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Phospholipid asymmetry in renal brush-border membranes

Catherine Venien and Christian Le Grimellec

INSERM U.251, Département de Physiologie, Faculté de Medecine Xavier-Bichat, Paris (France)

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The topological distribution of phospholipids between the inside and the outside of rabbit kidney brush-border membranes has been investigated by incubating membrane vesicles with sphingomyelinase, phospholipases A_2 from bee venom and hog pancreas, phospholipases C and D, and trinitrobenzene sulfonate. Orientation and integrity of vesicles upon phospholipase treatment was determined by using two monoclonal antibodies recognizing an extracytoplasmic and a cytoplasmic domain, respectively, of the neutral endopeptidase (EC 3.4.24.11). It is shown that the transbilayer distribution of phospholipids is highly asymmetrical in kidney brush-border membranes: sphingomyelin accounted for 75% of the phospholipids present in the external leaflet, whereas phosphatidylethanolamine and phosphatidylserine plus phosphatidylinositol were found to comprise the majority of the inner layer of the membrane.

Introduction

Using isolated brush-border and basolateral membranes it has been demonstrated that, in transporting epithelia, chemical composition and function of apical and basolateral domains of the plasma membrane differ markedly [1–3]. This polarity extends to the lipid constituents as well as to the fluidity properties of the plasma membrane [4–7]. From experiments on epithelial cells in culture it has been shown that tight junctions constitute a barrier to the diffusion of lipids in the outer, but not in the inner, plasma membrane leaflet [8,9]. This suggests the existence, for epithelial cells, of a transmembrane asymmetry in the distribution of their plasma membrane lipids. Conclusive evidence for such an asymmetric distribution of phospholipids across the membrane has been obtained from red blood cells [10–12], but, except for a recent study on the distribution of PC and PE in rabbit intestinal brush-border membranes [13], and partial results concerning the localization of PC [14], for dog, and sphingomyelin for rat kidney brush-border membranes [15], little is known about lipid topology in epithelial plasma membranes.

Because they contain very large amounts of glycolipids, in the intestine [16,17], or of sphingomyelin, in the kidney [7,14], two polar lipid species whose transbilayer movements are generally extremely slow [18,19], brush borders might constitute an useful model for studies on the maintenance of lipid asymmetry in membranes.

In the present experiments we have therefore investigated the topological distribution of the major phospholipids of rabbit kidney brush-border membranes. Bee venom and hog pancreas phos-

Abbreviations: PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; TNBS, 2,4,6-trinitrobenzene sulfonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FITC, fluorescein isothiocyanate.

Correspondence: C. Le Grimellec, INSERM U.251 Faculté Xavier Bichat, 16, rue Henri Huchard, 75018 Paris, France.

pholipases A_2, sphingomyelinase, phospholipases C and D and TNBS were used as membrane probes.

Orientation of vesicles, checked by monoclonal antibodies directed against both the extracytoplasmic and the cytoplasmic domains of the endopeptidase 24.11, was predominantly (86%) right-side-out. The results demonstrate that the transbilayer distribution of phospholipids in kidney brush-border membranes is highly asymmetrical: SPH accounted for 75% of the phospholipids present in the external leaflet, the other phospholipid classes, PC, PE and PS plus PI representing 15%, 7% and 3%, respectively. Corresponding values for the cytoplasmic leaflet were 9%, 24%, 38% and 28%, respectively. Phospholipase A_2 experiments further indicated a rate of transbilayer migration faster for PE and PS + PI than for PC.

Experimental procedures

Membrane preparation. Brush-border membrane vesicles from the kidney cortex of male New Zealand white rabbits (2–2.5 kg body weight) were isolated as previously described [5] using a $MgCl_2$ method developed by Booth and Kenny [20]. Membranes were suspended (5 mg protein/ml) in 150 mM NaCl/20 mM Hepes (pH 7.4) and used in topology experiments the day of their preparation. In brush-border vesicle preparations, activities of alkaline phosphatase and γ-glutamyltranspeptidase were enriched 9–12-fold over those of cortical homogenate, compared to less than 0.7-fold for Na^+/K^+-ATPase, less than 0.6-fold for succinate dehydrogenase, less than 0.4-fold for glucose-6-phosphatase and less than 0.2-fold for glucosaminidase.

Vesicle orientation. Orientation of vesicles was determined using two monoclonal antibodies, 23B11 and 2B12, a gift of Dr. P. Crine (Department of Biochemistry, University of Montreal, Canada), recognizing a cytoplasmic and an extracytoplasmic domain, respectively, of the neutral endopeptidase-24.11, a protein present in large amount in kidney brush borders. Vesicles (100 μg protein), either intact or permeabilized by digitonin (3 mg/mg of protein), were incubated for 1 h at 37 °C with an excess (8 μg) of antibodies.

After two washes in phosphate-buffered saline (pH 7.4), pellets were resuspended in phosphate-buffered saline containing anti-mouse IgG conjugated to FITC in excess. Unbound antibody was removed by three washes in phosphate-buffered saline and the pellet was solubilized by adding 200 μl of sodium dodecyl sulfate 20 mM. Fluorescence was measured after complete solubilization and 10-fold dilution by water. Vesicles without 23B11 and 2B12 and second antibody treatment and samples from which monoclonal antibodies were omitted were run in parallel to serve as a control for nonspecific binding and light scattering, respectively. The percentage of sealed, right-side-out vesicles was calculated from the increase in 23B11 coupled fluorescence induced by digitonin. That of inside-out vesicles was obtained in a similar way using 2B12, whereas the percentage of unsealed vesicles was calculated by difference.

Lipid analysis. Lipids were extracted using the method of Bligh and Dyer [21]. Extraction was repeated three times to obtain a 100% recovery [14]. Extracts were pooled, evaporated to dryness under nitrogen and solubilized in chloroform/methanol mixture (2 : 1, v/v). Thin-layer chromatography was done on precoated silica gel thin-layer plates (Whatman K5) using chloroform/methanol/water/acetic acid (65 : 25 : 4 : 1, v/v) as developing solvent. Individual components were detected by exposure to iodine vapor and identified by comparison with authentic standards. The spots were scraped off and transferred into acid-washed test-tubes. Known amounts of cholesterol and defined phospholipids were treated in the same way to be used as internal standards. The phosphorus content of phospholipid classes was determined according to Mrsny et al. [22]. Cholesterol and cholesterol esters were determined enzymatically [23].

Phospholipase treatment. Phospholipases A_2 from bee venom and from hog pancreas, phospholipase C and sphingomyelinase from *Bacillus cereus* and phospholipase D from cabbage were purchased from Boehringer-Mannheim (France).

For experiments with phospholipases A_2, phospholipase C and sphingomyelinase, brush-border membrane vesicles were dispersed in 20 mM Hepes/150 mM NaCl (pH 7.4) at 1 mg of protein/ml. Acetate buffer (100 mM sodium

acetate/50 mM NaCl (pH 5.6)) replaced Hepes buffer in phospholipase D experiments. Incubations were carried out at 37°C with gentle shaking. The amount of enzyme and the concentration of divalent cations to be added to vesicles for a good resolution of the biphasic character of hydrolysis curves (see Results) were determined in preliminary experiments. Best results were obtained with 1 U sphingomyelinase/mg protein in presence of 0.25 mM $MgCl_2$, 0.5 U phospholipase A_2 (bee venom/mg protein) plus 10 mM $CaCl_2$, 0.25 U phospholipase A_2 (hog pancreas)/mg protein with addition of 1 mM $CaCl_2$ and 5% bovine serum albumin, and 2.5 mg phospholipase D/mg protein in presence of 40 mM $CaCl_2$. Using phospholipase C, concentrations as low as 0.02 U/mg of protein in 0.1 mM $CaCl_2$ were found to be lytic for the vesicles. Reactions were started by adding the appropriate amount of enzyme to the vesicle suspension. After various incubation times, aliquots were taken and the reaction was stopped by the addition of an excess of EDTA. Lipids were immediately extracted. For each experiment, a sample treated identically but with the phospholipase omitted was run in parallel to serve as a control. Maximal accessibility of phospholipids to sphingomyelinase, phospholipase A_2 (hog pancreas), and phospholipases C and D was achieved by solubilization with 0.1% (w/v) Triton X-100. When using phospholipase A_2 from bee venom vesicles were disrupted by 0.4% (w/v) deoxycholate: membranes were preincubated for 10 min at 0°C with the enzyme and deoxycholate, in the absence of calcium. Phospholipase was then activated by the addition of 10 mM calcium and by raising the temperature to 37°C, an experimental condition which resulted in the precipitation of deoxycholate.

Labelling by TNBS. Color-free TNBS was purchased from Eastman Kodak and neutralized as described by Grunberger et al. [24]. Chemical labelling of amino groups was done by incubating the brush-border vesicles in 2 mM TNBS/20 mM Hepes/150 mM NaCl (pH 8.0) at 4°C in the dark. Reaction was stopped by addition of 1 M HCl (final pH 2.5). Unreacted TNBS was eliminated by centrifugation and washing. The pellet was resuspended in pH 2.5 buffer and lipids were extracted as above. Trinitrophenyl derivatives of PE and PS were separated by thin-layer chromatography. Estimation of the total amount of primary amino groups present in the preparation was made by adding 0.1% Triton X-100 to the vesicle suspension.

Enzyme and protein determination. Activities of the marker enzymes were determined as previously described [5,14]. Protein determinations were made by the method of Lowry et al. [26] after precipitation with 10% trichloroacetic acid and by the method of Bradford [26]. Bovine serum albumin was used as a standard.

Calculations. Distribution of phospholipids between external and cytoplasmic leaflets was calculated from phospholipase and TNBS data assuming similar reaction rates for both leaflets. Correction for the contribution of inside-out and unsealed vesicles was done using the formula:

$$\% \text{ modified lipid} = Ax + B(1 - x) + C$$

in which A, B and C represent the percentages of right-side-out, inside-out and unsealed vesicles and x the portion of the phospholipid present on the external leaflet.

Results

Orientation and lipid composition of vesicles

The availability of two monoclonal antibodies, 23B11 and 2B12, recognizing a cytoplasmic and an extracytoplasmic domain, respectively of the endopeptidase 24.11, a transmembrane protein present in large amount in kidney brush-border membranes [27,28], allowed us to determine the orientation of isolated vesicles: fluorescence intensities of anti-mouse IgG conjugated to FITC and bound to the monoclonal antibodies measured in intact and digitonin-permeabilized membranes indicated that, in freshly prepared vesicles, more than 85% of the vesicles had maintained their original orientation, i.e., were right-side-out (Table I). Vesicles inverted or permeable to the antibodies represented 9 and 5% of the population, respectively.

The phospholipid and cholesterol contents of rabbit kidney brush-border membranes are given in Table II. The amount of free cholesterol present in the brush border was significantly lower than

TABLE I

ORIENTATION OF ISOLATED BRUSH-BORDER MEMBRANE VESICLES

Brush-border membrane vesicles, intact or permeabilized by digitonin, were incubated for 1 h at 37 °C with an excess of the antibodies. After two washes, pellets were suspended in phosphate-buffered saline containing an excess of anti-mouse IgG conjugated to FITC. Fluorescence intensity was determined after solubilization of the pellet by sodium lauryl sulfate. The percentage of right-side-out, inside-out, and unsealed vesicles was calculated as described in Experimental procedures.

Experiment	Right-side-out (%)	Inside-out (%)	Unsealed (%)
1	95	4	1
2	87	11	3
3	84	8	8
4	81	11	8
5	84	10	6
Mean ± S.E.	86 ± 2	9 ± 1	5 ± 1

TABLE II

LIPID COMPOSITION OF RABBIT KIDNEY BRUSH-BORDER MEMBRANES

Total lipid phosphorus, cholesterol and cholesterol esters were determined from total lipid extracts (four different membrane preparations). The percentage of the different lipid classes was obtained from phosphorus determinations after separation by thin-layer chromatography ($n = 20$). Data presented are means ± S.E. Sph, sphingomyelin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI + PS, phosphatidylinositol plus phosphatidylserine.

Lipids	μmol/mg protein	mol% of the total
Cholesterol	0.37 ± 0.03	–
Cholesterol esters	0.02 ± 0.01	–
Lipid phosphorus	0.61 ± 0.02	–
Sph	0.23 ± 0.01	37.9 ± 1.3
PE	0.15 ± 0.01	24.8 ± 0.8
PC	0.12 ± 0.01	19.0 ± 0.7
PI + PS	0.11 ± 0.01	18.1 ± 0.7
Lyso PC	0.01 ± 0.003	1.1 ± 0.2

Cholesterol/phospholipid (mol · mol^{-1}) = 0.61 ± 0.06

that of total phospholipids (0.37 vs. 0.61 μmol/mg protein, respectively). Accordingly the ratio of free cholesterol to total phospholipids (C/P), was largely below unity (0.61) in these membranes. Examination of the distribution of major phospholipid classes revealed that sphingomyelin accounted for 38% of total phospholipids, followed by PE (25%), PC (19%) and PI + PS (18%). Lysophosphatidylcholine was present only in very small quantities. This lipid composition was very close to those reported from dog [14] and rat [7,29] kidney brush-border membranes.

Hydrolysis by phospholipases

Control incubations in absence of exogenous phospholipases revealed that, after 1 h at 37 °C,

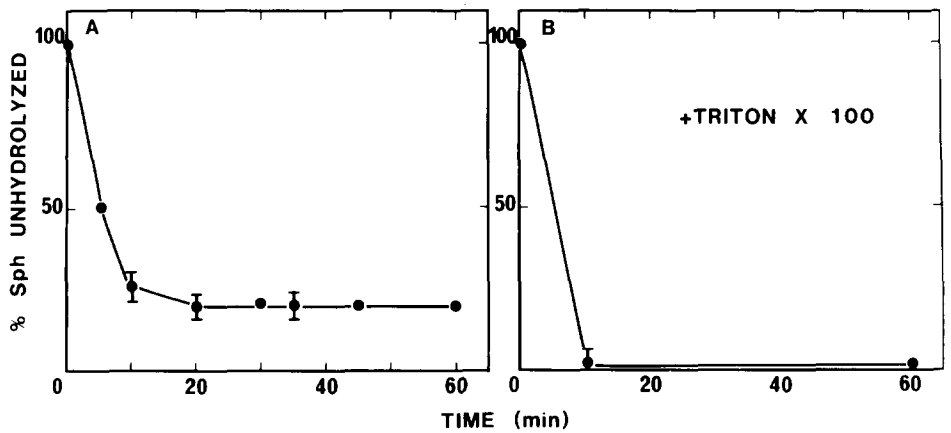

Fig. 1. Effect of sphingomyelinase on sphingomyelin content of kidney brush-border vesicles. Brush-border membrane vesicles were suspended (1 mg of protein/ml) in 20 mM Hepes/150 mM NaCl (pH 7.4). The reaction was started by adding sphingomyelinase (1 U/ml) and $MgCl_2$ (final concentration 0.25 mM). Incubation was carried out at 37 °C, hydrolysis was stopped by addition of an excess of EDTA and immediate extraction of lipids. Data obtained in the absence (A) or in the presence of 0.1% Triton X-100 (B) are illustrated. Values are mean ± S.E. of four membrane preparations.

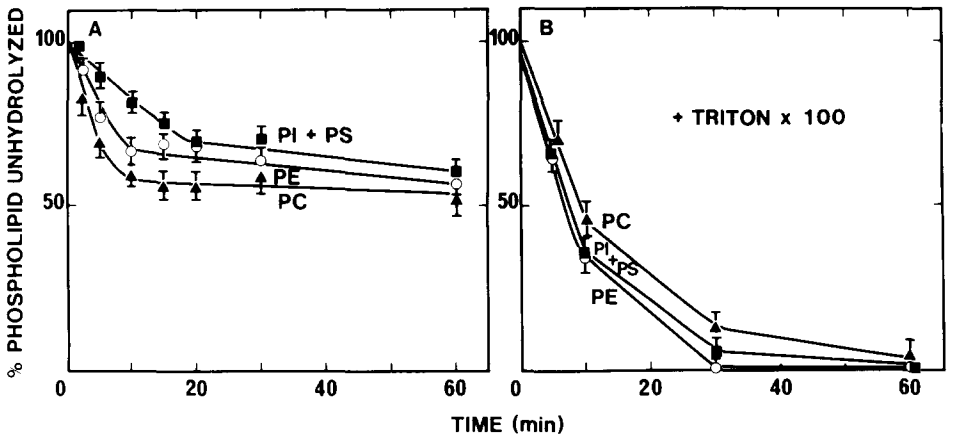

Fig. 2. Kinetics of phospholipid (PE, PE, PI + PS) hydrolysis in the presence of phospholipase A_2 from hog pancreas. Brush-border membrane vesicles were suspended (1 mg of protein/ml) in 20 mM Hepes/150 mM NaCl (pH 7.4) containing 5% of bovine serum albumin. Reaction was started by the addition of 0.25 U/mg of protein of phospholipase A_2 and of 1 mM $CaCl_2$. Incubation was carried out at 37 °C. Each point is the mean ± S.E. of three measurements on different membrane preparations. (A) Native membranes. (B) Membranes treated by 0.1% Triton X-100. ▲, PC; ○, PE; ■, PI + PS.

recovery of the various phospholipid classes present in rabbit kidney brush-border membranes was close to 100%: 99 ± 3, 100 ± 1, 98 ± 3 and 98 ± 2% for Sph, PE, PC and PI + PS, respectively. These data demonstrate the absence of significant endogenous phospholipase activities under the experimental conditions adopted.

Sphingomyelinase treatment

Incubation of brush-border membrane vesicles with sphingomyelinase (1 U/mg protein) at 37 °C resulted in the hydrolysis of 80% of the sphingomyelin within 20 min (Fig. 1A). No further reduction in sphingomyelin content occurred when increasing the incubation time or when ad-

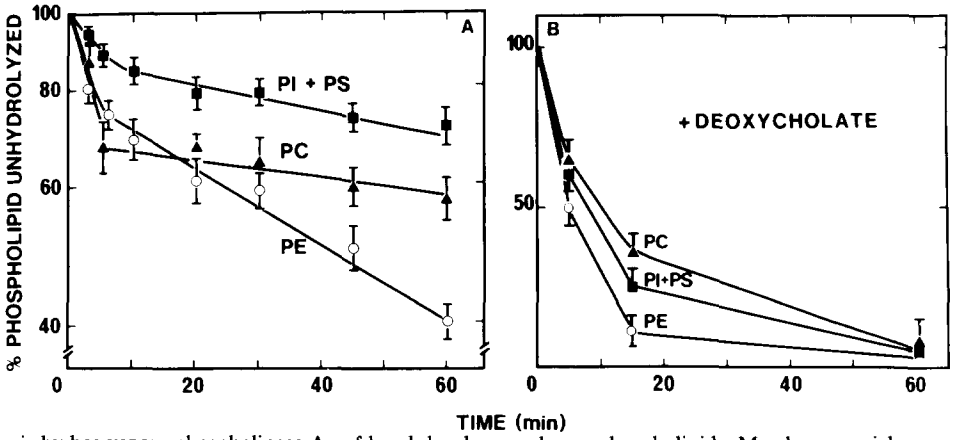

Fig. 3. Hydrolysis by bee venom phospholipase A_2 of brush-border membrane phospholipids. Membrane vesicles were suspended as described for Fig. 2 except that serum albumin was omitted from the incubation medium. Phospholipase A_2 and calcium were added at 0.5 U/mg of protein and 10 mM, respectively. To illustrate the biphasic character of the hydrolysis, data on native membranes are presented using a semi-logarithmic plot (A). In (B), membranes were preincubated for 10 min at 0 °C, with the enzyme and 0.4% (w/v) deoxycholate. Phospholipase was then activated by addition of $CaCl_2$, and by raising the temperature to 37 °C. Each point is the mean ± S.E. of three experiments on different membrane preparations. Symbols are as defined in Fig. 2.

ding at $t = 20$ min an extra amount (1 U/mg of protein) of enzyme. A small but significant degradation of other phospholipid classes was seen which accounted for 12% of PE, 13% of PC and 10% of PI + PS for 1 h incubations, an observation which can be explained by the presence of small amounts of contaminating phospholipases as reported in previous studies [15,30]. Fixation of mab 23B11, the monoclonal antibody recognizing the cytoplasmic epitope of the neutral endopeptidase, was not enhanced by 1 h sphingomyelinase treatment: in this series of experiments, fluorescence intensities measured in sphingomyelinase-treated vesicles represented 2–4% of the maximal values obtained after permeabilization of the brush border by digitonin. Hydrolysis of sphingomyelin was thus not associated with an opening of vesicles. As shown in Fig. 1B, addition of Triton X-100 to brush-border membranes resulted in a rapid and total hydrolysis of sphingomyelin. Addition of the detergent also increased the degradation of the other phospholipid classes (not shown).

Phospholipases A_2 treatment

Time-dependence of the hydrolysis of brush-border membrane phospholipids by hog pancreas or bee venom phospholipases A_2 is shown in Fig. 2 and Fig. 3. The use of a semi-logarithmic plot (Fig. 3A) confirmed the biphasic character of the hydrolysis process, indicating that PE, PC and PI + PS were distributed between two pools differing in their accessibility to the enzymes. Extrapolation to zero time of the straight-line portion of curves gave a rough estimate of the portions of the phospholipids which were available for a rapid hydrolysis. Using the hog enzyme, sizes of the readily accessible pools were $38 \pm 3\%$, $25 \pm 4\%$, and $18 \pm 3\%$ for PC, PE and PI + PS, respectively. Corresponding values obtained with the bee venom enzyme were $30 \pm 2\%$, $22 \pm 2\%$ and $13 \pm 4\%$. Sphingomyelin content was unaffected by these phospholipases. The percentage of sealed right-side-out vesicles detected by specific fixation of mab 23B11 to brush-border membranes following incubation with phospholipase A_2 was $82 \pm 3\%$, a value not significantly different from controls. Addition of Triton X-100 to the vesicles resulted in a rapid and complete hydrolysis of the glycerophospholipids by the hog pancreas enzyme (Fig. 2B). Bee venom phospholipase A_2 in the presence of deoxycholate (0.4%) hydrolyzed 95–100% of PC, PE and PI + PS (Fig. 3B).

The kinetic analysis of the hydrolysis of phospholipids by phospholipases A_2 is based on a model of two pools between which exchange of phospholipids takes place [12,31]. Using this model, in which the biphasic curve is fitted by the sum of two exponentials, sizes of the readily accessible (a), and less accessible (b) pools, the rate constant of hydrolysis, k_0, and the rate constant of phospholipid migration from the less accessible to the readily accessible pool, k_{ab}, can be calculated. The results of such calculations for PE, PC, and PI + PS, obtained through the use of hog

TABLE III

KINETIC PARAMETERS FOR THE HYDROLYSIS OF PHOSPHOLIPIDS BY PHOSPHOLIPASES A_2

The biphasic curves of phospholipid hydrolysis by phospholipases A_2 presented in Figs. 2 and 3 were fitted by the sum of two exponentials, $k_1 e^{-g_1 t} + k_2 e^{-g_2 t}$ [31]. In this table, a and b correspond to the pool size of the readily and less accessible pools respectively. k_0 is the rate constant of phospholipid hydrolysis, while k_{ab} represents the rate constant for lipid movement from inner to outer leaflet. Phospholipase A_2 sources are hog pancreas and bee venom, respectively.

Phospholipid hydrolyzed	Phospholipase A_2 source	Pool size (%)		Rate constant (h^{-1})		Half-time (min)
		a	b	k_0	k_{ab}	$t_{1/2}$
PE	hog	26 ± 3	74 ± 3	13 ± 5	0.33 ± 0.05	38 ± 5
	bee	21 ± 1	79 ± 1	44 ± 9	0.66 ± 0.03	15 ± 3
PI + PS	hog	21 ± 4	79 ± 4	6 ± 2	0.28 ± 0.04	40 ± 5
	bee	12 ± 5	88 ± 5	18 ± 2	0.24 ± 0.01	25 ± 2
PC	hog	43 ± 2	57 ± 2	15 ± 1	0.11 ± 0.04	116 ± 16
	bee	30 ± 1	70 ± 1	12 ± 2	0.22 ± 0.03	61 ± 2

pancreas and bee venom phospholipases are summarized in Table III. For each phospholipid class, k_0 exceeded k_{ab} by more than an order of magnitude, thus confirming that the limiting step was not the hydrolysis but the passage from the less to the readily accessible pool. Half-times for the migration of phospholipids were, independently of the phospholipase used, significantly higher for PC than for PE and PI + PS. On the other hand, half-times determined from bee venom experiments were reduced 2–3-fold when compared to hog pancreas data.

Hydrolysis by phospholipases C and D

Hydrolysis of the phospholipids present in kidney brush-border membranes by phospholipase C resulted in the loss of the integrity of the vesicles: the size of the readily accessible pool of the different phospholipid classes was a function of the phospholipase added for amounts varying between 4 and 0.02 units per ml, the concentration of calcium being kept constant at 0.1 mM. For example, 95% of PC, 80% of PE, 60% of PI + PS and 40% of sphingomyelin were hydrolysed following 20 min incubation with 3 U/ml phospholipase C. Increasing the incubation to 90 min resulted in the hydrolysis of 98% PC, 90% PE, 85% PI + PS and 45% sphingomyelin. Using 0.02 U/ml the extent of hydrolysis for $t = 20$ min was still of 75% for PC, 55% for PE, 40% for PI + PS and 25% for sphingomyelin.

On the other hand, even under optimal conditions (pH 5.6, 40 mM $CaCl_2$, 2.5 mg enzyme/ml), hydrolysis of phospholipids of kidney brush-border membranes by phospholipase D was incomplete in the presence of 0.1% Triton X-100. Accordingly, this phospholipase was not used further in topology experiments.

Treatment of vesicles with TNBS

In order to avoid modification of TNBS and labelling of the inner membrane leaflet, the reaction was carried out at 4°C, in the dark. As shown by Fig. 4, the extent of trinitrophenylation was significantly higher for PE than for PS and, for both phospholipids, increased as a function of time. Extrapolation of the curves to zero time indicated that approx. 9% and 24% of PS and PE, respectively, constituted a pool readily accessible

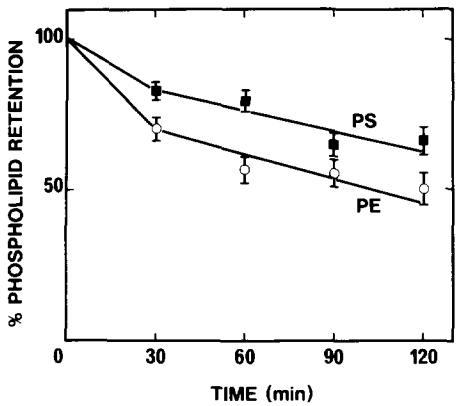

FIg. 4. Trinitrophenylation of amino-phospholipids of kidney brush-border membranes. Membrane vesicles were suspended (1 mg of protein/ml) in 20 mM Hepes/150 mM NaCl (pH 8.0). Incubation with TNBS (2 mM), was carried out at 4° C in the dark. The reaction was stopped by addition of 1 M HCl (final pH, 2.5), and unreacted TNBS was eliminated by centrifugation and washing. Values are mean ± S.E. of three experiments on different preparations. ■, PS; ○, PE.

to TNBS. Labelled PS and PE accounted for 33% and 50% of their corresponding species after 2 h incubations. When the TNBS treatment was done in the presence of Triton X-100, 90% of total PE and PS was derivatized within 1 h.

Discussion

The present experiments demonstrate that, in brush-border membrane vesicles isolated from rabbit kidney cortex, there is a pool of phospholipids readily accessible to sphingomyelinase and phospholipases A_2. As a first approximation, this pool accounts for 80% of total sphingomyelin, 30–38% of PC, 22–25% of PE and 13–18% of PI + PS.

Previous studies have stressed the importance of both the vesicle integrity and the relative rates of hydrolysis vs. transbilayer phospholipid movements (review in Ref. 11) when using phospholipases as a tool for the determination of phospholipids topology in biological membranes. The absence of any significant increase in the specific binding of the monoclonal antibody recognizing a cytoplasmic domain of the endopeptidase 24.11 during our experiments indicated that brush-border membrane vesicles had maintained their barrier properties towards these proteins. Ex-

amination of the migration rates of the different phospholipid classes from the inner to the outer membrane leaflet also revealed that hydrolysis proceeded at a rate more than one order of magnitude that of the transbilayer movement (Table III). In accordance with the data obtained on rat kidney by Haase et al. [32], using a different technique, brush-border membrane vesicles were predominantly (86%) oriented right-side-out, inside-out and unsealed vesicles representing 9% and 5% of the population, respectively.

Taking into account this orientation factor, it can be estimated (see Experimental procedures) that approx. 86% of the sphingomyelin, and 30% of the PC species were accessible from the external membrane leaflet. In terms of absolute amounts, sphingomyelin by itself accounted for 75% of the readily accessible pool of phospholipids of the external leaflet.

A preferential localization of sphingomyelin on the outer leaflet of plasma membranes has also been demonstrated for erythrocytes, blood platelets, LM cells (a transformed murine fibroblast cell line) [11], and recently, for brush-border isolated from rat kidney [15]. The highly asymmetric distribution of this phospholipid is of particular importance in kidney brush-border membranes because it represents 40% of their total phospholipids and because it undergoes broad phase transitions extending from 18–22 °C to 43–45 °C [14]. Thus, taking into account the high lipid order characteristic of sphingomyelin species [14,33], it is likely that the lipid order of the outer leaflet of brush-border membrane significantly exceeds that of the inner leaflet and that temperature will affect markedly its fluidity properties. It is noticeable that, between species as different as rat, rabbit and dog, very little variation exists in the phospholipid composition of the kidney brush-border membranes [7,14,29]. The results obtained for the localization of PC by the use of phospholipases A_2 are in close agreement with those reported for dog kidney brush-border membrane. Thus, the size of the rapidly exchangeable PC pool, determined by using a specific PC exchange protein, was found to represent 39% of total PC [14]. This would lead, assuming an orientation of vesicles similar to that determined in the present experiments, to a value of 35% for dog kidney. Therefore, like for erythrocyte membranes [11,12], choline-containing phospholipids are found to comprise the majority (approx. 85%) of the outer layer of the membrane. On the other hand, only 14% of the PE and 5% of the PI + PS (PI accounting for 10–20% of the total [7,14]), i.e., values comparable to those found in red blood cells [10,11], were accessible to phospholipases from the external membrane leaflet.

This low accessibility of PE and of the negatively charged phospholipid PS was confirmed using TNBS, a primary amino-group reagent: extrapolation to zero time of the trinitrophenylation curves gave values of 24% and 9% for the readily accessible pools of PE and PS, respectively, which, once corrected for the orientation of the vesicles, were in agreement with those issued from phospholipase A_2 experiments. Absolute amounts of sphingomyelin, PE, PC and PI + PS in the readily accessible pool were 0.21, 0.02, 0.04 and 0.01 μmol lipid phosphorus per mg of protein and thus account for only 45% of the total brush-border membrane phospholipids. The presence of glycolipids which, although much less abundant than in intestinal brush borders, may represent up to 15% of polar lipids [34] and are almost exclusively found on the outer leaflet of plasma membranes [35,36], can contribute to this observation. It is also possible that a small percentage of the phospholipids from the external leaflet interact strongly with proteins and are shielded from the action of phospholipases. Consequently, values calculated for the inaccessible pool, i.e., 0.02, 0.13, 0.08 and 0.10 μmol lipid phosphorus per mg of protein for sphingomyelin, PE, PC and PI + PS respectively, might also be overestimated by a few percent. In the absence of data on inverted vesicles, assignment of the less accessible phospholipid pool to the inner membrane leaflet is based essentially on the evidence derived from indirect results, i.e., the non-reactivity of some phospholipids, in combination with the assumption that polar lipids are distributed in a 1 : 1 ratio over inner and outer leaflets [10,11,13]. However, the observation that phospholipase C, by disrupting the vesicle integrity, allowed a quasi-complete hydrolysis of PC, PE and PI + PS strongly supported the identification of the less accessible pool as the inner leaflet. This membrane-disruptive effect observed with the

phospholipase C, whose products are in most case not lytic, resembles that described when erythrocytes are first treated by sphingomyelinase [37]. Both the presence of sphingomyelinase, as contaminant, and the large amount of sphingomyelin in the external leaflet might have contributed to our observation. It is noticeable that the preferential localization of PE and PI + PS on the cytoplasmic side of the brush-border membrane is in agreement with the distribution of these phospholipids in erythrocytes, blood platelets and in the plasma membrane of LM cells [11].

Comparison of the present localization data on renal brush borders with those recently reported concerning the topology of PE and PC in intestinal brush-border membranes [13], also reveals a similar distribution of these two phospholipids, in spite of profound differences in their lipid composition. The very high content in glycolipids characteristic of intestinal brush-border membranes is likely to play the same role in the asymmetry that the sphingomyelin in renal membranes.

It has been reported that for intestinal brush borders 75–85% of the vesicles are unsealed and permeable to molecules of molecular weight lower than 700 [38], including charged amino-group reagents. The correspondence between the results of localizations of PE and PI + PS by phospholipase A_2 attack and by TNBS treatment demonstrates that the resealing of brush-border vesicles from the kidney must be, except for the few percent detected by monoclonal antibodies, practically complete. Too long to be determined for sphingomyelin, half-times for the transbilayer migration, from the inner to the outer leaflet, calculated from phospholipase A_2 experiment, were significantly shorter for PE and PI + PS than for PC. Indeed, these data have to be handled very carefully. Thus, for PC, the half-time obtained in the present experiments is at least 3-fold shorter than that calculated from experiments, on dog kidney vesicles, using a specific PC exchange protein [14]. This difference probably results from the phospholipid depletion at one side of the membrane [11] and was enhanced when using the bee venom enzyme. The faster transmigration rates obtained with this enzyme likely resulted from differences in incubation media. The reasons for the difference in half-time of transbilayer migration recorded between aminophospholipids and PC are unknown, but might involve the presence of a specific protein translocator of the aminophospholipids like those identified in erythrocytes and platelets [39,40]. However, due to the very high sphingomyelin content of the external leaflet and because the transbilayer movements of this lipid are extremely slow, the maintainance of the lipid asymmetry in brush borders can hardly be accounted for by the activity of such a protein.

In conclusion, our data demonstrate that the distribution of phospholipids in kidney brush-border membranes is highly asymmetrical. Sphingomyelin accounts for 75% of the phospholipids present on the external leaflet, whereas aminophospholipids are the main constituents of the cytoplasmic side. The origin and the physiological role of this asymmetry remain to be elucidated.

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References

1 Heidrich, H.G., Kinne, R., Kinne-Saffran, E. and Hannig, K. (1972) J. Cell. Biol. 54, 232–245.
2 Fujita, M., Kawai, K., Asano, K. and Nakao, M. (1973) Biochim. Biophys. Acta 307, 141–151.
3 Sacktor, B. (1977) in Current Topics of Bioenergetics (Sanadi, R., ed.), pp. 39–81, Academic Press, New York.
4 Brasitus, T.A. and Schachter, D. (1980) Biochemistry 19, 2763–2769.
5 Le Grimellec, C., Giocondi, M.C., Carriere, B., Carriere, S. and Cardinal, J. (1982) Am. J. Physiol. 245, F227–F231.
6 Hise, M.K., Mantulin, W.W. and Weinman, E.J. (1984) Am. J. Physiol. 247, F434–439.
7 Molitoris, B.A. and Simon, F.R. (1985) J. Membr. Biol. 33, 207–215.
8 Dragsten, P.R., Blumenthal, R. and Handler, J.S. (1981) Nature 294, 718–722.
9 Van Meer, G. and Simons, K. (1986) EMBO J. 5, 1455–1464.
10 Renooij, W., Van Golde, L.M.G., Zwaal, R.F. and Van Deenen, L.L.M. (1976) Eur. J. Biochem. 61, 53–58.
11 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71.

12 Van Deenen, L.L.M. (1981) FEBS Lett. 123, 3–15.
13 Barsukov, L.I., Bergelson, L.D., Spiess, M., Hauser, H. and Semenza, G. (1986) Biochim. Biophys. Acta 862, 87–99.
14 Carmel, G., Rodrigue, F., Carriere, S. and Le Grimellec, C. (1985) Biochim. Biophys. Acta 857, 131–138.
15 Hise, M.K., Mantulin, W.W. and Weinman, E.J. (1986) J. Clin. Invest. 77, 768–773.
16 Forstner, G.G., Tanaka, K. and Isselbacher, K.J. (1968) Biochem. J. 109, 51–59.
17 Christiansen, K. and Carlsen, J. (1981) Biochim. Biophys. Acta 647, 188–195.
18 Boegheim, J.P.J., Jr., Van Linde, M., Op den Kamp, J.A.F. and Roelofsen, B. (1983) Biochim. Biophys. Acta 735, 438–44.
19 Zachowski, A., Fellman, P. and Devaux, P.F. (1985) Biochim. Biophys. Acta 815, 510–514.
20 Booth, A.G. and Kenny, A.J. (1974) Biochem. J. 142, 575–581.
21 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
22 Mrsny, R.J., Volwerk, J.J. and Griffith, O.H. (1986) Chem. Phys. Lipids 39, 185–191.
23 Omodeo Sale, F., Marchesini, S., Fishman, P.H. and Berra, B. (1984) Anal. Biochem. 142, 347–350.
24 Grunberger, D., Haimovitz, R. and Shinitzky, M. (1982) Biochim. Biophys. Acta 688, 764–774
25 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
26 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
27 Crine, P., Le Grimellec, C., Lemieux, E., Labonte, L., Fortin, S., Blachier, A. and Aubry, M. (1985) Biochem. Biophys. Res. Commun. 131, 255–261.
28 Kenny, J. (1986) Trends Biochem. Sci. 11, 40–42.
29 Schwertz, D.W., Kreisberg, J.I. and Venkatachalam, M.A. (1983) Arch. Biochem. Biophys. 224, 555–567.
30 Ikezawa, H., Mori, M., Ohyabu, T. and Taguchi, R. (1978) Biochim. Biophys. Acta 528, 247–256.
31 Bloj, B. and Zilversmit, D.B. (1976) Biochemistry 15, 1277–1283.
32 Haase, W., Schafer, A., Murer, H. and Kinne, R. (1978) Biochem. J. 172, 57–62.
33 Shinitzky, M. and Inbar, M. (1976) Biochim. Biophys. Acta 433, 133–49.
34 Zambrano, F., Fleischer, S. and Fleischer, B. (1975) Biochim. Biophys. Acta 380, 357–369.
35 Steck, T. and Dawson, G. (1974) J. Biol. Chem. 249, 2135–2142.
36 Leden, R.W. (1978) J. Supramol. Struct. 8, 1–17.
37 Zwaal, R.A., Roelofsen, B. and Colley, C.M. (1973) Biochim. Biophys. Acta 300, 159–182.
38 Gains, N. and Hauser, H. (1984) Biochim. Biophys. Acta 772, 161–166.
39 Zachowski, A., Favre, E., Cribier, S., Herve, P. and Devaux, P.F. (1986) Biochemistry 25, 2585–2590.
40 Sune, A., Bette-Bobillo, P., Bienvenue, A., Fellmann, P. and Devaux, P.F. (1987) Biochemistry 26, 2972–2978.