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ASYMMETRIC DISTRIBUTION OF PHOSPHATIDYLETHANOLAMINE IN THE ENDOPLASMIC RETICULUM DEMONSTRATED USING TRINITROBENZENESULPHONIC ACID AS A PROBE

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At pH 7.4 approximately one third of the phosphatidylethanolamine (PE) of rat liver microsomes is labelled by trinitrobenzenesulphonic acid (TNBS). The same fraction of the PE was labelled, when a fixed concentration of microsomes were incubated with concentrations of TNBS from 1.5 mM to 12 mM, or when the TNBS concentration was fixed at 3.0 mM and the microsomal protein varied between 1.2 and 12.0 mg. Microsomes incubated with TNBS remain closed indicated by retention of mannose-6-phosphatase latency, retention of labelled vesicular contents and by the appearance of the vesicles in the electron microscope. When the microsomal vesicles were opened by alkaline pH or after passage through the French pressure cell the % of PE labelled increased up to 90% of the total. The small % remaining unlabelled may be due to some vesicles remaining closed or to steric hindrance by the relatively bulky label on both phospholipid and protein. Phospholipase C hydrolyses approximately one third of the PE in closed microsomal vesicles. After treatment of microsomes with phospholipase C the % PE available for labelling by TNBS decreased and was inversely proportional to the % PE hydrolysed. These results suggest that the same pool of PE is available for either hydrolysis by phospholipase C or for labelling by TNBS, and that this pool is that of the outer leaflet of the microsomal membrane bilayer.

Introduction

The endoplasmic reticulum of rat hepatocytes is the major site of synthesis of membrane phospholipids [1] and plays a central role in the biogenesis of its own phospholipid bilayer and that of other cellular membranes. It is important, therefore, in order to understand the biogenetic interrelationships between membranes to determine the distribution of phospholipids in the bilayer of the endoplasmic reticulum. There have been several such investigations. However, these have yielded conflicting results. We have used phospholipase C as a probe and demonstrated that the phospholipids of the endoplasmic reticulum are distributed asymmetrically with approximately two-thirds of the phosphatidylcholine and sphingomyelin in the outer leaflet and approximately two-thirds of the phosphatidylethanolamine and phosphatidylserine in the inner leaflet [2,3]. Sundler et al. [4] have used phospholipase A of *Crotalus atrox* as a probe and showed that this enzyme causes the microsomal vesicles to be opened, but from the initial rate of hydrolysis concluded that there is no selective hydrolysis of individual phospholipids suggesting that there is no asymmetric distribution of these. Nilsson and Dallner [5,6] have also used

Abbreviations: PE, phosphatidylethanolamine; TNBS, trinitrobenzenesulphonate; TNP-PE, trinitrophenol phosphatidylethanolamine.

phospholipase A from *Naja naja*. In their hands, the microsomal vesicles remained closed after treatment with phospholipase A and their results suggested that the phospholipids are asymmetrically distributed with phosphatidylethanolamine and phosphatidylserine at a higher concentration in the outer leaflet and phosphatidylcholine distributed evenly. We have not been able to repeat these experiments, and in our hands phospholipase A from a variety of sources causes complete hydrolysis of microsomal phospholipids. There have been no other investigations of liver endoplasmic reticulum, although studies of intestinal mucosa membranes have indicated that phosphatidylethanolamine is at a higher concentration in the inner leaflet of the membrane bilayer [7].

In order to resolve the conflict between the reported observations of phospholipid distribution in the endoplasmic reticulum alternative probes to phospholipases are necessary. Phospholipid-exchange proteins, which do not result in a modification of the phospholipid composition of the bilayer are attractive probes for investigations of the transverse distribution of endoplasmic reticulum phospholipids [8–10]. As these may function in vivo to transfer or exchange phospholipids between cell membranous organelles it might be anticipated that they would not modify the structure of the membrane when used as probes in vitro. However, these catalyse complete exchange of all of the phospholipids of the endoplasmic reticulum and cannot, therefore, differentiate between the two leaflets of the membrane bilayer [11–13]. Trinitrobenzenesulphonic acid has been used successfully in studies of the distribution of aminophospholipids in erythrocytes [14–16], platelets [17], fibroblasts [18–20], bacteria [9,21,22], mitochondria [16], retinal rod outer segment disc membranes [23,24] and sarcoplasmic reticulum [25,26]. We have now investigated the use of trinitrobenzenesulphonate, as a probe of the distribution of phosphatidylethanolamine in microsomal membranes derived from the endoplasmic reticulum of rat liver. Under conditions in which the vesicles remain closed, approximately one third of the phosphatidylethanolamine is labelled, an observation consistent with our previous results.

Methods

General methods. Protein was determined by the method of Lowry et al. [27]. Phospholipid phosphorus was determined by the method of Bartlett [28] after digestion of the lipid with perchloric acid.

Preparation of rat liver microsomes. Rat liver microsomes having a low contamination with Golgi or plasma membrane vesicles were prepared as previously using an MSE Pegasus DP ultracentrifuge [29,30].

Incubation of microsomes with trinitrobenzenesulphonate. Microsomal pellets were resuspended using a Potter-Elvejhem homogenizer by hand in a medium containing 70 mM sucrose, 280 mM mannitol, 40 mM $NaHCO_3$, 1 mM $MgCl_2$ and 2 mM succinate, pH 7.4. Aliquots of the suspension were incubated with trinitrobenzenesulphonic acid (TNBS) in a total volume of 5 ml of the above medium. In most experiments microsomal concentrations of 5–8 mg proteins were incubated with 3.0 mM TNBS at 7°C. However, in preliminary experiments a range of TNBS and protein concentrations were investigated and incubations were at 7°C and 37°C.

Extraction of lipids. At the end of the incubation period 1.0 ml of 30% trichloroacetic acid was added. The precipitated microsomes were isolated by centrifugation in a bench centrifuge and the pellets resuspended in 1.0 ml of methanol. 8 ml of chloroform/methanol/conc. HCl (2 : 1 : 0.01, v/v) was added and the extracts allowed to stand for at least 60 min. 2 ml of 0.2 M KCl in 0.1 M HCl was added to separate the phases. The upper phase was discarded and the lower phase washed with a further 2 ml of 0.2 M KCl in 0.1 M HCl. The lower phase was taken to dryness under vacuum or under nitrogen.

In some experiments at the end of the incubation, the microsomes were pelleted by centrifugation at 40 000 rpm for 40 min in the 40 rotor of the Beckman 65 ultracentrifuge. The pellets were extracted in the same way as those precipitated with trichloroacetic acid. Essentially the same results were obtained with either procedure.

Analysis of phospholipids. The total lipid extracts were redissolved in chloroform/methanol (1 : 1, v/v). Aliquots were removed to determine

total phospholipid phosphorus and further aliquots separated on thin-layer plates (Merck 60 F 254) using a one-dimensional system chloroform/methanol/glacial acetic acid/water (60 : 50 : 1 : 4, v/v). In this system, phosphatidylethanolamine (PE) had an R_f of 0.65 and trinitrophenol phosphatidylethanolamine (TNP-PE) an R_f of 0.86 and these lipids were resolved from other components. TNP-PE was visible as a yellow spot or band and after this was removed, PE was detected either by iodine vapour, or by spraying the plate with ninhydrin (3% in acetone) followed by heat to develop the pink colour characteristic of aminophospholipids.

The TNP-PE spot was scraped and eluted with chloroform/methanol (1 : 1, v/v) and diluted to an appropriate concentration for determination (see below).

The PE spot was scraped and the lipid digested with perchloric acid, distilled water added and the silica gel removed by centrifugation. Phosphorus was determined on the supernatant.

To prepare a standard curve for estimation of TNP-PE a range of concentrations of PE (Lipid Products, Nutfield Nurseries, Crab Hill Lane, South Nutfield, Surrey, U.K.) in 1 ml of chloroform/methanol (1 : 1, v/v) were reacted with TNBS at room temperature for 120 min. The lipids were extracted as above, redissolved in chloroform/methanol (1 : 1, v/v) and absorbance read at 337 nm. Under these conditions, all of the PE was converted to TNP-PE demonstrated by thin-layer chromatography. To determine TNP-PE reacted in microsomal membranes the yellow spot was eluted and diluted to give a value on the linear part of the curve.

In order to determine the % of microsomal PE labelled with TNBS, μmol of TNP-PE and PE were determined and the TNP-PE expressed as a % of the total recovery. Alternatively, total PE was determined in unreacted microsomes and the TNP-PE expressed as a % of total PE corrected for small differences in the recovery of total phospholipid of each individual sample. Either method gave the same % of PE reacted with TNBS.

Determination of integrity of microsomal vesicles. The integrity of microsomal vesicles was determined by measurement of mannose-6-phosphatase latency [30,31], or, by determination of loss of vesicular protein contents labelled with [^{3}H]leucine in vivo [2,32].

Electron microscopy of microsomes. After incubation of microsomes with or without TNBS, these were fixed overnight by addition of 0.2 vol. of 12.5% glutaraldehyde in cacodylate buffer, 0.1 M, pH 7.4 containing 0.25 M sucrose. The microsomes were pelleted by centrifugation at $10\,000 \times g$ in the 40 rotor of the Beckman 65 ultracentrifuge. The pellets were fixed overnight with 2% osmium tetroxide in 0.1 M Michaelis veronal buffer pH 7.4, rinsed with several changes of Michaelis buffer and stained en bloc with uranyl acetate 0.5% in distilled water overnight. The pellets were dehydrated and embedded in Araldite. thin sections were cut stained for 2 min with lead citrate and examined in a Philips 301 electron microscope.

Results

Reaction of microsomal PE with TNBS

At pH 7.4 approximately one third of the total PE of the microsomal membranes reacted with

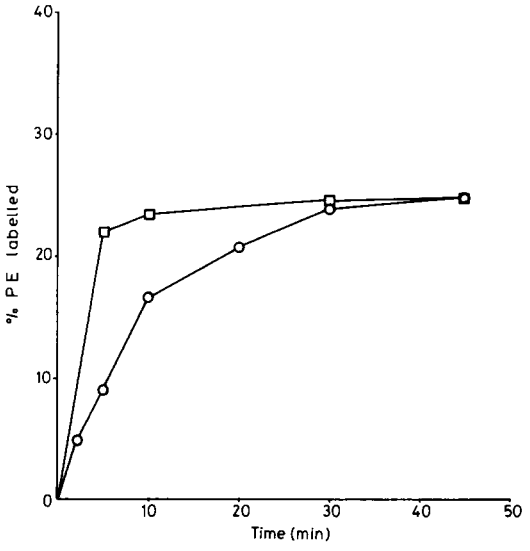


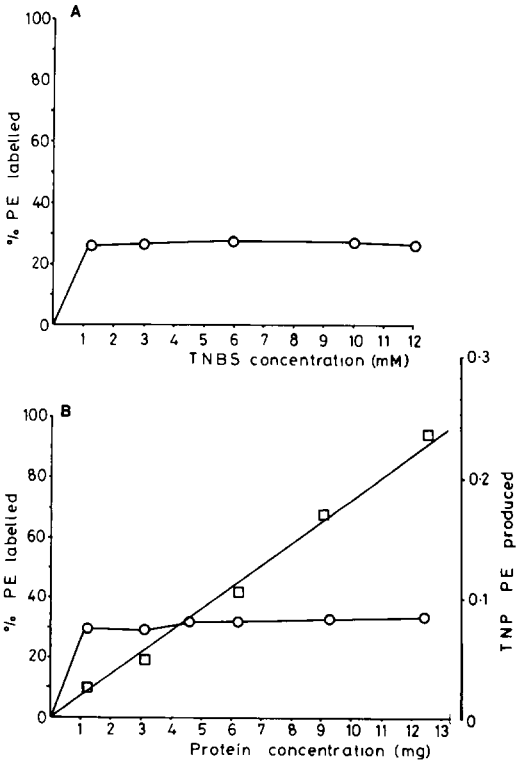
Fig. 1. Reaction of microsomal PE with TNBS. Microsomes (10 mg protein) were incubated for a range of times with TNBS (3.0 mM) at 37°C (□———□) and 7°C (○———○) and the microsomal PE labelled determined as described in Methods. Points plotted are averages of duplicate determinations at each time point.

TABLE I

REACTION OF MEMBRANE PE WITH TNBS IN MICROSOMAL VESICLES OPENED UNDER DIFFERENT CONDITIONS

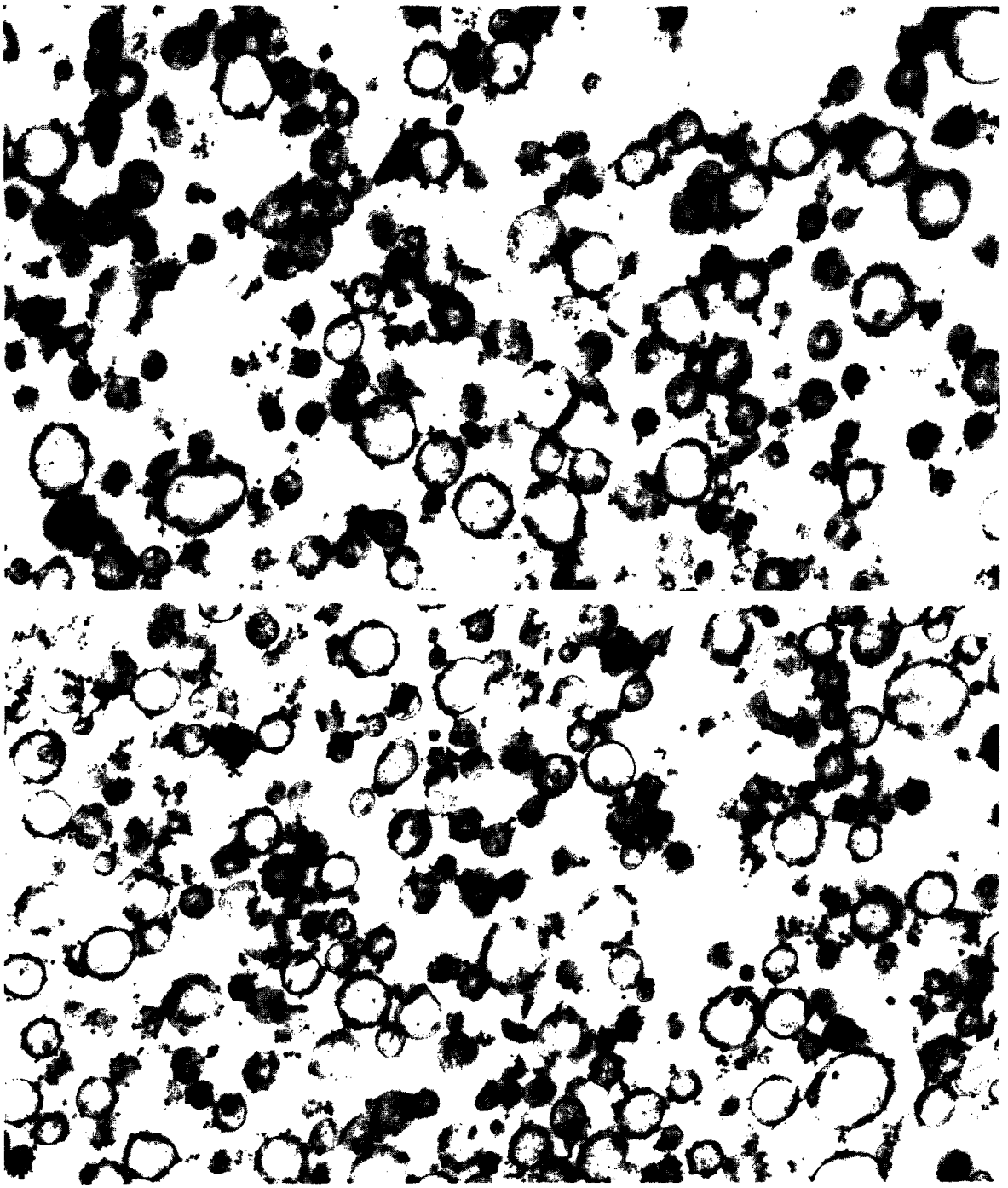
Microsomes (5–8 mg protein) were incubated for 30 min at 7°C with TNBS (3.0 mM) and the % PE labelled determined as described in Methods. French pressure cell treated microsomes were passed through the press in the presence of TNBS followed by incubation at 7°C for the necessary time to bring the total to 30 min. At alkaline pH values microsomes were incubated with TNBS in mannitol buffer at pH 8.2 and pH 9.0. For determination of mannose-6-phosphatase, the microsomes were isolated from the incubation medium by centrifugation, and resuspended in Tris buffer at pH 7.4 [30]. In parallel experiments, microsomes having labelled contents were incubated under the above conditions and the loss of labelled protein determined as described in Table I. Results plotted are averages ± S.D. (number of observations) for TNBS-labelled PE and averages of triplicate determinations of mannose-6-phosphatase latency or loss of labelled contents.

Treatment	% PE labelled	% mannose-6-phosphatase latency	% loss of labelled contents
No treatment	34.8 ± 5.4(8)	89.3	8.4
French pressure			
One passage	51.7 ± 10.9(8)	48.5	
Three passages	85.3 ± 4.7(4)	10.9	83
Incubation			
pH 8.2	80.0 ± 0.5(4)	91.8	30.1
pH 9.0	78.9 ± 4.3(4)	82.3	37.3
Taurocholate 0.4%	11.3 ± 2.4(4)		



TNBS. At 7°C the reaction reached completion within 30 min, while at 37°C labelling was more rapid and was complete within 10 min (Fig. 1). Over eight separate experiments 34.8 ± 5.4% (S.D.) of the PE was labelled at 7°C with 30 min incubation (Table I). When the TNBS concentration was fixed at 3.0 mM the % of the microsomal PE labelled remained constant over a range of microsomal protein concentrations from 1.2 to 12.4 mg and the TNP-PE produced was linear with membrane protein and phospholipid concentration (Fig. 2B). Similarly with a fixed protein concentration the same % of the microsomal PE was labelled over a range of TNBS concentrations from 1.2 to 12 mM (Fig. 2A). These observations suggest that

Fig. 2. Reaction of PE at a range of TNBS concentrations and microsomal protein concentrations. Microsomes were incubated with TNBS and PE labelled determined as described in Methods. In A, microsomal protein was fixed at 8 mg and % PE labelled is plotted against TNBS concentration. In B, TNBS concentration was 3.0 mM; % of PE labelled is plotted against microsomal protein concentration (○——○) and TNP-PE produced is plotted against microsomal protein concentration (□——□). Points plotted are averages of duplicate determinations at each protein concentration.



Figs. 3 and 4. Microsomes (10 mg protein) were incubated with and without TNBS (3.0 mM) for 30 min at 7°C and fixed and embedded for electron microscopy as described in Methods.

Fig. 3. Morphology of TNBS labelled microsomes. The preparation consists of closed vesicles many of which have bound ribosomes. There is no detectable difference between this preparation and microsomes incubated in the absence of TNBS. ×36000.

Fig. 4. Morphology of control microsomes incubated in the absence of TNBS. ×36000.

there are two pools of PE in microsomal membranes, approximately one third of the total PE is available to TNBS in the external medium, while two thirds are unavailable. TNBS at 3.0 mM is in excess at least up to 12 mg of microsomal protein and increasing this concentration of the labelling reagents by a factor of four does not increase the proportion of the membrane PE labelled.

Integrity of microsomal vesicles during reaction with TNBS

The validity of investigations of the transverse distribution of phospholipids depends on the membrane vesicles remaining impermeable to the probe used. To check this, soluble protein contents of microsomes were labelled by injection of [^{3}H]leucine 30 min prior to killing. A small percentage of labelled proteins associated with microsomes appeared in the incubation medium under the conditions used for labelling experiments without TNBS. This may represent adsorbed cytoplasmic proteins which are released on incubation, or may be due to some leakage from vesicles. On treatment of the microsomes with TNBS 8.5 ± 0.56% of the labelled proteins appeared in the incubation medium suggesting that the vesicles are not opened by this treatment. Consistent with this, latency of mannose-6-phosphatase was 91.65 ± 5.32% in untreated microsomes and 92.83 ± 4.21% after treatment with TNBS.

Morphology of TNBS treated microsomes

The microsomal preparation contained both rough and smooth vesicles. After incubation with TNBS there was no detectable change in the morphology of the preparation (Figs. 3 and 4). The vesicles remained closed consistent with retention of both labelled contents and latency of mannose-6-phosphatase.

Reaction of PE of opened microsomal vesicles with TNBS

When microsomal vesicles were opened partially by one passage through the French pressure cell, mannose 6 phosphatase latency fell from 90% to 40% and the membrane PE labelled increased to approx. 50% (Table I). Three passages through the French pressure cell decreased mannose-6-phosphatase latency to 10% and increased labelling of the total PE to more than 85%. The increased availability of PE for reaction with TNBS is therefore related to the proportion of vesicles opened, as indicated by loss of latency of mannose-6-phosphatase.

Most methods published for labelling membrane PE with TNBS perform the incubation at pH 8.2. In preliminary experiments we found that

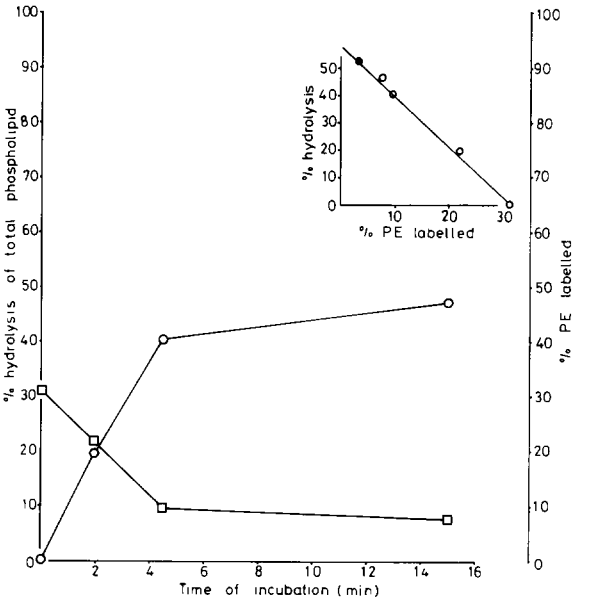


Fig. 5. Reaction of microsomal PE with TNBS after hydrolysis of membrane phospholipid with phospholipase C. Microsomes (40 mg protein) were resuspended in 0.14 M NaCl adjusted to pH 7.4 with bicarbonate and containing 1 mM $CaCl_2$. Aliquots (4 ml) were incubated for a range of times at 37°C with phospholipase C (10 units). One group of samples were incubated without phospholipase C. At the end of the incubation period 10 ml of the same medium lacking calcium but containing 1 mM EGTA was added to stop the reaction. The microsomes were isolated by centrifugation, and the pellets rinsed with and resuspended in mannitol buffer and incubated at 7°C with TNBS (3.0 mM) for 30 min. The reaction was stopped by addition of trichloroacetic acid and the lipids extracted and analysed as described in Methods. The % hydrolysis of total phospholipids (○——○) and the % of the total PE available for labelling by TNBS (□——□) are plotted against time of incubation with phospholipase C. In the inset figure % hydrolysis of phospholipids is plotted against % labelling of PE. An additional point (●) is included. In this experiment, microsomes (24 mg protein in 4 ml) were incubated with phospholipase C (40 units) for 5 min and no EGTA was added to stop the reaction before centrifugation. All points plotted are means of four determinations.

at pH values 8.2 and 9.0, approx. 80% of the membrane PE was labelled compared with 35% at pH 7.4 (Table I). At alkaline pH however the microsomal vesicles and other intracellular membrane vesicles are leaky [3,33,34]. Loss of labelled contents from microsomes is increased to 30.1% and 37.3% on incubation at pH values 8.2 and 9.0, respectively. The vesicles are therefore at least partially opened sufficient to allow relatively large protein molecules to leave. Latency of mannose-6-phosphatase is a measure of the entry of a relatively small molecule into the vesicles. However, it was not possible to measure this activity at high pHs and when microsomes were resuspended at pH 7.4 after incubation at alkaline pH and isolation by centrifugation the vesicles apparently resealed and mannose-6-phosphatase latency was high.

Taurocholate is effective at opening microsomal vesicles, however, in the presence of 0.4% taurocholate labelling of PE by TNBS was reduced to 11% suggesting that the reaction is inhibited by this detergent (Table I).

Comparison of phospholipase C and TNBS as probes for PE of microsomal vesicles

Phospholipase C hydrolysis approx. 30% of microsomal PE. When microsomes were treated with phospholipase C prior to labelling with TNBS, the % of the membrane PE labelled fell from 31% to 3%. The PE available for labelling by TNBS was proportional to the hydrolysis of the phospholipids (Fig. 5). Phosphorylethanolamine lost by hydrolysis is therefore the same as that labelled by TNBS. These observations support our previous conclusions that phospholipase C hydrolyses phospholipids of the outer leaflet of the membrane bilayer and also indicates that hydrolysis of these phospholipids does not result in any major rearrangement of the PE.

In the reverse experiments in which microsomes were labelled with TNBS prior to treatment with phospholipase C TNP-PE was not hydrolysed although hydrolysis of membrane phospholipids took place. Labelled PE is apparently not a substrate for phospholipase C and cannot be probed using this enzyme.

Discussion

Investigations of the distribution of phospholipids in vesicular membranes must fulfil two major criteria. The probe used should not have access to the interior of the microsomal vesicles unless the latter are opened experimentally, and the membrane structure should remain unperturbed by the treatment used. Microsomal vesicles are not opened by incubation with TNBS. This is indicated by retention of mannose-6-phosphatase latency, retention of labelled vesicular protein contents and the appearance of TNBS-labelled microsomes in the electron microscope. Under conditions in which the vesicles remain closed judged by these three criteria one third of the membrane PE is labelled. Phosphatidylserine also reacts with TNBS and approx. 27% of this phospholipid is labelled under the same conditions. However, as this phospholipid is present in only small amounts in microsomal membranes it is difficult to obtain accurate assays of its distribution using TNBS. Our previous investigations using phospholipase C [2,3] and phospholipase D [30] as probes have indicated that a similar proportion of PE is available for hydrolysis in closed microsomal vesicles to that labelled by TNBS. Judged by the criteria indicated above microsomal vesicles also remain closed after phospholipase C treatment. It is more difficult to demonstrate that the membrane structure is not perturbed by probes used to investigate the distribution of phospholipids. Perturbation of the structure may be small or drastic and methods used to assess the vesicle integrity will generally probably detect only major alterations in membrane structure. However, hydrolysis of PE by phospholipase C removes the PE labelled by TNBS in closed vesicles and there is a linear relationship between phospholipid hydrolysis and PE labelled. The PE labelled is therefore the same pool as that hydrolysed suggesting that with either type of probe used at least this phospholipid does not move from one side of the bilayer to the other. Phospholipase C produces diacylglycerol in the outer leaflet of the membrane bilayer and probably reduces the charge in this leaflet, while TNBS adds fairly bulky negatively charged groups to the surface PE. It is improbable that these two modifications would produce the same membrane structure perturbation.

When microsomal vesicles were opened by the French pressure cell or incubation at alkaline pH the PE labelled increased up to 90% of the total. Other investigators have also reported that not all membrane PE is reactive on incubation with TNBS [14,15,35,36]. One explanation of this is that TNBS introduces a bulky electronegative group into the membrane lipids and proteins and this prevents the reaction going to completion [36,37]. However, microsomal vesicles are extremely difficult to open completely. After three passages through the French pressure cell 10% of the mannose-6-phosphatase remained unexpressed. These closed vesicles may at least in part account for incomplete labelling of PE.

The present observations are consistent with our previous investigations of the transverse distribution of phospholipids in microsomal membranes. They indicate that PE has an asymmetric distribution with approximately one third in the outer leaflet of the membrane bilayer. The same pool of PE labelled by TNBS is hydrolysed by phospholipase C.

These observations confirm by an independent experimental approach our results from investigations using phospholipase C [2,3,30,38] and also support the validity of this enzyme as a probe for the transverse distribution of phospholipids in the endoplasmic reticulum.

Acknowledgments

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