The transverse distribution of phospholipids in the membranes of Golgi subfractions of rat hepatocytes

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The transverse distribution of phospholipids in the membranes of subfractions of the Golgi complex was investigated by using phospholipase C and 2,4,6-trinitrobenzenesulphonic acid as probes. In trans-enriched Golgi membranes, 26% of the phosphatidylethanolamine is available for reaction with trinitrobenzenesulphonate or for hydrolysis by phospholipase C, and 72% of the phosphatidylcholine is hydrolysed by phospholipase C. In cis-enriched Golgi membranes, 45% of the phosphatidylethanolamine is available for reaction with trinitrobenzenesulphonate and for hydrolysis by phospholipase C, and 95% of the phosphatidylcholine is hydrolysed by phospholipase C. Under the conditions used with either probe the contents of the Golgi vesicles labelled with either [^3H]palmitic acid or [^{14}C]leucine were retained. Galactosyltransferase activity of the membrane vesicles was partially inhibited by the experimental procedures used to investigate the transverse distribution of phospholipids. However, the residual activity was latent, suggesting that the vesicles remained closed. Trinitrobenzenesulphonic acid caused no detectable morphological change in either Golgi fraction. Phospholipase C treatment caused morphological changes, including fusion of vesicles and the appearance of 'signet-ring' profiles in some vesicles; however, the vesicles remained closed and the bilayer was retained. It appears, therefore, that neither probe causes major disruption of the Golgi vesicles nor gains access to the inner surface of the membrane bilayer. These observations suggest that phospholipids have a transverse asymmetry in Golgi membranes, that this distribution differs in trans and cis membranes, and that the phospholipid structure of Golgi membranes is inconsistent with a simple flow of membrane bilayer from endoplasmic reticulum to Golgi membranes to plasma membrane.

The role of hepatocyte Golgi membranes as an intermediate site in the elaboration, secretion and processing of plasma proteins and very-low-density lipoproteins is well established (Jones *et al.*, 1967; Stein & Stein, 1967; Claude, 1970; Redman & Cherian, 1972; Farquhar & Palade, 1981; Yokata & Fahimi, 1981). A functional continuity between the endoplasmic reticulum, the Golgi membranes and the plasma membrane therefore exists during secretion. Investigations of the Golgi complex in different cells have indicated that this organelle is also involved in lysosome formation, in the transfer of plasma-membrane proteins to the cell surface, in glycosylation or sulphation of membrane or secretory proteins and in the recycling of membrane retrieved from the cell surface (Farquhar & Palade, 1981). However, despite their diversity of function and complex interrelationships with other membranes, Golgi membranes

retain their individual specificity. There is evidence that individual cisternae differ from each other and that each may consist of a mosaic of membrane domains having different functions (Farquhar & Palade, 1981).

The phospholipids of Golgi membranes and plasma membrane are synthesized in the endoplasmic reticulum (Van den Bosch, 1974; Van Golde *et al.*, 1974). These membrane components must therefore be transferred from their site of synthesis to their final destination. The mechanism of this transfer and its controls are unknown, but in view of the heterogeneity of the Golgi membranes the formation of the phospholipid bilayer of these membranes must be complex. We have in recent years investigated the transverse distribution of phospholipids in the rough and the smooth endoplasmic reticulum and the plasma membrane of hepatocytes (Higgins & Dawson, 1977; Higgins &

Evans, 1978; Bollen & Higgins, 1980; Higgins & Pigott, 1982). The results of these studies indicated that both the phospholipid composition and their transverse distribution are inconsistent with a simple movement of membrane bilayer from endoplasmic reticulum. In the present investigation a similar study of the Golgi complex has been made, with the use of phospholipase C and 2,4,6-trinitrobenzenesulphonic acid as probes. The results of these investigations suggested that the architecture of the phospholipid bilayer of Golgi membranes resembles that of the endoplasmic reticulum, and indicate that if a flow of membrane from endoplasmic reticulum to Golgi to plasma membrane takes place there must be a re-arrangement and modification of the bilayer in the different membrane compartments.

Methods

Materials

[³H]Palmitic acid, [¹⁴C]leucine and UDP-[¹⁴C]-galactose were purchased from Amersham International. Glucose 6-phosphate, AMP, UDP-galactose, ovalbumin, phospholipase C (type I) and 2,4,6-trinitrobenzenesulphonic acid were purchased from Sigma Chemical Co. Other chemicals and solvents were purchased from BDH Chemicals.

Preparation of Golgi subfractions

The subfractions of Golgi membranes were isolated from livers of untreated rats by a modification of the method described by Bergeron (1979), which is based on that originally described by Ehrenreich *et al.* (1973). Livers were removed from male Sprague-Dawley rats of 150–200 g body weight. These were homogenized in 0.25 M-sucrose in a Potter-Elvehjem homogenizer at a concentration of 20 g of wet liver to 100 ml final volume. The homogenate was centrifuged at 10000 rev./min (7500 g) for 10 min in the 8 × 35 ml rotor of an MSE Pegasus DP ultracentrifuge. The pellet was resuspended in half the volume of 0.25 M-sucrose and re-centrifuged at the same speed. The supernatants of both runs were pooled and centrifuged at 40000 rev./min (150000 g) for 35 min in the 8 × 35 ml rotor. The pellets were resuspended by gentle homogenization in the Potter-Elvehjem homogenizer, in 1.15 M-sucrose at a concentration of less than 7 g starting weight of liver per 10 ml final volume. The suspension was layered beneath 10 ml of 0.86 M-sucrose and 12 ml of 0.25 M-sucrose. The gradient was centrifuged at 24000 rev./min (85000 g) for 2 h 20 min in the 6 × 38 ml swing-out rotor of an MSE Pegasus DP ultracentrifuge. Bands formed at the 0.25 M/0.86 M-sucrose interface and the 0.86 M/1.15 M-sucrose interface, ma-

terial remained in the 1.15 M-sucrose layer and a pellet formed. The floating fractions were removed, diluted to 0.25 M-sucrose and isolated by centrifugation at 40000 rev./min (150000 g_{av.}) for 45 min.

Separation of Golgi membranes and content

The method described by Howell & Palade (1982a) was selected as most satisfactory in the present studies. G₁ and G₂ fractions were resuspended in 100 mM-Na₂CO₃, pH 11.0, at concentrations of below 1 mg of protein/ml determined by the method of Lowry *et al.* (1951). These were left on ice for at least 30 min, and the membranes were separated from the content by centrifugation at 50000 rev./min (185000 g_{av.}) for 45 min.

Labelling of Golgi contents

Golgi secretory-protein contents were labelled by injection of [¹⁴C]leucine (5 μCi/100 g body wt.) in 0.9% NaCl, and lipids were labelled by injection of [³H]palmitate bound to bovine serum albumin (50 μCi/100 g body wt.). Both labelled compounds were injected into the portal vein of anaesthetized rats 10 min before removal of the liver and isolation of Golgi fractions.

Enzyme assays

Glucose-6-phosphatase, 5'-nucleotidase and UDP-galactose galactosyltransferase were assayed as described previously (Higgins, 1976) on freshly isolated Golgi fractions.

Investigations of the distribution of phospholipids in Golgi fractions

Phospholipase C treatment. Golgi fractions from two or three livers were resuspended in 0.14 M-NaCl adjusted to pH 7.4 with NaHCO₃ and containing 1 mM-CaCl₂. Samples were incubated for 10 min at 37°C with or without phospholipase C at a final concentration of 10 units/ml as described previously (Higgins & Dawson, 1977; Bollen & Higgins, 1980), except that because the yields of these fractions are small the volume of the incubation mixture was decreased to 0.1 ml for G₁ and 0.2 ml for G₂.

Labelling with trinitrobenzenesulphonate. Golgi fractions from two or three livers were resuspended in a medium containing 70 mM-sucrose, 280 mM-mannitol, 40 mM-NaHCO₃, 1 mM-MgCl₂ and 2 mM-succinate, pH 7.4. Samples were incubated with and without trinitrobenzenesulphonic acid (3 mM) in a final volume of 2.5 ml as described previously for 30 min at 7°C (Higgins & Pigott, 1982).

Extraction and analysis of lipids

At the end of the incubation with phospholipase C or trinitrobenzenesulphonate, lipids were ex-

tracted as described previously (Higgins & Dawson, 1977; Higgins & Pigott, 1982). In experiments in which loss of labelled lipid content into the supernatant was determined, the Golgi membranes were separated from the medium by centrifugation at 40 000 rev./min (150 000 $g_{av.}$) for 60 min, the pellets extracted with chloroform/methanol/conc. HCl (2:1:0.02, by vol.) and the supernatants extracted by the method of Bligh & Dyer (1959).

Portions of the lipid extract were taken for determination of total phospholipid, and larger portions were separated on thin layers of silica gel (Merck 60F 254) with a one-dimensional system (chloroform/methanol/acetic acid/water, 60:50:1:4, by vol.). In experiments in which phosphatidylethanolamine was labelled by trinitrobenzenesulphonate, the yellow derivative was scraped off and unmodified phosphatidylethanolamine detected by spraying the plate with 3% ninhydrin in acetone followed by gentle heat. In other experiments the phospholipid classes were detected by I_2 vapour.

All phospholipids were scraped from t.l.c. plates, and the phosphate was determined by the method of Bartlett (1959) after digestion with 70% (w/v) $HClO_4$. Neutral-lipid-containing spots were scraped off, eluted with several changes of chloroform/methanol (1:1, v/v) and the esters determined on the extract (Snyder & Stephens, 1959).

The hydrolysis of individual phospholipids by phospholipase C was determined by comparing untreated with phospholipase C-treated samples incubated in parallel. The percentage labelling of phosphatidylethanolamine was determined by measurement of loss of this lipid and appearance of its trinitrophenol derivative and by comparing untreated with trinitrobenzenesulphonate-labelled samples incubated in parallel.

Results

Preparation of Golgi fractions

In initial investigations we used a modification of the method described by Bergeron (1979) to prepare Golgi membranes from livers of untreated rats. This method was based on that of Ehrenreich *et al.* (1973), and yielded three Golgi-enriched fractions at the 0.25M/0.6M-sucrose, 0.6M/0.86M-sucrose and 0.86M/1.15M-sucrose interfaces of a discontinuous sucrose gradient; these were designated light, intermediate and heavy fractions respectively. The light fraction was too small for our investigation, and therefore the gradient was modified to produce a pooled light and intermediate fraction at the 0.25M/0.86M-sucrose interface and a heavy fraction at the 0.86M/1.15M-sucrose interface. A similar pooled light and intermediate fraction has been used in a number of investigations by Palade and co-workers (Ito &

Palade, 1978; Howell *et al.*, 1978; Howell & Palade, 1982a,b). These fractions have been designated G_1 and G_2 in order of increasing density. Vesicular material also remained in the 1.15M-sucrose layer, and a pellet formed. These are designated S and R respectively, in accordance with the convention of Bergeron (1979).

Viewed in the electron microscope, G_1 consisted predominantly of large vesicles filled with lipoprotein droplets, together with some large empty membrane vesicles and some flattened lamellae (Fig. 1). G_2 consisted of large vesicles, of flattened lamellae frequently associated in stacks and of smaller smooth surfaced vesicles (Fig. 1). S contained the same elements observed in G_2 , but having a larger proportion of small smooth vesicles, and R consisted predominantly of small vesicles, most of which had bound ribosomes (not illustrated). These morphological observations are consistent with those made by Bergeron (1979) and Ehrenreich *et al.* (1973). These authors have suggested that the light fraction is enriched with trans elements of the Golgi complex and that the heavy fraction is enriched with cis elements, on the basis of their appearance and enzyme characteristics (Farquhar & Palade, 1981). In liver the trans elements are equivalent to secretory granules of other secretory cells, whereas the cis elements probably are derived from the flattened stacked Golgi cisternae.

G_1 contained 6% of the galactosyltransferase of the microsomal fraction, and this enzyme was enriched 33-fold compared with the total homogenate (Table 1). Glucose-6-phosphatase activity was very low in this fraction and in some preparations was not detectable. 5'-Nucleotidase activity was also very low in G_1 (Table 1). Both of these enzymes, although used as markers for endoplasmic reticulum and plasma membrane, have been demonstrated as components of Golgi elements (Howell *et al.*, 1978; Ito & Palade, 1978). It is not possible therefore to assess the degree of contamination of G_1 with other membranes. However, assuming that all of the glucose-6-phosphatase and 5'-nucleotidase are due to contamination, it can be calculated that endoplasmic reticulum and plasma membrane each contribute less than 8% of the G_1 fraction.

G_2 contained 46% of the galactosyltransferase of the microsomal fraction with an enrichment of 14-fold compared with the homogenate (Table 1). Some 3.5% of the glucose-6-phosphatase of the microsomal fraction occurred in G_2 and the specific activity was approximately one-third of that of R or S (Table 1). However, Howell *et al.* (1978) have demonstrated that the specific activity of supposed endoplasmic-reticulum marker enzymes of isolated Golgi fractions can be as great as

Fig. 1. Electron micrographs of G_1 (a and b) and G_2 (c and d) subfractions
 G_1 consists predominantly of large vesicles filled with lipoprotein droplets and large empty vesicles. G_2 consists of flattened lamellae in which fenestrations are apparent in oblique section, large dilated vesicles and small vesicles. Magnification: (a) and (c) 31 500; (b) 63 000; (d) 72 500.

Table 1. Specific activities of marker enzymes in Golgi and microsomal subfractions isolated from rat liver

G₁, G₂, S and R fractions were prepared from pooled livers of two rats. Enzymes and protein were assayed as described in the Methods section immediately after preparation of the fractions. The results are means $\pm$ S.D. of results from four preparations. Specific activities are given in the following units: galactosyltransferase, nmol of galactose transferred/min per mg of protein; glucose-6-phosphatase, nmol of phosphate released/min per mg of protein; 5'-nucleotidase, nmol of phosphate released/min per mg of protein. Percentage distribution indicates activity in the fraction as a percentage of that in the microsomal fraction. Relative specific activity indicates specific activity of the enzyme as a fraction of that of the original homogenate.

Fraction	Protein (mg/8 g of liver)	Galactosyltransferase			Glucose-6-phosphatase			5'-Nucleotidase		
		Specific activity	Percentage distribution	Relative specific activity	Specific activity	Percentage distribution	Relative specific activity	Specific activity	Percentage distribution	Relative specific activity
G ₁	0.39 $\pm$ 0.1	5.9 $\pm$ 0.9	6.06	33	0.02 $\pm$ 0.02	0.05	0.4	0.039 $\pm$ 0.010	0.59	0.9
G ₂	5.97 $\pm$ 2.3	2.5 $\pm$ 1.2	45.87	14	0.09 $\pm$ 0.02	3.53	1.7	0.077 $\pm$ 0.008	18.10	1.9
S ₂	25.30 $\pm$ 2.2	0.62 $\pm$ 0.40	40.91	3.5	0.28 $\pm$ 0.03	42.50	4.9	0.045 $\pm$ 0.010	44.9	1.1
R	32.13 $\pm$ 1.9	0.08 $\pm$ 0.03	7.13	0.4	0.25 $\pm$ 0.03	53.90	4.9	0.029 $\pm$ 0.017	36.6	0.7

30% that of the microsomal fraction, provided that assays are performed on freshly prepared fractions. On storage of the fractions the specific activity of the Golgi enzymes decreases more rapidly than that of the endoplasmic reticulum. Ito & Palade (1978) have also demonstrated by immunoadsorbance techniques that morphologically recognizable Golgi elements contain the endoplasmic-reticulum marker NADPH-cytochrome *c* reductase. I assayed marker enzymes immediately after preparation of Golgi fractions. The glucose-6-phosphatase activity of G₂ could therefore largely be accounted for by Golgi activity rather than contaminating endoplasmic reticulum. The 5'-nucleotidase activity of G₂ was also higher than that of G₁. Bergeron (1979) noted a similar enrichment of this marker enzyme in this fraction, suggesting that plasma-membrane-derived vesicles may be a contaminant. However, 5'-nucleotidase has also been demonstrated to be a component of Golgi preparations by cytochemical techniques (Farquhar *et al.*, 1974; Little & Widnell, 1975). It is therefore difficult to assess the purity of G₂. This fraction probably has some content of both endoplasmic-reticulum- and plasma-membrane-derived vesicles, although these may be a small proportion of the total. As G₂ contained a large fraction of the galactosyltransferase of the homogenate and is morphologically different from G₁, this fraction was also used in subsequent investigations.

A large fraction of the microsomal galactosyltransferase was found in the S fraction, consistent with its morphology (Table 1). However, this fraction was also enriched in glucose-6-phosphatase. The pellet R contained little galactosyltransferase and was largely composed of rough-endoplasmic-reticulum vesicles (Table 1).

Preparation of opened Golgi membranes

For control experiments it was necessary to prepare opened Golgi membranes. We investigated a number of procedures, including the French pressure cell and osmotic shock, but found that the procedure reported by Howell & Palade (1982a) during the course of these investigations was the most effective method for separating Golgi membranes and contents. Treatment of G₁ and G₂ with 100 mM-Na₂CO₃ resulted in loss of 82% and 74% of the ³H-labelled protein contents of G₁ and G₂ respectively and 65% and 49% of the [³H]palmitate labelled triacylglycerol contents (Table 2). The membranes were recovered as flat sheets with no trace of lipoprotein droplets as content.

Approx. 34% of the total protein, 76% of the triacylglycerol and 21% of the phospholipid were lost from G₁ on treatment with Na₂CO₃ (Table 2). Similarly 53% of the total protein, 46% of the triacylglycerol and 5% of the total phospholipid were

Table 2. *Loss of components of G₁ and G₂ on treatment with Na₂CO₃*

G₁ and G₂ fractions were prepared from livers of rats after injection of [¹⁴C]leucine or [³H]palmitic acid or unlabelled leucine or palmitic acid. These fractions were separated into membranes and contents as described in the Methods section. Loss of protein was calculated by determination or radioassay of measured portions of membrane or content. In experiments to determine loss of triacylglycerol the membrane pellets and content supernatants were extracted and separated by t.l.c., and the triacylglycerol spot was eluted and either its radioactivity counted or determined as described in the Methods section. Phospholipid was determined on measured portions of the total lipid extracts of membrane and content. Loss of each component into the medium was determined as a percentage of recovery and as a percentage of untreated sample. These values were close and were averaged. Results are the means $\pm$ S.D. for four determinations.

	G ₁	G ₂
% loss of labelled protein content	82.4 $\pm$ 1.4	74.5 $\pm$ 0.4
% loss of labelled triacylglycerol content	65.8 $\pm$ 1.6	49.0 $\pm$ 2.2
% loss of protein	33.9 $\pm$ 5.0	52.8 $\pm$ 3.9
% loss of triacylglycerol	75.8 $\pm$ 0.4	45.5 $\pm$ 0.8
% loss of phospholipid	21.5 $\pm$ 0.4	4.7 $\pm$ 0.8

lost from G₂ (Table 2). As Howell & Palade (1982a) observed, the phospholipid released from the Golgi fractions consisted of phosphatidylcholine, which accounted for 77% and 81% of the content phospholipid of G₁ and G₂ respectively, and sphingomyelin, which accounted for 23% and 19%. There was no detectable phosphatidylethanolamine in the supernatants of G₁ or G₂, although this lipid comprised 20% of the total phospholipid of these fractions. Howell & Palade (1982a) have suggested that phosphatidylethanolamine might be a marker for Golgi membrane. Our observations support this possibility.

Phospholipid composition of Golgi fractions

Both G₁ and G₂ had comparable phospholipid compositions (Table 3): phosphatidylcholine accounted for approx. 50% of the total phospholipid and phosphatidylethanolamine for approx. 20%. The composition of these fractions was comparable with that reported by other investigators and is similar to that of most biological membranes.

Transverse distribution of phospholipids of Golgi fractions

Hydrolysis of the phospholipids of G₁ was rapid and reached a plateau after 5 min incubation, when 35% of the phospholipid was hydrolysed (Table 4). However, as 21% of the phospholipid is in the

Table 3. *Composition of phospholipid of Golgi fractions*
G₁ and G₂ fractions were prepared and the lipids extracted, separated by t.l.c. and assayed as described in the Methods section. The composition of the phospholipids was determined as percentages of the total recovery. Results are the means $\pm$ S.D. for five determinations.

	G ₁	G ₂
Sphingomyelin	12.1 $\pm$ 2.9	12.9 $\pm$ 4.9
Phosphatidylcholine	46.5 $\pm$ 2.2	49.5 $\pm$ 5.6
Phosphatidylserine	9.7 $\pm$ 1.8	10.9 $\pm$ 0.1
Phosphatidylinositol	9.2 $\pm$ 2.4	7.0 $\pm$ 2.0
Phosphatidylethanolamine	21.4 $\pm$ 2.7	20.9 $\pm$ 2.2

Table 4. *Transverse distribution of phospholipids of Golgi fractions*

G₁ and G₂ were prepared and the phospholipid available for reaction with trinitrobenzenesulphonate or for hydrolysis by phospholipase C determined as described in the Methods section. Results are the means $\pm$ S.D. for four determinations.

	G ₁	G ₂
% of phosphatidylethanolamine labelled by trinitrobenzenesulphonate	26.0 $\pm$ 4.5	45.1 $\pm$ 3.5
% of phospholipid hydrolysed by phospholipase C		
Phosphatidylethanolamine	26.4 $\pm$ 6.5	45.5 $\pm$ 1.5
Phosphatidylcholine	72.2 $\pm$ 5.4	95.0 $\pm$ 1.0
Total phospholipid	34.5 $\pm$ 1.7	53.3 $\pm$ 2.4

vesicle content, hydrolysis of the membrane phospholipid is approx. 44%. After making a similar correction for the composition of the membrane content hydrolysis of membrane phosphatidylcholine can be calculated to be 88%, and hydrolysis of membrane phosphatidylethanolamine, which is not a component of the content, was 26% (Table 4). The remaining phospholipids hydrolysed by phospholipase C, sphingomyelin and phosphatidylserine, are minor constituents of the membranes and, because yields of G₁ and G₂ are low, these lipids cannot be accurately assayed after hydrolysis, and data for these are not included.

Some 54% of the total phospholipid of G₂ was hydrolysed at equilibrium by phospholipase C (Table 4). Only 5% of the phospholipid of this fraction is in the content and, corrected for this, hydrolysis of the total phospholipid is 57%. A greater proportion of G₂ membrane phospholipids is therefore available for hydrolysis by exogenous phospholipase C than of G₁ phospholipid; 95% of the phosphatidylcholine of G₂ was hydrolysed and 46% of the phosphatidylethanolamine (Table 4).

Trinitrobenzenesulphonic acid reacts with amino-phospholipids. Labelling reached a plateau after 20 min incubation, when 26% and 45% of the

phosphatidylethanolamine of G_1 and G_2 had reacted (Table 4). This is in good agreement with the results from investigations with phospholipase C.

Integrity of Golgi fractions during experimental procedures

Retention of contents. The validity of experiments in which probes are used to determine the transverse distribution of membrane phospholipids is dependent on the membrane vesicles under investigation remaining impermeable. One measure of vesicle permeability is the retention of labelled contents. [^{14}C]Leucine injected short times before preparation of Golgi fractions labels predominantly the soluble-secretory-protein content of the vesicles (Howell & Palade, 1982a; Kreibich *et al.*, 1973). When the Golgi vesicles are opened by Na_2CO_3 treatment the bulk of the proteins labelled in this way are lost into the incubation medium (Table 2). When G_1 and G_2 were labelled with trinitrobenzenesulphonic acid there was a small loss of labelled protein into the incubation medium (Table 5). This was similar to that lost on incubation in the absence of trinitrobenzenesulphonate, and may represent adsorbed labelled protein lost from the vesicle surface on incubation at 37°C or rupture of a small proportion of the vesicles. When the vesicles were treated with phospholipase C there was a small increase in the loss of label into the medium (Table 5), but this was considerably less than that lost when the vesicles were opened by using Na_2CO_3 (Table 2).

[^3H]Palmitic acid bound to albumin injected intraportally is rapidly incorporated into both phospholipid and triacylglycerol of G_1 and G_2 , with approx. 70% of the label in neutral lipid, 20% in phosphatidylcholine and 6% in phosphatidylethanolamine. When the fractions were treated with Na_2CO_3 , 66% and 49.0% of the labelled triacylglycerol were lost from G_1 and G_2 respectively. There was also loss of labelled phospholipid, suggesting that a large proportion of the labelled lipids

are located in the content of the vesicles. When G_1 and G_2 were incubated with trinitrobenzenesulphonate or phospholipase C, very little radioactivity appeared in the incubation medium (Table 5). The relatively large lipoprotein droplets are therefore retained, as are the protein contents, suggesting that the membrane vesicles remain closed during experiments to probe the distribution of phospholipids.

Latency of galactosyltransferase. Galactosyltransferase was assayed in Golgi fractions by transfer of [^3H]galactose from UDP-galactose to ovalbumin. With this acceptor Triton X-100, or an alternative detergent, is required for expression of enzyme activity. The detergent apparently opens the vesicles to allow ovalbumin access to the enzyme at the inner surface of the membrane. In the absence of Triton X-100 only 10–12% of the galactosyltransferase of G_1 or G_2 was expressed (Table 6). Bergeron *et al.* (1982) have demonstrated that this enzyme will transfer galactose to endogenous acceptors in the absence of Triton. This activity, which is a few percent of that observed in the presence of Triton and exogenous acceptor, could account for part of the activity that we observed without Triton. The latency of galactosyltransferase in the absence compared with the presence of Triton X-100 with ovalbumin as an acceptor may potentially be used as an index of the permeability of the Golgi vesicles. The validity of this would be established by the demonstration that when the vesicles are opened the latency is abolished. Unfortunately the most satisfactory method of opening the vesicles, 100 mM- Na_2CO_3 , also completely inhibited galactosyltransferase activity. However, with an endogenous acceptor galactosyltransferase does not need Triton X-100 for expression. It is unlikely that the detergent is unmasking the enzyme, but more probable that it serves to open the vesicle to large-molecular-mass acceptors.

After treatment of G_1 or G_2 with trinitrobenzenesulphonic acid or phospholipase C, galactosyl-

Table 5. *Loss of labelled content of Golgi fractions on treatment with phospholipase C or trinitrobenzenesulphonic acid* G_1 and G_2 fractions were prepared either having ^{14}C -labelled protein contents or [^3H]palmitate-labelled lipid contents. These were treated with trinitrobenzenesulphonate or phospholipase C as described in the Methods section. The membranes were isolated from the incubation medium by centrifugation, and the radioactivities of measured portions of supernatants, pellets and untreated fractions were counted. Results expressed are means $\pm$ S.D. for four determinations of the percentage of label appearing in the incubation medium.

	G_1			G_2		
	No treatment	Trinitrobenzenesulphonate	Phospholipase C	No treatment	Trinitrobenzenesulphonate	Phospholipase C
% loss of ^{14}C -labelled protein	6.1 ± 0.9	7.2 ± 1.3	11.5 ± 2.3	6.2 ± 0.7	5.6 ± 2.4	10.9 ± 1.0
% loss of [^3H]palmitic acid	0.8 ± 0.4	1.0 ± 0.8	4.2 ± 2.0	1.1 ± 0.4	1.0 ± 0.2	2.1 ± 0.5

Table 6. Latency of galactosyltransferase of Golgi fractions treated with trinitrobenzenesulphonate or phospholipase C. G_1 and G_2 fractions were prepared and treated with trinitrobenzenesulphonate or phospholipase C, as described in the Methods section. The Golgi fractions were isolated by centrifugation and the galactosyltransferase activity was determined with and without Triton X-100 as described in the Methods section. The specific activity is nmol of [14 C]galactose transferred to trichloroacetic acid-insoluble material in the presence of Triton. Percentage latency is the specific activity in the presence of Triton minus the specific activity in the absence of Triton as a percentage of activity in the presence of Triton. Results are averages for two determinations on three preparations of Golgi subfractions.

	G_1		G_2	
	Specific activity	Percentage latency	Specific activity	Percentage latency
Untreated	6.43	87.8	1.98	89.3
Trinitrobenzenesulphonate-treated	1.64	95.5	0.66	99.2
Phospholipase C-treated	0.35	99.9	0.1	99.2

transferase was inhibited, suggesting that, although this enzyme has a cisternal location, it may be modified by probes of the phospholipids or protein at the cytoplasmic side of the membrane. However, the residual activity in each case required Triton X-100, suggesting that the vesicles remain impermeable to ovalbumin (Table 6).

Morphology of Golgi subfraction after phospholipase C or trinitrobenzenesulphonate treatment

After incubation with trinitrobenzenesulphonic acid G_1 showed little morphological change. The fraction still consisted of large membrane envelopes filled with lipoprotein droplets and some empty vesicles. Some of the envelopes tended to be dilated, so that the lipoprotein particles were more clearly defined. However, similar changes were seen after incubation of G_1 without trinitrobenzenesulphonate (Fig. 2).

G_2 after incubation tended to form smaller vesicles, and, although some lamellae were retained, these did not associate in stacks as in the unincubated fraction. Incubation of G_2 with trinitrobenzenesulphonate caused no further change in the fraction (Fig. 2).

In contrast with trinitrobenzenesulphonate treatment, incubation with phospholipase C caused significant morphological changes in both G_1 and G_2 (Fig. 3). Although G_1 vesicles for the most part retained their size and content, they tended to adhere and fuse so that several vesicles pooled their content. In addition, some vesicles exhibited a 'signet-ring' structure similar to that I have observed previously in microsomal vesicles treated with phospholipase C (Higgins, 1982). We have interpreted these observations in phospholipase C-treated microsomal preparations as being due to an increase in the diacylglycerol of the outer leaflet of the bilayer. This would result in a shrinkage of this leaflet relative to the inner leaflet, which

might be stabilized by fusion of the vesicles. Similar suggestions have been made for shape changes in the erythrocyte membrane after phospholipase C treatment (Allan *et al.*, 1975). The membranes of the Golgi vesicles retained the bilayer structure when seen in appropriate section.

G_2 tended to form smaller vesicles on incubation with or without phospholipase C. The changes observed after phospholipase C treatment were similar to those observed in microsomal preparations (Higgins, 1982). The vesicles tended to adhere, and some vesicles exhibited a 'signet-ring' structure (Fig. 3).

Although phospholipase C undoubtedly causes morphological changes in both G_1 and G_2 , the vesicular structure is apparently retained and the bilayer appearance of the membrane is not disrupted.

Availability of phospholipids of Na_2CO_3 -treated membranes to phospholipase C or trinitrobenzenesulphonate

When G_1 or G_2 was opened by Na_2CO_3 treatment before hydrolysis with phospholipase C, hydrolysis was increased to 80–85%. Phosphatidylcholine hydrolysis increased to 90%, and hydrolysis of phosphatidylethanolamine also increased, to 60–70% (Table 7). Similarly 70–80% of the phosphatidylethanolamine of G_1 and G_2 was available to trinitrobenzenesulphonate in 'opened' Golgi membranes. The phospholipids of the open Golgi fractions thus behave as a more uniform pool, when probed with either phospholipase C or trinitrobenzenesulphonic acid (Table 7).

Discussion

The validity of investigations to determine the transverse distribution of phospholipids is dependent on several criteria. The probe used should not gain access to the inner surface of the vesicle and

Fig. 2. Electron micrographs of trinitrobenzenesulphonate-labelled G_1 and G_2 subfractions
 G_2 [(a) and (b)] exhibits morphological changes compared with unincubated G_2 (Figs. 1c and 1d). Although some large dilated vesicles and flattened lamellae are retained, many of these appear to have formed smaller vesicles. This was also observed in control incubated fractions and is probably a consequence of the incubation rather than the labelling procedure. The bilayer structure of the membrane is retained when viewed in appropriate section (arrow). G_1 [(c) and (d)] exhibits no marked morphological change compared with the untreated subfraction (Figs. 1a and 1b), and the membrane bilayer structure is retained in this fraction (arrow). Magnification: (a) and (c) 39 400; (b) and (d) 77 000.

Fig. 3. Electron micrographs of phospholipase C-treated G_1 and G_2 subfractions

Both G_1 [(a)–(c)] and G_2 [(d) and (e)] subfractions exhibit morphological changes after phospholipase C treatment. G_1 [(a)–(c)] contained some apparently unchanged vesicles, some vesicles with associated lipid droplets giving a 'signet-ring'-like appearance (arrow head) and some vesicles that appeared to fuse, sharing contents or having a common bilayer membrane (arrows). In all of the vesicles the bilayer was retained (double arrows). G_2 [(d) and (e)] formed small vesicles as in incubation controls. Profiles were observed that suggested that these vesicles fuse and adhere. 'Signet-ring'-like profiles were also apparent (arrow head). The bilayer structure was also retained in these vesicles (double arrows). Magnification: (a) and (e) 78 200; (b) and (c) 61 000; (d) 42 400.

Table 7. Availability of phospholipids of 'open' Golgi fractions to trinitrobenzenesulphonate or phospholipase C
 G_1 and G_2 fractions were isolated and 'opened' with Na_2CO_3 as described in the Methods section. The 'open' membranes were treated with trinitrobenzenesulphonate or phospholipase C as described in the Methods section. Results are averages for three determinations.

	G_1	G_2
% of phosphatidylethanolamine labelled by trinitrobenzenesulphonate	78	77
% of phospholipids hydrolysed by phospholipase C		
Total phospholipid	85	80
Phosphatidylcholine	99	90
Phosphatidylethanolamine	75	67

should not cause a re-arrangement of the phospholipids of the membrane. In the present investigation several pieces of evidence suggest that Golgi

vesicles remain impermeable during treatment with either phospholipase C or trinitrobenzenesulphonate: (a) the protein contents labelled with [^{14}C]leucine and the triacylglycerol contents labelled with [^3H]palmitate are retained during treatment, although the bulk of these are lost if the vesicles are opened with Na_2CO_3 ; (b) residual galactosyltransferase activity remains latent in Golgi vesicles after experimental manipulation; (c) the morphology of Golgi subfraction is unchanged after trinitrobenzenesulphonate labelling, and although there are morphological changes after phospholipase C treatment the Golgi vesicles apparently remain closed and the bilayer is retained.

It is difficult to demonstrate that there is no re-arrangement of membrane phospholipid on treatment with phospholipase C or trinitrobenzenesulphonate. These procedures undoubtedly cause a modification of the phospholipid bilayer, but it is critical that this modification does not cause trans-

verse movement of the phospholipids. The bilayer structure of the membrane is retained after either phospholipase C or trinitrobenzenesulphonate, indicating that there is no gross re-arrangement of the membrane structure. Both probes used act on the same percentage of phosphatidylethanolamine, and we have demonstrated that in microsomal vesicles the pool of phosphatidylethanolamine hydrolysed by phospholipase C is the same pool as that labelled by trinitrobenzenesulphonic acid (Higgins & Pigott, 1982), and similar results were obtained in investigations of Golgi subfractions. It seems unlikely that hydrolysis of the bases of the outer-leaflet phospholipids would produce the same perturbation of membrane structure as the addition of a fairly bulky negatively charged molecule to the phosphatidylethanolamine of the outer leaflet.

The anatomic origin of Golgi subfractions used in the present investigation is not completely clear. G_1 appears to be enriched with trans elements and has little contamination with endoplasmic reticulum and plasma membrane. However, it is possible that some of the vesicles form by pinching from the ends of cis lamellae. G_2 appears to be enriched with cis elements, but contains a significant, although small, contamination with other smooth membranes. A clear-cut separation of Golgi elements is not therefore achieved. However, investigations of the transverse distribution of phospholipids measure the average pattern and therefore provide information about the bulk of the membrane in each fraction. A small contamination of the Golgi subfractions would not significantly modify conclusions from such studies.

The observations reported in the present paper indicate that the phospholipids of Golgi membranes have an asymmetric distribution. G_1 has 85% of the phosphatidylcholine and 25% of the phosphatidylethanolamine in the cytoplasmic leaflet, whereas G_2 has 95% of the phosphatidylcholine and 46% of the phosphatidylethanolamine in the cytoplasmic leaflet. A greater proportion of the membrane phospholipid of G_2 is available for hydrolysis than that of G_1 , suggesting that more phospholipid is in the cytoplasmic leaflet of G_2 . This might be due to differences in the concentration and transverse distribution of either protein or cholesterol. In secretory cells there is evidence from freeze-fracture studies that the intramembranous (protein) particles decrease from the cis to the trans side of the complex and that cholesterol detected by filipin increases in the same direction (Orci *et al.*, 1981a,b).

A comparison of the distribution of phospholipids with that previously reported for rough and smooth microsomal membranes and for subfractions of plasma membrane yields several points of

Fig. 4. Distribution of phospholipids in subfractions of rat liver

The transverse distribution of phosphatidylcholine (▨) and phosphatidylethanolamine (□) in the bilayer of endoplasmic reticulum, Golgi membranes and plasma-membrane subfractions is illustrated. For each fraction the phospholipid in the 'outer' leaflet is shown at the top of the histogram. This corresponds to the cisternal leaflet of endoplasmic reticulum and Golgi subfractions and the external leaflet of the plasma-membrane subfractions.

interest (Fig. 4) (Higgins & Dawson, 1977; Higgins & Evans, 1978; Bollen & Higgins, 1980; Higgins & Piggott, 1982). All of these fractions have the bulk of the phosphatidylcholine in the outer leaflet of the vesicle membrane. This corresponds to the external surface of the plasma membrane and the cytoplasmic surface of the Golgi and endoplasmic-reticulum membranes. These observations indicate that a simple flow of membrane bilayer from endoplasmic reticulum to Golgi elements to the sinusoidal plasma membrane does not occur. If there is a net transfer of membrane bilayer during secretion, there is at the same time a re-arrangement of the transverse distribution of phospholipid and small changes in the composition of the phospholipids. The mechanisms and control factors involved in this re-arrangement are unknown. Transfer of phospholipids, for example by phospholipid-

exchange proteins, may also play a role. There is evidence from cytochemical experiments on isolated Golgi membranes and plasma-membrane fractions that 5'-nucleotidase shifts from the outside of cis elements of the Golgi fractions to the inside of the trans elements and the outside of the plasma-membrane vesicles (Farquhar *et al.*, 1974; Little & Widnell, 1975). Re-arrangement of the transverse distribution of the membrane is not restricted to phospholipids, therefore, but may also involve enzyme components.

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