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The involvement of cytoskeletal proteins in the maintenance of phospholipid topology in renal brush-border membranes

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When incubated for 14 h at 37°C in the absence of energy supply, brush-border membrane vesicles from rabbit kidney cortex maintain, as judged by the use of sphingomyelinase and trinitrobenzene sulfonate as membrane probes, their highly asymmetrical phospholipid distribution. In particular, sphingomyelin still accounts for 75% of the phospholipids present on the outer membrane leaflet. Pretreatment of the vesicles with 5 mM diamide resulted in extensive crosslinking of membranous and cytoskeletal proteins. Although it had no immediate effect on the topology of phospholipids, this crosslinking resulted in a limited but significant increase in the amount of aminophospholipids present on the outer membrane leaflet after 14-h incubations. Degradation of aminophospholipids, upon incubation with hog pancreas and bee venom phospholipases A_2, was also enhanced by diamide. However, this enhanced hydrolysis was observed immediately after the diamide treatment. A similar increase in degradation of aminophospholipids was obtained when vesicles were incubated with dihydrocytochalasin B. Our results strongly suggest that cytoskeletal proteins, via interactions with aminophospholipids, stabilize the lipid bilayer of the brush-border membrane. It is also suggested that, due to a low transbilayer migration rate, sphingomyelin may play an important role in the maintenance of the lipid asymmetry in these membranes.

Introduction

The topological distribution of phospholipids of kidney brush-border membranes is highly asymmetric: amino-containing species, phosphatidylethanolamine (PE) and phosphatidylserine (PS) are primarily localized in the inner membrane leaflet. On the other hand, sphingomyelin accounts for more than 75% of the phospholipids present in the outer leaflet [1].

The cytoskeletal apparatus associated with the brush-border surface of epithelial cells is among the most highly ordered arrays of actin and associated proteins found in a non-muscle cell [2–4]. Each microvillus contains a central core of actin filaments, held into a tight bundle by two cross-linking proteins, fimbrin and villin, which is attached laterally to the surrounding membrane by membrane microfilament linkages. These interrelations between cytoskeleton and membrane are maintained during the purification of brush-border membrane vesicles [5].

Evidence is accumulating that the cytoskeleton, by interacting with the inner leaflet of the membrane, may stabilize the asymmetric distribution

Abbreviations: DTT, dithiothreitol; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PE, phosphatidylethanolamine; PS, phosphatidylserine; TNBS, 2,4,6-trinitrobenzene sulfonate.

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of phospholipids. Thus, in red blood cell and platelet membranes [6–9], both interaction with membrane skeletal proteins and an ATP-dependent translocation process are involved in the maintenance of the asymmetric distribution of aminophospholipids.

In kidney cells, however, membrane constituents are continuously renewed and the processes involved in the maintenance of a phospholipid asymmetry in the plasma membrane may differ.

In this paper, we have investigated the relative importance of ATP-dependent and -independent processes, including membrane cytoskeleton interactions, in the maintenance of the phospholipid asymmetry of kidney brush-border membranes. To assess the influence of ATP on the process, vesicles were incubated for 14 h at 37°C, in the absence of the nucleotide. Diamide, a sulfhydryl oxidizing agent, was used to disturb the membrane skeletal network [6]. Changes in phospholipid organization were investigated by determination of their accessibility to exogenous phospholipases and to 2,4,6-trinitrobenzene sulfonate (TNBS) [1]. The results show that brush-border vesicles maintain their phospholipid asymmetry even after 14-h incubations. They support the view that membrane cytoskeleton interactions have a stabilizing effect on the lipid bilayer and also suggest that, due to a low transbilayer migration rate, sphingomyelin plays an important role in the maintenance of the transbilayer asymmetry.

Materials and Methods

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 [10] using an $MgCl_2$ method developed by Booth and Kenny [11]. Membranes were suspended (5 mg of protein/ml) in 150 mM NaCl/20 mM Hepes (pH 7.4) (Buffer A), and used in topology experiments on the day of their preparation. In brush-border vesicle preparations, activities of alkaline phosphatase and γ-glutamyl transpeptidase were enriched 9–12-fold over that 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 and tightness

Orientation of vesicles was determined as described [1] using a monoclonal antibody 23B11, a gift of Dr. P. Crine (Department of Biochemistry, University of Montreal, Canada), recognizing a cytoplasmic domain of the neutral endopeptidase 24.11, a protein present in large amount in kidney brush borders [12]. Briefly, vesicles (100 μg of protein), either intact or permeabilized by digitonin, were incubated for 1 h at 37°C with an excess of the antibody. After two washes, pellets were resuspended in phosphate-buffered saline (PBS) (pH 7.4) containing anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) in excess. Unbound antibody was removed by three washes in PBS.

Fluorescence was measured after complete solubilization by sodium dodecyl sulfate (20 mM) and ten-fold dilution by water. Vesicles without 23B11 and second antibody treatment and samples from which monoclonal antibody was omitted were run in parallel to serve as a control for non-specific 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.

Lipid analysis

Lipids were extracted using the Bligh and Dyer method [13]. Extraction was repeated three times to obtain a 100% recovery [14]. Extracts were pooled, evaporated to dryness under nitrogen, and solubilized in a 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 vapors and identified by comparison with authentic standards. The spots were scraped off and transferred into acid-washed test tubes. Known amounts of defined phospholipids were treated in the same way to be used as internal standards. The phosphorus content of phospholi-

pid classes was determined according to Mrsny et al. [15].

Phospholipase treatment

Phospholipases A_2 from bee venom and from hog pancreas and sphingomyelinase from *Bacillus cereus* were purchased from Boehringer Mannheim (France).

Brush-border membrane vesicles were dispersed in Buffer A at 1 mg of protein/ml. Incubations were carried out at 37°C with gentle shaking. Phospholipases were used as follows: 1 U/mg of protein of sphingomyelinase in the presence of 0.25 mM $MgCl_2$; 0.5 U/mg of protein of bee venom phospholipase A_2 plus 10 mM $CaCl_2$ and 5% bovine serum albumin; 0.25 U/mg of protein of hog pancreas phospholipase A_2 plus 1 mM $CaCl_2$ and 5% bovine serum albumin. 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 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.

Labeling by TNBS

Color-free TNBS was purchased from Eastman Kodak and neutralized as described by Grunberger et al. [16]. Chemical labeling 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.

Diamide treatment

Diamide was purchased from Sigma. Brush-border membrane vesicles were dispersed for various times in 20 mM Hepes/150 mM NaCl (pH 8) containing 5 mM diamide. Brush-border membranes were washed twice and then incubated with phospholipases or TNBS. 14-h incubations at 37°C, following diamide treatment and washing, were done in penicillin (100 U/ml) and streptomycin (100 μg/ml) containing Buffer A.

Dihydrocytochalasin B treatment

Dihydrocytochalasin B (Janssen Chemica, Pantin, France) dissolved in dimethyl sulfoxide (DMSO) was added (10 μg/ml final concentration) to brush-border membranes vesicles (1 mg of protein/ml Buffer A). The concentration of DMSO was adjusted to 0.005% in all incubations, but had no significant effect on phospholipid distribution.

Polyacrylamide gel electrophoresis

All products were obtained from Bio-Rad Laboratories. Sodium dodecyl sulfate (SDS) electrophoresis was done according to the method of Laemmli [17] on 10% gels. Samples for electrophoresis were solubilized in 2% SDS at a concentration of 2 mg of protein/ml and heated for 5 min at 100°C. Following addition of 25% (v/v) glycerol; 0.001% (w/v) bromophenol blue and 0.125 M Tris (pH 7.5), final concentrations, 15–20 μg of proteins were applied to each lane. Carbonic anhydrase (31 000), actin (43 000), ovalbumin (45 000), bovine serum albumin (66 200) and phosphorylase *B* (92 500) were used as molecular weight standards. Gels were stained in 0.5% (w/v) Coomassie brilliant blue R, 10% (v/v) acetic acid/50% (v/v) methanol and washed in 10% (v/v) acetic acid/45% (v/v) methanol. The relative intensity of protein bands was determined by densitometry, with a C5930 Shimadzu apparatus (Japan).

Enzyme and protein determination

Activities of the marker enzymes were determined as previously described [10,14]. Protein determinations were made by the method of Lowry et al. [18] after precipitation with 10% trichloroacetic acid. Bovine serum albumin was used as a standard.

Results

Maintenance of asymmetry after long-duration incubations

Because sphingomyelin accounts for more than 75% of the phospholipids present in the outer

TABLE I

HYDROLYSIS OF SPHINGOMYELIN IN BRUSH-BORDER VESICLES

Control or diamide-treated vesicles were incubated with sphingomyelinase immediately or 14 h after the pretreatment (see Materials and Methods). Values are mean ± S.E. of three different experiments.

Treatment with diamide (min)	Sphingomyelin hydrolysed (%)	
	fresh preparation	after 14-h incubation
0	80±2	79±3
15	75±2	74±3
30	74±3	73±4
60	74±3	75±2

leaflet of kidney brush-border membranes [1], the effect of long incubations, at 37 °C, in the absence of energy supply on its distribution was investigated. As shown by Table I, the amount of sphingomyelin hydrolysed by sphingomyelinase after 14 h incubation was identical to that degraded in fresh preparations. Trinitrophenylation of aminophospholipids by TNBS was also identical in fresh and 14-h-incubated preparations (Table II). This NH_2-group-specific reagent permeates only very slowly in brush-border membranes and was used before in the estimation of the PE and PS present on the outer membrane leaflet [1,19]. The use of fluorescamine as an NH_2-group probe was excluded by preliminary experiments that indicated a 70–80% labeling of aminophospholipids in intact vesicles, i.e., a rapid migration of the reagent across the membrane.

Effects of diamide treatment on lipid asymmetry

In both fresh and 14-h-incubated brush-border preparations, the amount of sphingomyelin degraded by sphingomyelinase was slight but not significantly decreased by pretreatment with diamide (Table I). 15–60-min diamide pretreatment also had no effect on the trinitrophenylation of PE and PS in fresh preparations (Table II). On the other hand, the relative amount of PE and PS labeled by TNBS (30 min treatment) increased from 27 to 38% and from 15 to 24%, respectively, in diamide-treated vesicles incubated for 14 h. The effect was similar for vesicles pretreated by diamide for 15 or 60 min. Fixation of the monoclonal antibody 23B11, a marker of the cytoplasmic end of the endopeptidase 24.11 [12], was not affected by either the duration of the incubation or the treatment with diamide: in fresh preparations, sealed right-side-out vesicles represented 83% of the total as compared to 82% in diamide-treated vesicles incubated for 14 h.

TABLE II

TRINITROPHENYLATION OF AMINOPHOSPHOLIPIDS IN BRUSH-BORDER MEMBRANES

Vesicles were preincubated with 5 mM diamide for 15 or 60 min at 37 °C and pH 8. After washing, vesicles were immediately (fresh vesicles) or after 14 h incubation at 37 °C (pH 7.4) incubated with TNBS (2 mM) at 4 °C, in the dark, for 5, 10 or 30 min. The extent of the reaction is expressed as the average (mol%) of aminophospholipids remaining underivatized. Values are mean ± S.E. of three determinations, each determination being itself the average value of duplicates.

TNBS treatment (min):	Fresh vesicles			14-h incubated vesicles		
	5	10	30	5	10	30
No diamide						
PE unlabeled (%)	93±2	90±2	70±3	94±2	90±2	73±2
PS unlabeled (%)	96±2	94±2	85±2	96±2	95±2	85±2
15 min diamide						
PE unlabeled (%)	92±3	89±2	67±2	74±3 **	65±2 **	62±2 *
PS unlabeled (%)	93±3	92±3	87±2	87±2 *	78±2 **	76±2 *
60 min diamide						
PE unlabeled (%)	90±3	91±3	70±3	79±4 *	80±3 *	60±2 *
PS unlabeled (%)	98±2	90±2	85±3	82±3 *	81±3 *	75±4 *

* $P < 0.05$; ** $P < 0.01$.

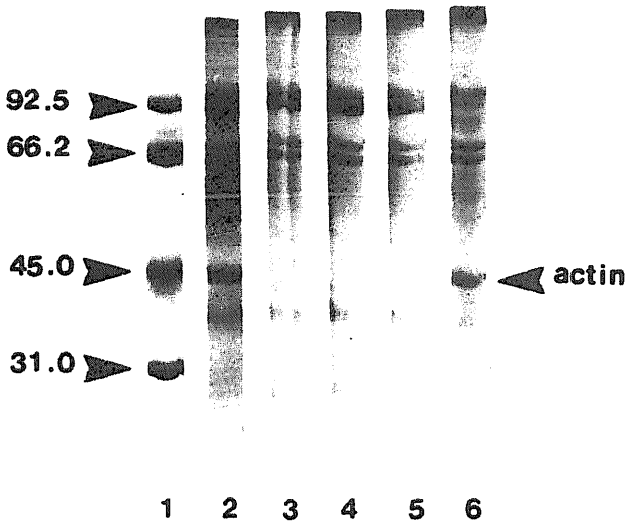

Fig. 1. Gel electrophoretic profiles of brush-border membranes treated with diamide. Membrane vesicles were incubated (pH 8, 37°C) with 5 mM diamide for 15.30 and 60 min. When used, DTT (2 mM) was added after washing of the vesicles. Lanes: 1, molecular weight standards (kDa); 2, control; 3, diamide 15 min; 4, diamide 30 min; 5, diamide 60 min; 6, diamide 60 min + DTT.

One-dimensional polyacrylamide gel electrophoresis revealed that treatment of brush-border membrane vesicles with diamide resulted in a marked decrease in the staining of several bands, whereas crosslinked proteins appeared at the top of the gels (Fig. 1). On the basis of their apparent molecular weights and by comparison with previous studies [4,21], the bands corresponding to villin (95 kDa), fimbrin (68 kDa) and actin (43 kDa) were among those most affected by the treatment. The effects of diamide were reversed by adding dithiothreitol (DTT), an SH-group reducing agent, to treated vesicles (Fig. 1, lane 6). Densitometric analysis of the gels further indicated that the crosslinking of these proteins was practically completed within the first 15 min of diamide treatment (Table III).

Hydrolysis of phospholipids in diamide-treated vesicles

Hydrolysis of brush-border membrane phospholipids by hog pancreas and bee venom phospholipases A_2 was determined in control vesicles and in vesicles treated by diamide just prior to the enzyme attack. The extent of the lipid degradation

TABLE III

DENSITOMETRIC ANALYSIS OF CYTOSKELETAL PROTEINS IN DIAMIDE-TREATED VESICLES

Brush-border membrane vesicles were preincubated with 5 mM diamide for 15, 30 or 60 min at 37°C and pH 8. The ratio of principal cytoskeletal proteins and oligomer which appeared at the top of the gel is expressed as the percentage of total intensity of the bands. Values are mean ± S.E. of six determinations.

Protein	Relative intensity (% of the total) at time (min)			
	0	15	30	60
Actin	5.67 ± 0.34	2.09 ± 0.21	2.07 ± 0.34	1.21 ± 0.34
Villin	3.32 ± 0.23	1.20 ± 0.42	0.59 ± 0.28	0.56 ± 0.32
Fimbrin	5.21 ± 1.52	2.3 ± 0.69	2.9 ± 0.65	2.52 ± 0.50
Oligomer	0	19.8 ± 3.6	25.2 ± 4.2	23.9 ± 3.3

was determined for 20-min incubations with the enzymes, a time sufficient, under our experimental conditions, to allow the complete conversion into their lysoderivatives of the PC, PE and PI + PS species localized on the outer membrane leaflet [1]. As shown by Fig. 2, for the hog pancreas enzyme, a 15-min pretreatment with diamide was associated with an increase from 21 to 29% of the total amount of hydrolysed phospholipids, a value not significantly modified by longer preincubation periods. This increase was accounted for by the

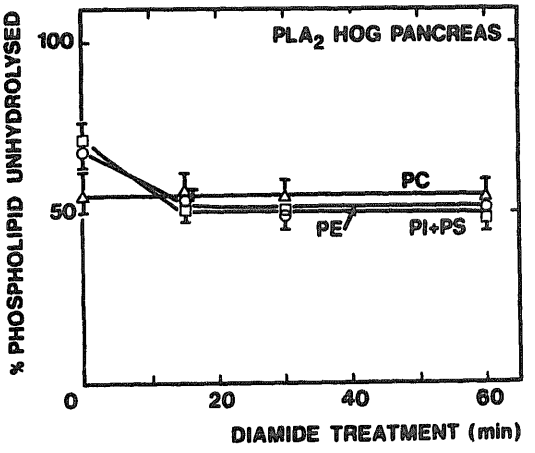

Fig. 2. Effect of diamide on lipid hydrolysis by hog pancreas phospholipase A_2 (PLA_2). Brush-border membranes were pretreated with 5 mM diamide for 15, 30 or 60 min at 37°C (pH 8). Vesicles were washed and incubated with hog pancreas phospholipase A_2 (0.25 U/mg of protein) for 20 min at 37°C (pH 7.4). Values are mean + S.E. of three determinations. Each determination is the mean of duplicates. PE (○); PS + PI (□); PC (△).

TABLE IV

EFFECTS OF DIHYDROCYTOCHALASIN B ON LIPID HYDROLYSIS BY PHOSPHOLIPASES A_2

PLA_2, phospholipase A_2; C, control vesicles; E, vesicles preincubated for 1 h with 10 μg/ml dihydrocytochalasin B. Incubations were done as described in Materials and Methods. Values are mean ± S.E. of three different experiments.

	Phospholipid species	Phospholipids hydrolysed (%) at incubation time			
		5 min		30 min	
		C	E	C	E
Hog pancreas PLA_2	PC	30±4	32±2	42±2	46±3
	PI+PS	10±2	10±4	30±2	38±2 *
	PE	13±3	14±3	35±2	50±3 *
Been venom PLA_2	PC	32±2	35±2	36±3	42±2
	PI+PS	11±3	20±3	16±5	35±3 *
	PE	26±3	40±4	41±2	51±3 *

* $P < 0.05$.

enhanced hydrolysis of PE and PI + PS, PC being unaffected. Comparable results were obtained with phospholipase A_2 from bee venom that hydrolysed 19% of total phospholipids in untreated membrane as compared to 26, 28, and 28% after 15, 30 and 60 min diamide pretreatment, respectively. For the 15-min diamide pretreatment, 40 ± 2% of total PI + PS and 44 + 2% of total PE were digested vs. 18 + 3% and 35 + 2% in the controls, respectively.

Treatment of vesicles with dihydrocytochalasin B

60-min preincubations with dihydrocytochalasin B (10 μg/ml), a ligand specific for the positive end of actin [20], resulted in a significant increase in the amount of PE and PI + PS hydrolysed by phospholipases A_2 after 30-min incubations with the enzymes (Table IV). As for the diamide pretreatment, the hydrolysis of PC was unaffected by dihydrocytochalasin B. Although still slightly depressed (74 + 2 vs. 79 + 1%), the pool of sphingomyelin accessible to sphingomyelinase was not significantly modified.

Discussion

The present experiments demonstrate for the first time that incubation of brush-border membrane vesicles from kidney cortex for 14 h at 37°C has no effect on the transbilayer distribution of the sphingomyelin and aminophospholipid species. To explain the in vitro maintenance, for such a duration, of the marked asymmetry these phospholipids exhibited in brush borders, two hypotheses from the work done on erythrocytes can be advanced: (a) asymmetry is maintained by an active process, ATP-dependent, involving the presence of an active 'flippase' similar to that described in red blood cells and platelets [8,9]; (b) the transbilayer movement of the various phospholipid species is extremely slow. Membrane cytoskeleton interaction might, at least partly, contribute to this slow rate [6,22].

The former hypothesis can be ruled out because the prolonged incubation at 37°C was done in the absence of any ATP-regenerating system, and because the intravesicular concentration of ATP in vesicles prepared by this method is very low, i.e., less than 0.1 μM (Ref. 23 and Vénien, unpublished observations).

On the other hand, pretreatment with diamide after 14-h incubations at 37°C resulted in a significant increase, from 27 to 38% for PE and from 15 to 24% for PS, in the amount of aminophospholipids labeled by TNBS ($t = 30$ min). Diamide, like in platelets and in red blood cells [6,7], crosslinked both cytoskeletal and membranous proteins of brush-border vesicles. The crosslinking was reversed by DTT and was essentially completed within 15 min treatment. The increase in aminophospholipid labeling was not observed during the first 0.5-h incubations which ruled out the possibility of large defects in the bilayer structure following diamide treatment. According to our previous experiments in control brush-border membranes, these conditions of labeling ($t = 30$ min with TNBS) overestimate by only a few percent the amount of PE and PS accessible at the membrane surface [1]. Thus, although highly significant, the changes in phospholipid topology, taking into account the absolute amounts of PE and PS in kidney brush-border membranes [1,14], affect less than 5% of total membrane phospholipids. Consequently, our data indicated that diamide-treated vesicles maintained a marked lipid asymmetry. This view was supported by the fact that sphingomyelinase still hydrolysed 75% (vs. 80% in control) of the

sphingomyelin present in these vesicles. Both this extent of sphingomyelin hydrolysis, and the absence of significant modification in the binding of the monoclonal antibody directed against the cytoplasmic domain of the endopeptidase 24.11, argued against a significant opening of the diamide-treated vesicles. Taken together, our data strongly suggest that the enhanced labeling by TNBS in diamide-treated vesicles incubated for 14 h was due to a net movement of the aminophospholipids from the inner to the outer leaflet.

In control vesicles, bee venom or hog pancreas phospholipase A_2 hydrolysed the totality of the PC, PE and PI + PS present on the external leaflet in less than 20 min [1]. Pretreatment with diamide increased the amount of PE and PI + PS degraded in 20 min by both phospholipases. This occurred immediately after the pretreatment, i.e., under conditions where the phospholipid distribution, as judged by TNBS experiments, was not altered. Similar results, i.e., an increase in the hydrolysis of aminophospholipids by phospholipase A_2 not associated with an in situ loss of phospholipid asymmetry, have been reported in diamide-treated erythrocytes [22]. The enhanced hydrolysis indicated an accelerated transfer from inner to outer monolayer in diamide-treated membranes. This movement would result from a partial destabilization of the lipid bilayer by the diamide treatment [22,24].

The relative importance of cytoskeletal vs. membrane protein crosslinking in the enhanced movement of aminophospholipids across the bilayer was assessed by comparing the effect of diamide with that of dihydrocytochalasin B, a ligand highly specific for the positive end of actin [20]. As shown by phospholipase experiments, diamide and dihydrocytochalasin B had a comparable effect on the rate of transfer of PE and PI + PS across the bilayer. Thus our data strongly suggest that, as proposed by Haest et al. for the erythrocyte membrane [6,24], phospholipid asymmetry in renal brush border is, to some degree, stabilized by an interaction between aminophospholipids and the cytoskeleton.

This stabilizing action, however, does not fully account for the observation that phospholipid membrane asymmetry remained for a large part preserved after 14-h incubations. Kidney brush-border membranes are characterized by a high sphingomyelin content, which can account for up to 40% of the total phospholipids present in the membrane [1,14,25], and a low glycolipid content [26]. The transbilayer migration rate of sphingomyelin is generally extremely low [27,28]. Because sphingomyelin accounts for 75% of the phospholipids of the external membrane leaflet [1], its flip to the inner layer might be the rate-limiting step for the loss of the transbilayer asymmetry. If one assumes the $t_{1/2}$ of PE and PS transbilayer migration to be, like in ATP-depleted erythrocytes [27], of the same order as the $t_{1/2}$ of PC, i.e., 6.5 h in kidney brush border [14], this would explain why aminophospholipids remain predominantly localized on the inner leaflet. Substituting glycolipids for sphingomyelin, a similar mechanism, would occur in intestinal brush-border membranes.

In contrast with red blood cells, constituents of the plasma membrane of epithelial cells are continuously renewed. In kidney cells, $t_{1/2}$ for degradation of membrane phospholipids is between 24 and 60 h [29]. Accordingly, it can be hypothesized that both membrane–cytoskeleton interactions and a very slow flip of sphingomyelin into the inner leaflet are major factors in the maintenance of the phospholipid asymmetry in kidney brush-border membranes.

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