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PHOSPHOLIPID METHYLATION OF KIDNEY CORTEX BRUSH BORDER MEMBRANES

EFFECT ON FLUIDITY AND TRANSPORT

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Exposure of intact brush border membrane vesicles of hog kidney cortex to cholesterol oxidase resulted in 24% oxidation of membrane cholesterol compared with more than 95% oxidation of cholesterol in lipids isolated from membranes, showing that cholesterol is asymmetrically distributed in membranes. Phospholipase C, hydrolyzed 76% of phosphatidylcholine and 10–12% phosphatidylethanolamine while phosphatidylserine was not hydrolyzed, thus indicating that majority of phosphatidylcholine is present on the outer surface of these vesicles while phosphatidylethanolamine and phosphatidylserine are present on the inner surface. Methylation of phospholipids in brush border membrane with *S*-adenosyl-[*methyl*-^{3}H]methionine resulted in the formation of phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine from endogenous phosphatidylethanolamine. The K_m for *S*-adenosylmethionine was $1 \cdot 10^{-4}$ M with an optimum pH 9.0 for the formation of all three methyl derivatives. Mg^{2+} was without any effect between pH 5 to 10. Addition of exogenous mono- and dimethylphosphatidylethanolamine derivatives enhanced methyl group incorporation by 4–5-fold as compared to the addition of phosphatidylethanolamine. The conversion of endogenous phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine or addition of exogenous phosphatidylmonomethylethanolamine to brush border membrane did not result in a change in bulk membrane fluidity as determined by fluorescence polarization of diphenylhexatriene. Methylation of phosphatidylethanolamine in brush border membrane did not affect the Na^+-dependent uptake of either D-glucose or phosphate, although the accessibility of cholesterol in membrane to cholesterol oxidase was diminished by 21%, presumably due to altered flip-flop movement of cholesterol in the membrane.

Introduction

Recent studies of Axelrod and his coworkers [1,2] have shown that methylation of phospholipids results in an enhancement in the coupling of β-adrenergic receptors of reticulocytes with the adenylate cyclase. Methylation of erythrocyte membrane phospholipids has been shown to increase markedly the bulk membrane fluidity with a concomitant increase in Ca^{2+}-ATPase activity and flux of Ca^{2+} [3,4]. In addition, studies have shown that methylation of phospholipids affects chemotaxis in bacteria and neural activity [2]. Since methylation of phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine in red blood

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Abbreviations: PE, phosphatidylethanolamine; PME, phosphatidyl-*N*-monomethylethanolamine; PDE, phosphatidyl-*N*,*N*-dimethylethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid.

cell membrane has been shown to result in altered fluidity and increased Ca^{2+} flux, studies were undertaken to determine whether such methylation reactions occur in plasma membrane of mammalian cells which are endowed with phosphatidylethanolamine as one of the constituent of membrane and thus may affect transport process of solutes.

In an effort to delineate how in vivo alteration of fluidity affects membrane bound enzymatic activity and transport of various solutes, studies were undertaken in kidney cortex brush border membrane vesicles. Since little is known about the topology of phospholipids and cholesterol in brush border membranes of kidney cortex, studies were carried out to delineate their orientation. There have been numerous reports on the lipid composition of brush border membranes of different origin [5–7] but studies on their topology are lacking.

The results presented in this communication show that phospholipids and cholesterol are asymmetrically distributed in the renal hog kidney cortex vesicles. Studies also show that methylation of endogenous phosphatidylethanolamine to phosphatidylcholine, in brush border membrane is mediated by two or more methyltransferases located asymmetrically in the membrane. Endogenous synthesis of phosphatidylmonomethylethanolamine in brush border membrane or addition of phosphatidylmonomethylethanolamine, unlike in red blood cell membrane, did not affect either the microviscosity or Na^+ dependent uptake of D-glucose and phosphate.

Materials and Methods

Preparation of brush border membrane vesicles. Brush border membrane vesicles were prepared from hog kidney cortex essentially according to the procedure of Beck and Sacktor [8]. All procedures were carried out at 4°C. Renal cortices from freshly obtained hog kidneys were dissected from the medulla, minced into small pieces and homogenized in 10 vol. (w/v) of ice-cold 50 mM mannitol containing 5 mM Hepes/Tris buffer, pH 7.3, using 4–5 strokes of a Potter-Elvehjem Teflon pestle at 1200 rpm. The suspension was further homogenized using Polytron (Brinkman Instruments) equipped with a PT-10 probe at a power setting of 4 and running for 15 min. The homogenate was stirred continuously for 20 min in the presence of 10 mM $MgCl_2$ and centrifuged at $5000 \times g$ for 20 min. The resulting loose pellet was manually homogenized in 300 mM mannitol containing 2 mM Hepes/Tris buffer, pH 7.5, using 5 strokes of tight fitting glass homogenizer and centrifuged at $27000 \times g$ for 10 min. The supernatant was discarded and fluffy portion of the pellet (mostly brush border membranes) was carefully removed leaving small densely packed portion of the pellet (primarily mitochondria). The membranes were suspended in buffer and centrifuged at $27000 \times g$ for 10 min, repeating the procedure three to four times, till the densely packed contaminating portion of the pellet almost diminished. Brush border membranes were resuspended in equal volume of 300 mM mannitol buffered with 1 mM Hepes/Tris, pH 7.5, manually homogenized and passed four times through a 26-gauge needle. The suspension was centrifuged at $2000 \times g$ for 5 min followed by another centrifugation at $27000 \times g$ for 10 min. The pellet was suspended in buffered mannitol medium to a protein concentration of approx. 10 mg/ml. The quality of brush border membrane vesicles was routinely determined by assay of specific marker enzymes, alkaline phosphatase [9] and γ-glutamyltransferase [10] (brush border membranes); glucose-6-phosphatase [11] (microsomes); ouabain sensitive (Na^+, K^+)-ATPase [9] (baso-lateral membrane) and succinate dehydrogenase [12] (mitochondria). In the brush border membrane preparations alkaline phosphatase and γ-glutamyltransferase were enriched 7–8-fold compared to the homogenate similar to that observed by Lin et al. [13]. Contamination with baso-lateral membranes, mitochondria and microsomes was insignificant.

Lipid extraction. Lipids of brush border membranes were extracted by the method of Folch et al. [14]. Phospholipids were separated on Silica gel G plates using solvent system chloroform/methanol/water (65:25:4, v/v). Free and esterified cholesterol were separated in petroleum ether/diethyl ether/glacial acetic acid (90:10:1, v/v) on Silica gel G plates.

Uptake experiments. Sodium dependent uptake of D-[^{14}C]glucose and [^{32}P]phosphate were mea-

sured by the Millipore filteration technique [15,16]. The uptake buffer contained either 300 mM mannitol or 100 mM mannitol plus 100 mM NaCl, where indicated. In all uptake studies, the osmolarity of the buffer was finally maintained at 300 mosM by adjusting the concentration of mannitol and pH to 7.5 with Tris or HCl. Uptake into brush border membrane vesicles was initiated by adding 30 μl of membrane suspension (100 to 300 μg protein) to 60 μl of mannitol medium containing the required radioactive isotope and incubated at 20°C for the indicated time intervals. The incubation was stopped by the addition of 2 ml of ice cold termination medium (154 mM NaCl in 1 mM Hepes/Tris buffer, pH 7.5, for glucose uptake and in addition containing 5 mM arsenate [16] for phosphate uptake studies). The suspension was then rapidly filtered through 0.45 μm Millipore filter, washed twice with 2 ml of buffer and radioactivity on the dried filter paper was determined using liquid scintillation spectrometer (Beckman, Model LS-8000). All incubations were carried out in duplicate with fresh brush border membrane vesicles. Each experiment was repeated at least three times with different membrane preparations.

Assay of phospholipid methylation. Brush border membranes (approx. 3 mg protein) in either 50 mM Tris-acetate buffer (pH 7.4 or pH 9.0) or 300 mM mannitol containing 5 mM Hepes/Tris, pH 7.5, were incubated at 37°C with 100 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine (2 μCi) in a total volume of 500 μl. Unless otherwise indicated, the incubation was carried out for 60 min. The reaction was terminated by adding 3 ml of chloroform/methanol/2 M HCl (6:3:1, v/v) or 1 ml of 10% trichloroacetic acid followed by extraction with chloroform/methanol (2:1, v/v). The chloroform phase was washed with 1 ml of 0.5 M KCl in 50% methanol as described [1]. The chloroform phase was dried at room temperature under a stream of N_2 gas and the residue was dissolved in 150 μl of chloroform. A 100 μl sample was then applied to Silica gel G plate and chromatograms were developed in chloroform/propionic acid/*n*-propyl alcohol/water (2:2:3:1, v/v) for phospholipid separation [1]. The spots were visualized by exposure to iodine vapors, scrapped and the radioactivity counted in 5 ml of Aquamix (West Chem Products, San Diego, CA). Corrections were made for the quenching, due to silica gel, in all the data expressed.

Treatment of brush border membranes with phospholipase C and sphingomyelinase. Brush border membrane vesicles (2–4 mg protein) in 300 mM mannitol containing 5 mM Tris/Hepes, pH 7.4, 5 mM $MgCl_2$ and 5 mM $CaCl_2$ were incubated with either 20 units of phospholipase C type I from *Bacillus cereus* (Sigma) or 20 units of sphingomyelinase from human placenta. At the indicated time, sample was washed with the same buffer. Lipids were extracted with chloroform/methanol (2:1, v/v) and phospholipids separated on Silica gel G plate using chloroform/methanol/water (65:25:4, v/v).

Trypsin treatment of brush border membranes. Brush border membranes (2–3 mg protein) in 5 mM Tris-acetate buffer, pH 7.4 containing 300 mM mannitol were incubated with 400 μg trypsin either in the presence or absence of 0.05% Triton X-100 at 37°C for 30 min. At the end of the incubation time 800 μg of trypsin inhibitor was added.

Fluorescence polarization measurement. Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene in brush border membrane vesicles was measured with an MPF-4 Perkin Elmer Spectrofluorometer as described [17].

Treatment of brush border membranes with cholesterol oxidase. Brush border membranes (1 mg protein) in 5 mM Hepes/Tris, pH 7.5, containing 300 mM mannitol were incubated with 15 μl of cholesterol oxidase (2.5 I.U./ml). The procedure of Dole [18] as modified by Moore et al. [19] was used for termination of reaction and extraction of neutral lipids. Cholestenone was assayed spectrophotometrically at 235 nm [19]. Blanks consisted of all reagents except the enzyme.

Analytical methods. Phospholipid phosphorus was estimated by the colorimetric procedure after perchloric acid digestion [20] and cholesterol was determined by the procedure of Zlatkis et al. [21]. Protein was estimated according to the method of Lowry et al. [22].

Materials. *S*-Adenosyl-L-[*m*ethyl-^{3}H]methionine was purchased from New England Nuclear (Boston, MA). Phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine were obtained from Calbiochem.-Behring Corp.

(LaJolla, CA). Cholesterol oxidase, *S*-adenosylmethionine and *S*-adenosyl-L-homocysteine were purchased from Boehringer-Mann. Phospholipase C, sphingomyelinase and trypsin were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

Results

Hydrolysis of brush border membrane vesicle phospholipids by phospholipase C and sphingomyelinase

The phospholipid composition of hog kidney cortex brush border membrane was determined by techniques given in Materials and Methods. As shown in Table I, there are three major phospholipids in brush border membranes consisting largely of phosphatidylcholine (32%), phosphatidylethanolamine (31%) and sphingomyelin (25%). The other minor component was the acidic phospholipid phosphatidylserine (11%). When brush border membrane vesicles were incubated at 37°C with phospholipase C, only 30% of the total membrane phospholipids were hydrolyzed in 2 h (Fig. 1). Incubation of membranes at 37°C with twice the amount of enzyme and prolonged incubation (6 h) did not result in further degradation of remaining phospholipids. Analysis of phospholipids of phospholipase C-treated membrane vesicles revealed that 76% of total phosphatidylcholine was degraded by phospholipase C, while only 10–12% of phosphatidylethanolamine was hydrolyzed. Other phospholipids in the membrane were not hydrolyzed under these conditions. It should be pointed out that phospholipase C cleaved 80% of phosphatidylserine, 90% phosphatidylcholine and 92% phosphatidylethanolamine, when membrane vesicles were opened with 0.05% Triton X-100 (data not shown). Treatment of brush border membrane vesicles with sphingomyelinase at 37°C resulted in hydrolysis of 90–95% sphingomyelin.

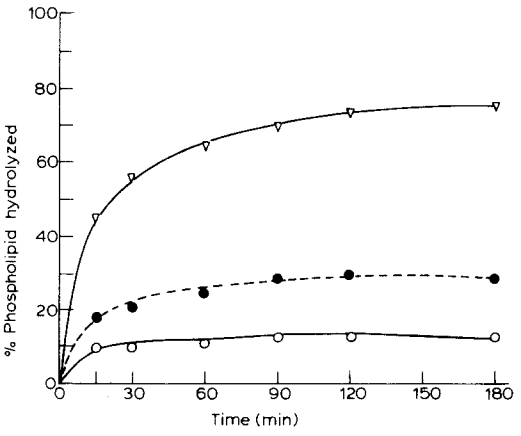

Fig. 1. Hydrolysis of brush border membrane phospholipids by phospholipase C. Brush border membrane (2–4 mg protein) were incubated with 20 I.U. of phospholipase C (*B. cereus*) in 300 mM mannitol buffered with 5 mM Hepes/Tris, pH 7.5, containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$. At the indicated time, reaction was terminated by the addition of *O*-phenanthroline and EDTA (final concentration 12 mM each) followed by the addition of 10% trichloroacetic acid. Lipids were extracted and analyzed. ●- - - - -●, Total phospholipids; ○——○, phosphatidylethanolamine; ▽——▽, phosphatidylcholine.

TABLE I

LIPID COMPOSITION OF HOG KIDNEY BRUSH BORDER MEMBRANES

Brush border membrane lipids were extracted, different classes of lipids were separated and quantitated. The percentage of phospholipids refers to individual phospholipid percentage in total phospholipids. Results are expressed as mean ± S.E. of five experiments.

Lipids	μg/mg protein	% phospholipids
Total cholesterol	91 ± 5	–
Free cholesterol	75 ± 4	–
Esterified cholesterol	16 ± 2	–
Total phospholipids	375 ± 11	–
Phosphatidylserine	42 ± 4	11 ± 1
Sphingomyelin	96 ± 5	25 ± 2
Phosphatidylcholine	120 ± 7	32 ± 2
Phosphatidylethanolamine	114 ± 7	31 ± 2

Accessibility of cholesterol pool in brush border membrane vesicles to cholesterol oxidase

The chemical composition of neutral lipids revealed that 80% of the total cholesterol pool in brush border membranes was present as free cholesterol while remaining 20% was in the esterified form (Table I). Total oxidizable cholesterol in brush border membrane vesicles was determined after extraction of lipids from the membrane followed by treatment with cholesterol oxidase in the presence of 10% sodium taurodeoxycholate.

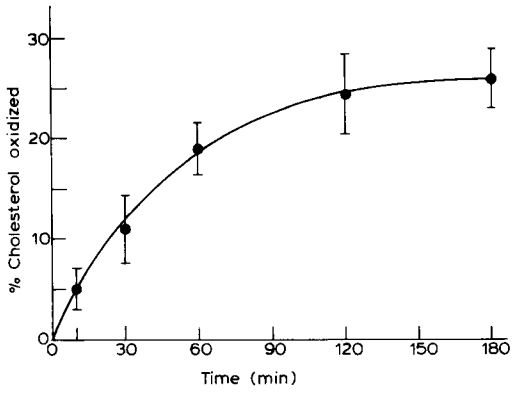

Fig. 2. Time course of oxidation of cholesterol in brush border membrane. Brush border membrane vesicles (1 mg protein) in mannitol buffer, pH 7.5, were incubated with 15 μl of cholesterol oxidase (2.5 I.U./ml) at 37°C for indicated time periods. Neutral lipids were extracted with 2 ml of Dole's reagent. Cholestenone was assayed spectrophotometrically at 235 nm. Each value is the mean ± S.D. of four experiments.

The amount of cholesterol determined by cholesterol oxidase method was same as that estimated by chemical method. When intact brush border membranes were treated with cholesterol oxidase only 26% of the free cholesterol pool in the membrane was accessible to oxidation (Fig. 2).

Incorporation of methyl groups from S-adenosyl-L-[methyl-^3H]methionine into brush border membrane phospholipids

Studies have shown that methylation of phosphatidylethanolamine to phosphatidylcholine is catalyzed by two methyltransferases in microsomal fraction of bovine adrenal medulla [1] and erythrocytes [3]. The first methyltransferase which catalyzes the methylation of phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine, has a pH optimum of 6.5, a low K_m for *S*-adenosylmethionine (4 μM) and an absolute requirement for Mg^{2+}. The second methyltransferase has been shown to catalyze the two successive methylations of phosphatidyl-*N*-monomethylethanolamine to phosphatidyl-*N*,*N*-dimethylethanolamine and to phosphatidylcholine, and has a pH optimum of 10 and a high K_m for *S*-adenosylmethionine [1]. As shown in Fig. 3 we compared the methylation of phospholipids in brush border membranes at different pH values, from 5 to 10. There was optimum incorporation of [^{3}H]methyl

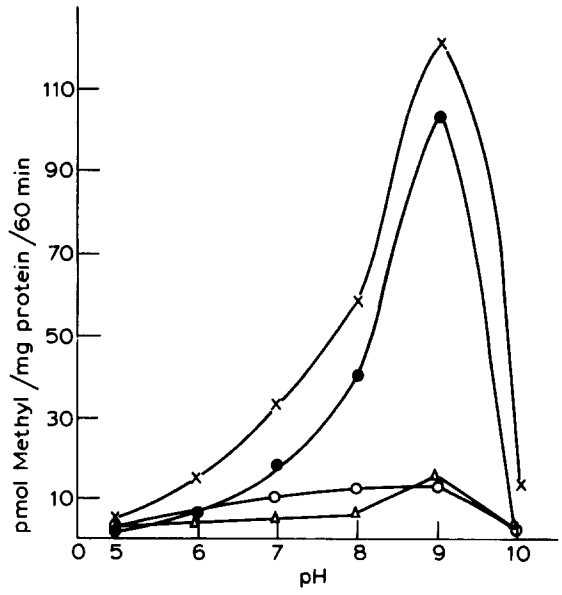

Fig. 3. Effect of pH on the enzymatic *N*-methylation of phosphatidylethanolamine. Brush border membranes (2–3 mg protein) were incubated at 37°C with *S*-adenosyl[*methyl*-^{3}H]methionine (100 μM) in the presence of different buffers. The buffers used were, 50 mM sodium acetate pH 5 and 6, 50 mM Tris-acetate, pH 7, 8 and 9 and 50 mM sodium borate, pH 10. After 60 min the reaction was terminated by addition of 10% trichloroacetic acid. The reaction products were extracted. Thin-layer chromatography was performed with a solvent system consisting of chloroform/propionic acid/*n*-propyl alcohol/water (2:2:3:1, v/v). Values are expressed as pmol [^{3}H]methyl groups incorporated into phospholipids per mg protein. Methyl incorporation into: ×———×, total phospholipids; ○———○, phosphatidylmonomethylethanolamine; △———△, phosphatidyldimethylethanolamine; ●—●, phosphatidylcholine.

groups into phosphatidylethanolamine at pH 9.0. At low concentration (4 μM) of *S*-adenosylmethionine in the presence of 5 mM Mg^{2+}, the extent of incorporation into phosphatidylethanolamine at pH 6.0–6.5 was 20-times less as compared to 100 μM concentration of *S*-adenosylmethionine. The extent of incorporation of [^{3}H]methyl groups into phosphatidylethanolamine was not affected by Mg^{2+} or EGTA at either pH 6 or pH 9 (data not shown). The K_m value of *S*-adenosylmethionine at pH 6 and pH 9 was similar, i.e., 100 μM. At pH 6, with 100 μM concentration of *S*-adenosylmethionine, there was greater incorporation of [^{3}H]methyl groups into phosphatidyl-*N*-monomethylethanolamine than into phos-

TABLE II

EFFECT OF EXOGENOUSLY ADDED PHOSPHOLIPIDS ON [^{3}H]METHYL GROUPS INCORPORATION INTO *N*-METHYL DERIVATIVES OF PHOSPHATIDYLETHANOLAMINE

Brush border membranes (2–3 mg protein) were preincubated with phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME) and phosphatidyldimethylethanolamine (PDE) at indicated concentrations for 10 min. These phospholipids were suspended in 50 mM Tris-acetate, pH 7.5 buffer containing 0.05% Triton X-100 by sonication at 40°C for either 15 min (PME and PDE) or 40 min (PE). Methylation was carried out for 10 min by the addition of *S*-adenosyl[*methyl*-^{3}H]methionine (100 μM) and the reaction was terminated by addition of 10% trichloroacetic acid. Phospholipids were extracted and separated. Values are mean ± S.E. of four experiments.

Addition	pmol [^{3}H]methyl incorporated per mg protein per 10 min			
	Total incorporation	PME	PDE	PC
None	15.0 ± 1.50	5.2 ± 0.95	3.6 ± 0.51	6.1 ± 0.87
PE (50 μg)	13.2 ± 0.90	4.0 ± 0.60	3.7 ± 0.40	5.5 ± 0.37
PE (500 μg)	13.8 ± 0.88	5.7 ± 0.49	3.5 ± 0.42	4.6 ± 0.39
PME (50 μg)	20.9 ± 2.70	6.2 ± 0.80	5.5 ± 0.29	9.0 ± 0.92
PME (500 μg)	61.9 ± 4.00	6.2 ± 0.72	25.5 ± 2.30	30.1 ± 3.10
PDE (50 μg)	19.4 ± 1.20	4.5 ± 0.20	3.5 ± 0.22	11.4 ± 0.97
PDE (500 μg)	58.8 ± 3.60	6.0 ± 0.45	7.1 ± 0.40	45.6 ± 3.81
Triton X-100 (0.05%)	11.0 ± 0.86	5.5 ± 0.30	2.0 ± 0.19	3.6 ± 0.47

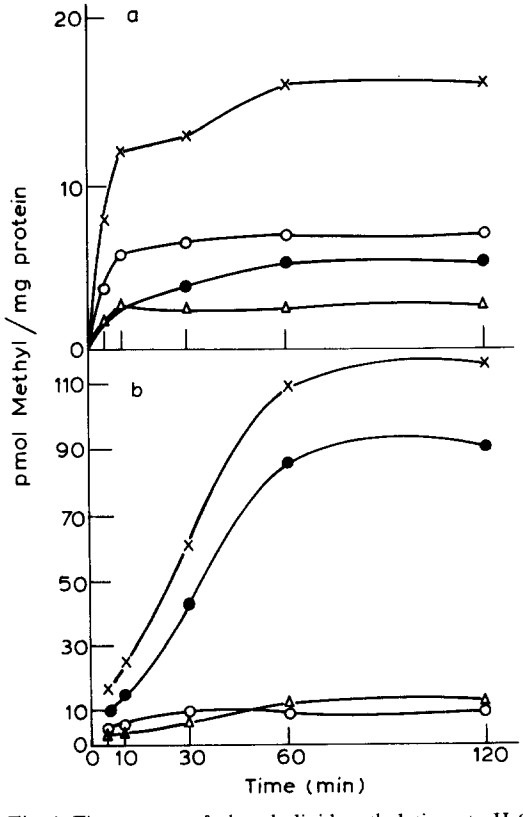

Fig. 4. Time course of phospholipid methylation at pH 6.0 and pH 9.0. Brush border membranes (2–3 mg protein) were incubated with *S*-adenosyl[*methyl*-^{3}H]methionine (100 μM) in presence of either (a) 50 mM sodium acetate (pH 6.0) or (b) 50 mM Tris-acetate buffer (pH 9.0). At the indicated time, reaction was terminated, phospholipids were extracted and separated. Values are expressed as pmol [^{3}H]methyl groups incorporated into phospholipids per mg protein. × ——— ×, Total phospholipids; ○ ——— ○, phosphatidyl-*N*-monomethylethanolamine; △ ——— △, phosphatidyl-*N*,*N*-dimethylethanolamine; ● ——— ●, phosphatidylcholine.

phatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine (Fig. 4). However, phosphatidyl-*N*-monomethylethanolamine was not the predominant labelled species as has been observed in microsomes of adrenal medulla [1]. At pH 9.0 the incorporation of [^{3}H]methyl groups into phosphatidylcholine was greater than in phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*,*N*-dimethylethanolamine.

Effect of exogenously added phospholipid substrates on methyltransferase activity

Since methylation of phosphatidylethanolamine in brush border membrane vesicles exhibited pH optimum of 9.0 and did not require Mg^{2+}, it was possible that a single methyltransferase enzyme catalyzed the conversion of phosphatidylethanolamine into phosphatidylcholine. As shown in Table III, the effect of exogenously added phosphatidylethanolamine, phosphatidyl-*N*-monomethyl-

ethanolamine (PME) and phosphatidyl-*N*,*N*-dimethylethanolamine (PDE) was studied on the incorporation of [^{3}H]methyl groups from *S*-adenosyl[*methyl*-^{3}H]methionine. The incorporation into total phospholipids was 4-fold higher when PME and PDE were added as compared to PE (Table II). Exogenous PE did not enhance the incorporation of [^{3}H]methyl groups into phospholipids indicating that exogenous PE was not a substrate for transmethylation reaction. As shown addition of PME (500 μg) resulted in 7-fold increased incorporation, i.e., from 3.6 pmol to 25.5 pmol into PDE and a 5-fold increased incorporation into phosphatidylcholine. Similarly, addition of PDE (500 μg) resulted in 7-fold increased incorporation of [^{3}H]methyl groups into phosphatidylcholine.

Methylation of phospholipids after trypsin treatment of brush border membrane vesicles

In order to delineate the localization of the phospholipid methylating enzyme(s) in brush border membrane vesicles, proteolytic digestion with trypsin was studied. As shown in Table III, treatment with trypsin resulted in a reduction (69%) in the incorporation of [^{3}H]methyl groups into total phospholipids. On further analysis it was observed that the incorporation of [^{3}H]methyl into phosphatidyldimethylethanolamine and phosphatidylcholine was significantly reduced. However, the incorporation of ^{3}H-label into phosphatidylmonomethylethanolamine was not reduced. When the membranes were opened with Triton X-100 followed by trypsin treatment (which presumably will result in entry of trypsin and

TABLE III

EFFECT OF TRYPSIN TREATMENT ON THE INCORPORATION OF [^{3}H]METHYL GROUPS INTO *N*-METHYL DERIVATIVES OF PHOSPHATIDYLETHANOLAMINE

Brush border membranes (2–3 mg protein) were preincubated with trypsin (400 μg) at 37°C. Trypsin inhibitor (800 μg) was added after 30 min. Methylation was started by incubating the membrane with *S*-adenosyl[*methyl*-^{3}H]methionine (100 μM) in Tris-acetate buffer (pH 9). Phospholipids were extracted and separated. Values are mean ± S.E. of four experiments.

	pmol [^{3}H]methyl incorporated per mg protein per 60 min	% reduction in incorporated radio compared to control
Untreated sealed vesicles		
Total phospholipids	86 ± 4	–
PME	22 ± 2	–
PDE	23 ± 2	–
PC	43 ± 3	–
Trypsin-treated sealed vesicles		
Total phospholipids	27 ± 3	69
PME	23 ± 2	0
PDE	2 ± 1	91
PC	2 ± 1	95
Sealed vesicles opened with 0.05% Triton X-100		
Total phospholipids	72 ± 4	–
PME	23 ± 2	–
PDE	12 ± 1	–
PC	37 ± 2	–
Trypsin treatment of vesicles incubated with 0.05% Triton X-100		
Total phospholipids	22 ± 2	70
PME	14 ± 1	39
PDE	4 ± 1	67
PC	4 ± 1	89

attack on the inner side of the membrane vesicles) incorporation of [^{3}H]methyl groups into phosphatidylcholine and phosphatidyl-*N*, *N*-dimethylethanolamine was reduced along with less incorporation (39%) into phosphatidylmonomethylethanolamine (Table III).

Effect of trypsin treatment on methylation of exogenously added phosphatidylethanolamine derivatives

Studies were carried out to determine th topology of methyltransferase enzyme in brush border membranes which catalyzed the transmethylation of exogenously added phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine derivatives. Treatment of brush border membranes with trypsin prior to initiating methylation reaction with *S*-adenosyl[*methyl*-^{3}H]-methionine resulted in reduced [^{3}H]methyl incorporation (6%) when exogenous phosphatidylmonomethylethanolamine was used as substrate (data not shown). These results suggest that methyltransferase, which catalyzes the successive conversion of phosphatidylmonomethylethanolamine to phosphatidylcholine is oriented on the external side of the brush border membrane vesicles.

Effect of methylation of phospholipids on fluorescence polarization of diphenylhexatriene and uptake of phosphate and glucose in brush border membrane vesicles

Previous studies of Hirata and Axelrod [3] have shown that methylation of phosphatidylethanolamine to phosphatidylmonomethylethanolamine in erythrocyte membrane caused an increase (30%) in the fluidity of the membrane concomitant with significant altered Ca^{2+}-ATPase activity and ATP-mediated Ca^{2+} flux. In our studies with brush border membrane vesicles, methylation of phospholipids did not significantly affect the fluorescence polarization *P* value of diphenylhexatriene (Table IV). Moreover, the Na^+-dependent uptake of phosphate (Fig. 5a) and glucose (Fig. 5b) in methylated and untreated brush border membranes was similar. Since phosphatidylmonomethylethanolamine predominantly alters the fluidity in erythrocyte membrane, we studied the effect of exogenously added PE, PME and PDE on the fluorescence polarization of diphenylhexatriene and the uptake of phosphate and D-glucose. As shown in Table IV, addition of these phospholipids to the membrane did not alter the fluidity of the lipid bilayer of the brush border membranes and there was no affect on the uptake of phosphate and D-glucose (Figs. 5a and 5b).

Effect of methylation of phospholipids on the movement of cholesterol in brush border membrane vesicles

Recent studies [23] have shown that cholesterol exhibits transmembrane movement in the red blood cell membrane. Studies were undertaken to determine whether methylation of phospholipids in brush border membrane vesicles affected the accessibility of cholesterol to cholesterol oxidase. The amount of cholesterol available in the membrane to oxidation by cholesterol oxidase was reduced by 21% (from 21 ± 1 μg/mg protein to 16.5 ± 0.9 μg/mg protein) when methylation of membrane phospholipids with *S*-adenosylmethionine was carried out for 60 min. The amount of cholesterol available for oxidation to cholest-4-en-3-one remained constant from 30 min to 3 h indicating that either the transmembrane movement of cholesterol is too rapid for measurement or the methylation process causes overall masking of the available β-hydroxyl groups in cholesterol molecules for accessibility towards cholesterol oxidase.

TABLE IV

FLUORESCENCE POLARIZATION (*P*) OF DIPHENYL HEXATRIENE IN BRUSH BORDER MEMRBANES AT 25°C

Brush border membranes (4 mg protein) were either methylated with *S*-adenosylmethionine (100 μM) at pH 7.5 or incubated with indicated concentrations of sonicated phospholipids for 30 min. Membranes were washed and incubated with $2 \cdot 10^{-6}$ M diphenylhexatriene for 60 min. Fluorescence polarization was carried out at 25°C. *P* values are mean ± S.E. of three determinations.

Treatment	Fluorescence polarization (*P*)
None	0.23 ± 0.01
S-Adenosylmethionine	0.23 ± 0.01
PE (500 μg)	0.22 ± 0.01
PME (500 μg)	0.23 ± 0.01
PDE (500 μg)	0.22 ± 0.01

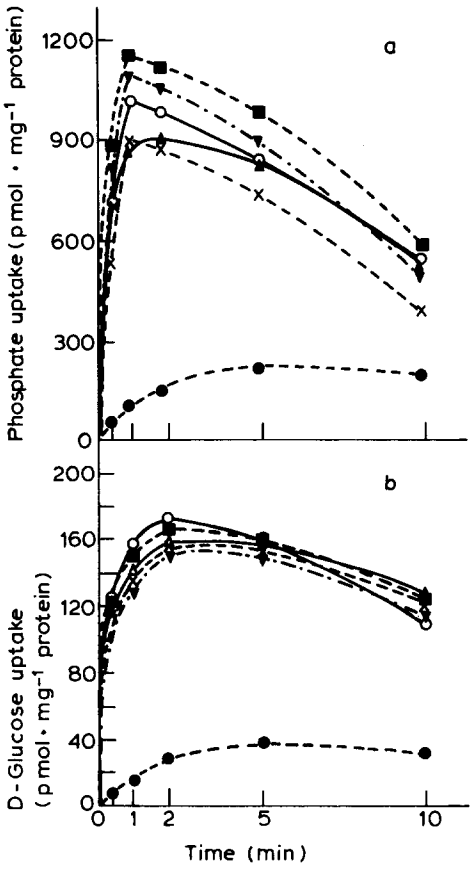

Fig. 5. Time course of the uptake of (a) phosphate and (b) D-glucose into brush border memrbane vesicles. Brush border membranes (100 to 300 μg protein) were incubated with either 25 μM of [^{32}P]phosphate or 50 μM D-[^{14}C]glucose (2 μCi/ml) solution. The extravesicular media contained either 100 mM buffered mannitol and 100 mM NaCl or 300 mM buffered mannitol. ○———○, Control brush border membranes; △———△, methylated brush border membranes; ■———■, brush border membranes preincubated with sonicated phosphatidylethanolamine, or ×------×, phosphatidyl-*N*-monomethylethanolamine, or ▼—·—·—▼, phosphatidyl-*N*,*N*-dimethylethanolamine derivatives. ●------●, indicates membranes incubated with 300 mM buffered mannitol without NaCl. The results shown are the means of three experiments.

Discussion

The results presented in this study using phospholipase C treatment show that in brush border membranes of hog kidney cortex, 76% of total phosphatidylcholine compared to 10% of phosphatidylethanolamine are localized in the outer surface of these vesicles. Studies have shown that phospholipase C treatment does not render the membrane leaky or lytic since complete hydrolysis of phosphatidylethanolamine and acidic phospholipids, presumably localized on the inner surface, was only observed when membranes were opened with Triton X-100. Studies utilizing sphingomyelinase revealed that majority of sphingomyelin (90–95%) was accessible to cleavage in brush border membranes thus indicating that phosphatidylcholine and sphingomyelin are present on the outer surface while phosphatidylethanolamine and phosphatidylserine are presumably present on the inner surface of brush border membrane vesicles. The existence of an asymmetric distribution of phospholipids across the bilayer has been observed in several other membranes [24,25] such as in red cells [26], rat liver plasma membrane [27], virus membranes [19,28] and bacterial membranes [29]. In contrast, in microsomal and Golgi membranes symmetrical distribution of phosphatidylinositol has been demonstrated [30].

Treatment of brush border membrane vesicles with cholesterol oxidase revealed that β-hydroxyl group of cholesterol was accessible to oxidation to cholest-4-en-3-one. However, approx. 26% of the free cholesterol pool in the sealed membrane vesicles was oxidized suggesting that either the cholesterol is asymmetrically distributed in the brush border membranes or free β-hydroxyl group of remaining (74%) cholesterol either sequestered in the lipid bilayer or shielded by membrane proteins thus blocking access of cholesterol oxidase to the substrate. Bittman and Rottem [31] utilzing binding of filipin to cholesterol as the assay system showed that in *Mycoplasma* membranes 66% of cholesterol was present on the outer half of the lipid bilayer. Based on the analysis of the cholesterol content in the two halves of the erythrocyte membrane obtained by freeze fracture, Fisher [32] showed that more cholesterol is present on the external side than on the inner half of the erythrocyte membrane. It is pertinent to mention that cholesterol in intact red blood cell membranes has been shown to be resistant to cholesterol oxidase digestion while enrichment of cells with exogenous cholesterol rendered the entire cholesterol pool susceptible to attack [23,33]. Similarly, cholesterol in intact vesicular stomatitis virus has been shown to be resistant to oxidation by

cholesterol oxidase, while removal of polar head groups by phospholipase C treatment prior to cholesterol oxidase digestion resulted in more than 90% oxidation of cholesterol to cholest-4-en-3-one [19]. These results have been interpreted by these authors that either the cholesterol is asymmetrically distributed in the outerleaflet of the virion bilayer or the symmetrically arranged cholesterol in the inner layer flip-flops across the bilayer upon phospholipase C treatment, and exchanges with the oxidizable cholesterol present in the outer layer [19].

Results presented in this study also show that methylation of endogenous phosphatidylethanolamine, localized on the inner surface of brush border membrane vesicles, resulted in the formation of phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N*, *N*-dimethylethanolamine and phosphatidylcholine. All three stepwise methylations of endogenous phosphatidylethanolamine were not affected by Mg^{2+}, EDTA or low concentrations (2–10 μM) of *S*-adenosylmethionine. Moreover, the extent of methylation was lower at pH 6.0 compared to 9.0, both at low (2–10 μM) and high concentrations (80–100 μM) of *S*-adenosylmethionine. In red blood cell membranes [3] and in microsomal membranes [34], the existence of two methyltransferase enzymes have been demonstrated which are involved in the methylation of phosphatidylethanolamine to phosphatidylcholine. The first methyltransferase in these membranes, which catalyzes the conversion of phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine has an optimum pH of 6.5, a low K_m for *S*-adenosylmethionine (1.4 μM), and an absolute requirement for Mg^{2+}. The second methyltransferase which catalyzes the two successive methylations of phosphatidyl-*N*-monomethylethanolamine to phosphatidyl *N*, *N*-dimethylethanolamine and phosphatidylcholine has been shown to exhibit optimum pH of 10, high K_m for *S*-adenosylmethionine (0.1 mM) and does not require Mg^{2+}.

In brush border membranes methylation of endogenous phosphatidylethanolamine by *S*-adenosylmethionine occurred at high pH and did not require Mg^{2+}, therefore it appears that either a single methyltransferase catalyzes the three successive methyl transfers or the specificity of enzymes are different in brush border membranes as compared to microsomal or red blood cell membranes. The possibility of a single enzyme rather than two or more methyltransferases catalyzing successive methylations of phosphatidylethanolamine to phosphatidylcholine was ruled out by the observation that trypsin treatment of brush border membranes, resulted in extensive diminished incorporation of [^{3}H]methyl groups from *S*-adenosyl-L-[*methyl*-^{3}H]methionine into phosphatidyl-*N*, *N*-dimethylethanolamine and phosphatidylcholine; while, the incorporation of radiolable into endogenous phosphatidylmonomethylethanolamine remained unchanged. These results suggest that at least two methyltransferases are involved in the methylation of endogenous phospholipids, one localized on the inner side and the second on the external side of the brush border membrane vesicles. This is also substantiated by the observation that exogenously added phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine stimulated several-fold the incorporation of methyl groups into phospholipids while exogenously added phosphatidylethanolamine did not affect the methyl incorporation. It appears that phosphatidylethanolamine probably cannot penetrate the membrane to become the substrate to methyltransferase-I localized on the inner side of the brush border membrane. Since, in trypsin treated membrane vesicles, incorporation of label into exogenously added phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine did not occur, it indicates that methyltransferase II is localized on the outer leaflet of the monolayer and thus available for proteolytic digestion. Thus specificity of methyltransferases in brush border membrane is different from that observed in microsomes where phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N*, *N*-dimethylethanolamine are synthesized by enzyme(s) located on the inner surface of the microsomal membranes [35].

Methylation of brush border membranes at both low pH (6.0) and high pH (9.0) did not result in changes in fluorescence polarization *P* value of diphenylhexatriene indicating that environment of the probe in the lipid bilayer remained similar before and after methylation. This could be due to formation of a small amount of monomethylphos-

phatidylethanolamine (0.015%) from the total pool of phosphatidylethanolamine present in membrane. However, in red blood cell membranes Hirata and Axelrod [3] observed a significant change (30%) in microviscosity when methylation was carried out at pH 6.0, which resulted in the formation of a very small fraction (0.0012%) of phosphatidylmonomethylethanolamine from total phosphatidylethanolamine present in these membranes. In our studies, the addition of phosphatidylmonomethylethanolamine lipid dispersion/liposomes to brush border membranes did not alter the microviscosity suggesting that in these membranes formation of phosphatidylmonomethylethanolamine in vivo may not play a role in regulating fluidity of the membrane. Moreover, studies show that methylation of endogenous phosphatidylethanolamine in brush border membranes does not affect active transport of solutes such as Na^+-dependent uptake of D-glucose and phosphate. However, methylation of phospholipids caused a reduction (21%) in the availability of free β-hydroxyl group of cholesterol in the membrane to oxidation by cholesterol oxidase. This may occur either (a) as a result of synthesis of newly formed phosphatidylcholine whose polar head group masks the β-hydroxyl group of cholesterol thus making it inaccessible to cholesterol oxidase or (b) the flip-flop rate of cholesterol from inner to outer bilayer is altered. However, we were unable to detect the transmembrane movement of cholesterol in brush border membranes in the time period range of (1–10 s) and (1–4 h). Thus either the rate of flip-flop of cholesterol is too fast, as has been observed in red blood cell membrane enriched with cholesterol [23] or is too slow (15–20 h) for measurement.

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