribution of phospholipids, except for phosphatidylcholine, which was slightly increased in the outer leaflet of the bilayer of the smooth fraction compared with the rough fraction ($P < 0.01$). This may account partly for greater hydrolysis of total phospholipid of the smooth fraction. Phosphatidylserine tended to be at a higher concentration in the inner leaflet of the rough fraction compared with the smooth. However, this is a small component of the membrane,

Table 4. *Hydrolysis of individual phospholipids of microsomal fractions from livers of untreated and phenobarbital-treated rats of phospholipase C*

Microsomal fractions were incubated with phospholipase C, and the lipids extracted and separated by t.l.c. as described in the Methods section. Results expressed are means $\pm$ s.d. for at least four determinations.

Rats	Microsomal fraction	Hydrolysis (%)			
		Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine	Sphingomyelin
Untreated	Total	77.4 $\pm$ 4.5	37.7 $\pm$ 0.6	21.3 $\pm$ 6.2	60.3 $\pm$ 2.5
	Rough	67.7 $\pm$ 5.3	39.7 $\pm$ 6.8	26.0 $\pm$ 7.6	57.7 $\pm$ 3.1
	Smooth	76.0 $\pm$ 7.4	39.8 $\pm$ 1.5	11.7 $\pm$ 5.4	63.0 $\pm$ 5.7
Phenobarbital-treated	Total	84.0 $\pm$ 6.5	33.3 $\pm$ 5.3	38.0 $\pm$ 2.0	66.0 $\pm$ 9.0
	Rough	78.3 $\pm$ 4.6	33.3 $\pm$ 7.0	34.0 $\pm$ 5.7	43.8 $\pm$ 4.9
	Smooth	86.7 $\pm$ 3.5	32.0 $\pm$ 8.4	15.0 $\pm$ 5.0	63.0 $\pm$ 8.9

Table 5. *Distribution of phospholipids across the bilayer of microsomal fractions*

The distribution of phospholipids on either side of the bilayer is calculated from the percentage hydrolysis of the individual phospholipids (data in Table 4) and the composition of the membranes (data in Table 1). Phosphatidylinositol is not a substrate for the phospholipase C used in these studies. Therefore its distribution is not given. Note that the inner surface of the microsomal membrane corresponds to the cisternal surface of the endoplasmic reticulum and the outer surface to the cytoplasmic surface.

Microsomal fraction	Rats	Surface	Distribution (%)				
			Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine	Sphingomyelin	
Smooth	Untreated	Inner	12.3	14.6	4.6	1.4	} 11.1
		Outer	38.9	9.55	0.6	3.4	
	Phenobarbital-treated	Inner	6.9	18.7	3.6	1.9	} 12.0
		Outer	45.0	8.9	0.6	2.4	
Rough	Untreated	Inner	16.9	17.0	3.7	2.3	} 12.3
		Outer	35.5	8.5	1.1	1.7	
	Phenobarbital-treated	Inner	11.3	15.7	3.2	4.2	} 14.3
		Outer	40.6	7.4	1.9	3.3	
Total	Untreated	Inner	11.4	14.5	5.5	2.0	} 12.5
		Outer	39.3	9.0	1.5	4.0	
	Phenobarbital-treated	Inner	6.9	16.7	3.5	0.9	} 13.9
		Outer	45.0	8.4	2.1	1.8	

and a slight difference in its distribution would have little effect on the overall composition of the membrane bilayers.

Microsomal fractions from phenobarbital-treated rats also exhibited an asymmetric distribution of phospholipids, with a similar pattern to that of the fractions from untreated rat liver (Table 5). However, phosphatidylcholine was increased in the outer leaflet of the bilayer of the smooth microsomal fraction ($P < 0.005$) and the rough microsomal fraction ($P < 0.001$), compared with preparations from untreated rat livers. The greater concentration of phosphatidylcholine in the outer leaflet of the smooth compared with the rough membranes was also maintained in fractions from phenobarbital-treated rats ($P = 0.005$).

The distribution of individual phospholipids in

terms of the composition of the microsomal membrane fractions is shown in Table 5. Although there is a small but significant shift of phosphatidylcholine from the inner to the outer leaflet of the membrane bilayer of both rough and smooth membranes after phenobarbital treatment, there is no equal reverse shift of other phospholipids to the inner leaflet. However, if more than one phospholipid were involved, the change may be too small to be detected.

Discussion

Partial hydrolysis of microsomal phospholipids by phospholipase C could be a result of two possible mechanisms. Hydrolysis of only the outer-leaflet phospholipids may occur, or half of the phos-

pholipids may be sequestered, not necessarily in the inner leaflet, and not available to phospholipase C. Several pieces of evidence suggest that the former is true. The microsomal fraction remains as closed vesicles, when examined in the electron microscope after phospholipase-C treatment (J. A. Higgins, unpublished work). Mannose 6-phosphatase is not increased by phospholipase C (Higgins, 1979), nor are the protein contents of the vesicles lost (Higgins & Dawson, 1977). The microsomal vesicles therefore appear to remain intact. When microsomal preparations are opened either mechanically, with detergents, or by raising the pH, hydrolysis of the membrane phospholipids increases and hydrolysis of individual phospholipids becomes more uniform (Higgins & Dawson, 1977). It is possible that the procedures used to open vesicles may also make sequestered phospholipids available; however, the variety of physical procedures used, the fact that each of these is known to open vesicles of the microsomal fraction, and the observations that unopened vesicles appear to remain closed after phospholipase C treatment, suggest that the sequestered pool is that of the inner leaflet of the bilayer.

Nilsson & Dallner (1977) used phospholipase A₂ to probe the outer leaflet of microsomal membranes, and concluded that phosphatidylcholine is evenly distributed and phosphatidylethanolamine is mainly in the outer leaflet of the bilayer. Sundler *et al.* (1977) also used phospholipase A₂, and demonstrated that this treatment opened microsomal fractions, but that before the vesicles opened there was no selective hydrolysis of any individual phospholipid. On this basis these investigators concluded that phospholipids are not asymmetrically distributed about the microsomal bilayer. However, lysophosphatidylcholine, one of the products of phospholipase A₂ activity, is a known lytic and membrane-perturbing agent, and may induce a micellar structure in the membrane before the vesicles become leaky (Weltzien, 1979). Krebs *et al.* (1979) have made a detailed study of the asymmetry of the mitochondrial inner membrane and demonstrated that phospholipase A₂, as used by Nilsson & Dallner (1977) and by Sundler *et al.* (1977), contains a lytic agent. Krebs *et al.* (1979) also obtained results for the transverse distribution of phospholipids across the mitochondrial membrane opposite to those of Nilsson & Dallner (1977). Our observations on microsomal membranes also suggest an opposite distribution of phospholipid to that found by Nilsson & Dallner (1977).

The rough- and smooth-endoplasmic-reticulum membranes of rat liver are continuous. However, there are differences between these membranes. The rough membranes contain ribophorins I and II, proteins specific for ribosome binding (Kreibich *et*

al., 1978) and have also been reported to contain GDP-mannosyltransferase, which is not found in the smooth membranes (Redman & Cherial, 1972). There are also quantitative differences in the specific activities of enzymes between the two membranes, especially in the phenobarbital-treated rat liver. The present results indicate that the phospholipid composition of the rough and smooth membranes is the same. However, there is more phospholipid available for hydrolysis by phospholipase C in the smooth membranes. This may reflect the absence of bound ribosomes and ribophorins at the cytoplasmic side of these membranes. In addition, the smooth endoplasmic reticulum is made up of tubules having curved surfaces, whereas the rough membranes are made up largely of planar sheets. These geometric differences between the membranes may result in a greater amount of phospholipid in the convex leaflet of the bilayer. A similar difference in distribution of total phospholipids was found between the planar contiguous plasma membranes of rat hepatocytes and the bile-canalicular and sinusoidal membranes, which have microvilli (Higgins & Evans, 1978). There is also slightly more phosphatidylcholine in the outer leaflet of the bilayer of the smooth membranes. However, apart from these fairly small quantitative differences the rough and smooth endoplasmic reticulum exhibit a similar pattern of phospholipid asymmetry.

After treatment for 4 days with phenobarbital there is up to a 4-fold increase in the specific volume of the smooth endoplasmic reticulum (Staebel *et al.*, 1969). These membranes are therefore made up largely of newly synthesized phospholipid. The phospholipid composition of both rough and smooth membranes, however, remains constant and an asymmetric distribution of phospholipid across the bilayer is maintained. The only significant difference between microsomal fractions from phenobarbital-treated rat liver and those from normal liver is a small shift of phosphatidylcholine to the outer leaflet of the microsomal bilayer of both rough and smooth membranes. The cytochrome P-450-NADPH-cytochrome *c* reductase enzyme system, which increases in response to injection of phenobarbital, is located on the cytoplasmic side of the endoplasmic-reticulum membrane (DePierre & Dallner, 1975; Morimoto *et al.*, 1976). This system has a requirement for 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (DePierre & Dallner, 1975). The increased concentration of this phospholipid in the outer leaflet of the membrane bilayer in smooth membranes and in response to phenobarbital may therefore represent phospholipid sequestered by the increased number of enzyme molecules in this bilayer. Stier & Sackmann (1973) have reported studies which suggest that cytochrome P-450 is enclosed in a halo of phospholipid molecules, which is more rigid than

the bulk of the microsomal phospholipids. The phospholipids of the microsomal membrane may thus be made up of a small pool, with a high concentration of phosphatidylcholine, distributed between rough and smooth membranes, in relation to their enzymic composition, and a large pool of mobile phospholipid, which is in equilibrium over the two-dimensional surface of the whole endoplasmic reticulum. The two halves of the membrane bilayer, however, behave as separate pools maintaining an asymmetric distribution.

These observations demonstrate that the phospholipid bilayers of rough- and smooth-endoplasmic-reticulum membranes are similar and that a modulation of the function of the liver resulting in a large increase in the smooth endoplasmic reticulum does not cause any marked change. The phospholipid bilayer therefore appears to be conserved even when the membranes proliferate 4-fold and when the protein composition and function of the membrane is modified.

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