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EFFECT OF PHOSPHOLIPID METHYLATION ON CALCIUM TRANSPORT AND $(Ca^{2+} + Mg^{2+})$-ATPase ACTIVITY IN KIDNEY CORTEX BASOLATERAL MEMBRANES

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Basolateral membranes isolated from hog kidney cortex, enriched 12- to 15-fold in $(Na^+ + K^+)$-ATPase activity, were 80% oriented inside-out as determined by assay of oubain-sensitive $(Na^+ + K^+)$-ATPase activity before and after opening of the membrane vesicle preparation with a mixture of deoxycholate and EDTA. In these membrane preparations 80% of total phosphatidylethanolamine was accessible to trinitrophenylation by trinitrobenzenesulfonic acid at 4°C, while at 37°C all of phosphatidylethanolamine fraction was chemically modified. Phospholipase C treatment resulted in hydrolysis of 80% phosphatidylethanolamine, 40% phosphatidylcholine and 35% of phosphatidylserine. Sphingomyelinase treatment resulted in 20% hydrolysis of sphingomyelin, presumably derived from right-side-out oriented vesicles. Results indicate that phosphatidylethanolamine is oriented exclusively on the outer leaflet of the lipid bilayer of inside-out oriented vesicles. Methylation of phospholipids in basolateral membranes with *S*-adenosyl[*methyl*-^{3}H]methionine resulted in the three successive methylation of ethanolamine moiety of phosphatidylethanolamine to phosphatidylcholine. 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 and 10. Basolateral membranes incubated in the presence of methyl donor, *S*-adenosylmethionine, exhibited increased (12–15%) $(Ca^{2+} + Mg^{2+})$-ATPase activity and increased ATP-dependent uptake of calcium. ATP-dependent calcium uptake in these vesicles was insensitive to oligomycin and ouabain but was abolished completely by 50 μM vanadate. The increase in ATP-dependent calcium uptake was due to an increase in V_{max} and not due to a change in K_m for Ca^{2+}. Preincubation of membranes with *S*-adenosylhomocysteine, a methyltransferase inhibitor, abolished the stimulatory effect of phospholipid methylation on calcium uptake. Phospholipid methylation at both low and high pH did not result in a change in bulk membrane fluidity as determined by the fluorescence polarization of diphenylhexatriene. These results suggest that phospholipid methylation may regulate transepithelial calcium flux in vivo.

Introduction

Several investigations have indicated that phospholipid methylation influence many membrane events such as membrane fluidity [1], lipid translocation [2], lectin induced lymphocyte mitogenesis [3] and coupling of β-adrenergic receptor with adenylate cyclase [4]. It has been shown that meth-

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Abbreviations: TNBS, 2,4,6-trinitrobenzenesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PE, phosphatidylethanolamine; PME, phosphatidyl-*N*-monomethylethanolamine; PDE, phosphatidyl-*N*,*N*-dimethylethanolamine; EGTA, ethyeleneglycol bis-(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride.

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ylation of erythrocyte membrane phospholipids, specifically conversion of phosphatidylethanolamine to phosphatidyl-*N*-monomethylethanolamine, results in an increase in bulk membrane fluidity with a concomitant increase (35–40%) in Ca^{2+}-ATPase activity and flux of Ca^{2+} [1,5]. In our previous studies [6] we observed that methylation of kidney cortex brush border membranes phospholipids did not alter the fluidity of the membrane and transport function such as Na^+-dependent uptake of D-glucose and phosphate. Since basolateral membrane is endowed with Ca^{2+}-ATPase activity which has been shown to mediate ATP-dependent uptake of Ca^{2+} in inside-out membrane vesicles [7], it was of interest to determine whether methylaton of phospholipids regulated Ca^{2+}-ATPase in basolateral membrane. It is pertinent to mention that calcium regulatory protein, calmodulin, has been implicated to regulate the flux of Ca^{2+} in basolateral membrane of rat small intestine [8]. In an effort to elucidate how methylation of phospholipids in vivo regulates transepithelial calcium flux, studies were initiated in isolated basolateral membrane preparations.

Materials and Methods

Preparation of basolateral membrane vesicles. Basolateral membrane vesicles were prepared from hog kidney cortex essentially according to the procedure of Kinsella et al. [9]. The cortex (30–40 g) was removed from the freshly obtained hog kidneys, minced with scissors in about 400 ml of 8% sucrose buffered with 5 mM Hepes/Tris (pH 7.4) followed by homogenization (10 strokes) in a Dounce glass homogenizer equipped with a tight-fitting pestle. The suspension was further homogenized in a Polytron homogenizer (Brikman Instruments) equipped with a PT-10 probe for 10 min at a power setting of 5. All operations were carried out at 4°C. The cortical homogenate was spun at $1000 \times g$ for 10 min and the supernatant obtained was further centrifuged at $9500 \times g$ for 10 min. The supernatant and the soft lighter portion of the pellet were removed, combined and centrifuged at $47\,000 \times g$ for 30 min. The supernatant was discarded, and the fluffy portion of the pellet (plasma membrane enriched fraction) was suspended in 300 mM mannitol buffered with 5 mM Hepes/Tris (pH 7.4) and homogenized using 5 strokes of tight fitting glass homogenizer. To this homogenate (6–8 mg protein/ml), 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$ were added and the suspension was kept at 4°C for 1 h, and centrifuged at $1400 \times g$ for 10 min. The pellet was re-extracted three times with buffered mannitol medium and the membranes were stirred for 30 min in this medium containing 5 mM EDTA at 4°C. After centrifugation at $26\,000 \times g$ for 20 min, the pellet was resuspended in buffered mannitol containing 0.1 mM PMSF. The suspension was dialyzed over-night at 4°C against 50 vol. of this buffer. The membrane suspension (5 ml) was layered over a discontinuous sucrose gradient comprising of 10 ml 31% (w/w) sucrose solution and 12 ml 8% sucrose solution and centrifuged at $90\,000 \times g$ for 60 min as described [9]. The basolateral membranes at 8–31% sucrose interface were collected and suspended in 300 mM mannitol buffered with 5 mM Hepes/Tris (pH 7.4). Sealed membrane vesicles were isolated by centrifuging the membrane suspension on 5% Dextran T-70 gradient [10] at $90\,000 \times g$ for 60 min.

The quality of basolateral membrane vesicles were routinely determined by assay of specific marker enzymes, oubain-sensitive $(Na^+ + K^+)$-ATPase [11] (basolateral membranes); alkaline phosphatase [12] and γ-glutamyltransferase [13] (brush border membranes); glucose-6-phosphatase [14] (endoplasmic reticulum) and succinate dehydrogenase [15] (mitochondria). In basolateral membrane preparations $(Na^+ + K^+)$-ATPase activity was enriched 12- to 15-fold compared to homogenate similar to that observed by Kinsella et al. [9]. Contamination with brush border membranes, mitochondria and microsomes was minimal.

Lipid extraction. Lipids of basolateral membranes were extracted by the method of Folch et al. [16]. 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.

Treatment of basolateral membranes with phospholipase C and sphingomyelinase. Basolateral membrane vesicles (1–2 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 or 20 units of sphingomyelinase, both from *Bacillus cereus* (Sigma). 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).

Treatment of basolateral membranes with cholesterol oxidase. Basolateral membranes (1 mg protein) in 5 mM Hepes/Tris (pH 7.4), containing 300 mM mannitol were incubated with 15 μl of cholesterol oxidase (2.5 I.U./ml). The procedure of Dole [17] as modified by Moore et al. [18] was used for termination of reaction and extraction of neutral lipids. Cholestenone was assayed spectrophotometrically at 235 nm [18]. Blanks consisted of all reagents except the enzyme.

Labeling of membrane vesicles with trinitrobenzenesulfonic acid. The procedure used for the labeling of membrane vesicles was essentially similar to that of Rothman and Kennedy [19]. Membrane vesicles (1.0 mg protein) were suspended in 0.5 ml of buffered manitol. To this was added 5 ml of 3 mM TNBS prepared in 200 mM mannitol containing 50 mM $NaHCO_3$ (pH 8.5) and incubated at 0–4°C for indicated intervals (30–120 min) of time. Reaction was terminated by the addition of chloroform/methanol (2 : 1, v/v). Lipids were extracted as described above. Phosphatidylethanolamine and trinitrophenyl-phosphatidylethanolamine were separated by thin-layer chromatography and quantified as described above.

Assay of phospholipid methylation. Basolateral membranes (approx. 1 mg protein) in buffered mannitol 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 2 h. The reaction was terminated by adding 3 ml of chloroform/methanol/2 M HCl (6 : 3 : 1, v/v). The chloroform phase was washed with 1 ml of 0.5 M KCl in 50% methanol [6]. The chloroform phase was dried and dissolved in 150 μl of chloroform. An aliquot (100 μl) was applied to Silica gel G plate and developed in chloroform/propionic acid/*n*-propyl alcohol/water (2 : 2 : 3 : 1, v/v) for phospholipid separation [4,6]. The spots were visualized by exposure to iodine vapours, 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 G, in all the data expressed.

Orientation of basolateral membrane vesicles. The orientation of basolateral membrane vesicles was ascertained by determining Ouabain-sensitive ATPase activity in intact vesicles and in disrupted vesicles (0.03% deoxycholate containing 1 mM EDTA) as described [20].

Assay of (Ca^{2+} + Mg^{2+})-ATPase activity. The (Ca^{2+} + Mg^{2+})-ATPase activity of basolateral membranes (0.3 to 0.5 mg protein) was measured at 37°C in a reaction mixture consisting of 80 mM histidine, pH 7.4, 33 mM KCl, 80 mM NaCl, 3.6 mM $MgCl_2$, 0.9 mM ouabain, 2.5 mM Tris-ATP and 50 μM $CaCl_2$. Assay of (Ca^{2+} + Mg^{2+})-ATPase activity in phospholipid methylated membranes was carried out after incubation of membrane vesicles with *S*-adenosylmethionine at pH 6.0 as described above. Where indicated, reaction mixture contained 2 μg/ml of oligomycin. After incubation for 30 min the reaction was terminated by the addition of 1 ml of ice-cold 10% trichloroacetic acid and centrifuged at 5000 × *g* for 10 min. Inorganic phosphate was determined in the supernatant [21].

Assay for calcium transport. Calcium transport in basolateral membrane vesicles (400 μg protein) was measured at 25°C in a reaction mixture containing 140 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes/Tris (pH 7.4) and 50 μM $^{45}CaCl_2$ ($8 \cdot 10^6$ cpm/ml) in a total volume of 0.5 ml. Uptake was initiated by the addition of Tris-ATP (5 mM). At indicated intervals of time 80 μl of reaction mixture was removed, immediately diluted in 5 ml of ice-cold termination medium i.e. uptake buffer containing in addition 1 mM EGTA, and filtered on 0.45 μm Millipore filter. The filter paper was washed twice with 5 ml of buffer and the radioactivity in the dried filter paper was determined using Betamex scintillation solvent (West Coast Scientific, La Jolla, CA) in a Beckman LS-8000 scintillation counter. Where indicated methylation of basolateral membranes was carried out at pH 6.0 for 1 h prior to transport assay. Inhibitors used in the transport assay were preincubated with the

membranes for 10–15 min. Each experiment was run in duplicate.

Fluorescence polarization measurement. Fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene in basolateral membrane vesicles (50 μg), in a total volume of 2.5 ml mannitol buffer, was measured with an MPF-4 Perkin-Elmer spectrofluorometer as described [22].

Analytical methods. Phospholipid phosphorus was estimated by the colorimetric procedure after perchloric acid digestion [23] and cholesterol was determined by the procedure of Zlatkis et al. [24]. Protein was estimated according to the method of Lowry et al. [25].

Materials. *S*-Adenosyl-L-[*methyl*-^{3}H]methionine and $^{45}CaCl_2$ were purchased from New England Nuclear (Boston, MA); phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine were procured from Calbiochem-Behring Corp. (La Jolla, CA); cholesterol oxidase, *S*-adenosylmethionine, *S*-adenosyl-L-homocysteine, sphingomyelinase from Boehringer-Mann; phospholipase C, oligomycin, and trinitrobenzenesulfonic acid from Sigma Chemical Co. (St. Louis, MO); lanthanum chloride from K and K Laboratories, Plainview, NY; sodium vanadate from Fisher Scientific Co. All other reagents were of analytical grade.

Results

Orientation of basolateral membrane vesicle preparation

$(Na^+ + K^+)$-ATPase activity in basolateral membrane preparation before and after disruption with detergent (0.03% deoxycholate) was determined. It was observed that after disruption, there was 20–25% increase in ouabain-sensitive ATPase activity (data not shown). If one assumes that ATP binding site on $(Na^+ + K^+)$-ATPase is accessible in natural membrane from inside, then it appears that basolateral membrane preparation are mostly (75–80%) oriented inside-out. Moreover, membrane vesicles and not sheets were obtained as vesicle preparation was separated on Dextran T-70 gradient.

Orientation of lipid components in basolateral membranes

Analysis of lipid components in this basolateral membrane preparation revealed that there are three major phospholipids consisting mainly of phosphatidylcholine (37%), phosphatidylethanolamine (29%) and sphingomyelin (24%) (Table I). The other minor component was phosphatidylserine (10%). When basolateral membranes were in-

TABLE I

LIPID COMPOSITION OF HOG KIDNEY BASOLATERAL MEMBRANE

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

Lipids	μg/mg protein	% phospholipids
Cholesterol	152 ± 12	–
Total phospholipids	460 ± 15	–
Phosphatidylserine	46 ± 4	10 ± 1
Sphingomyelin	111 ± 8	24 ± 2
Phosphatidylcholine	171 ± 9	37 ± 3
Phosphatidylethanolamine	134 ± 8	29 ± 2

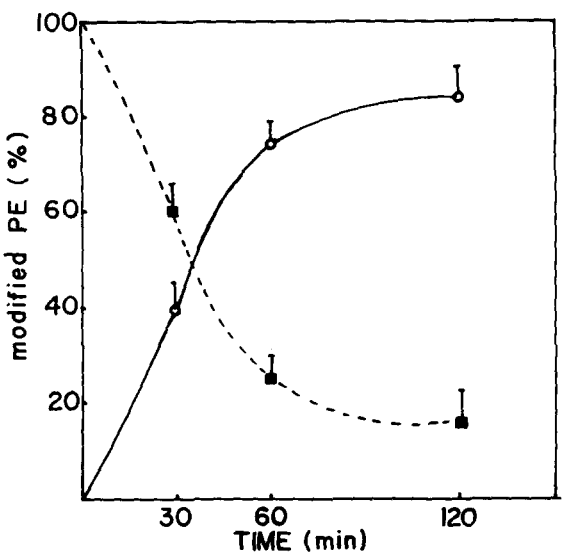


Fig. 1. Modification of phosphatidylethanolamine by trinitrobenzenesulfonic acid (TNBS). Basolateral membrane vesicles were incubated with 3 mM TNBS at 4°C. After indicated time intervals the reaction was stopped, the lipids were extracted and analyzed as described under Materials and Methods. Percentage of modified phosphatidylethanolamine (○——○) and unmodified phosphatidylethanolamine (■- - - -■) was calculated from the total amount of phosphatidylethanolamine present in the control experiments.

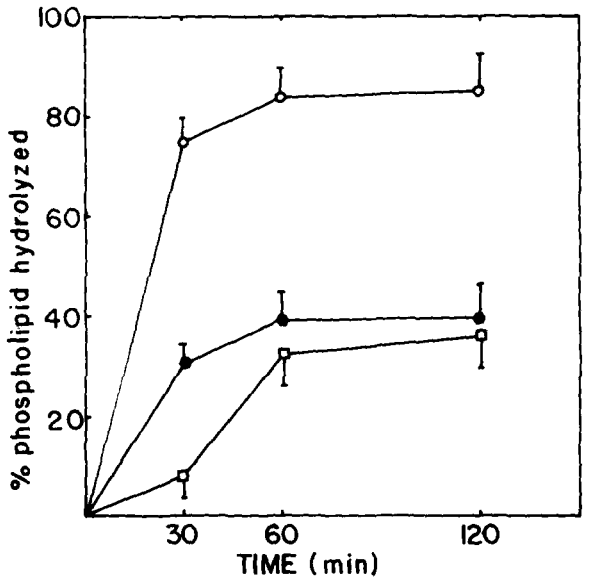


Fig. 2. Hydrolysis of basolateral membrane phospholipids by phospholipase C. Basolateral membrane vesicles (2–3 mg protein) were incubated with 20 I.U. of phospholipase C (*B. cereus*) in 300 mM mannitol buffered with 5 mM Hepes/Tris, (pH 7.4), containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$. At the indicated 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. O——O, Phosphatidylethanolamine; ●——●, phosphatidylcholine; □——□, phosphatidylserine.

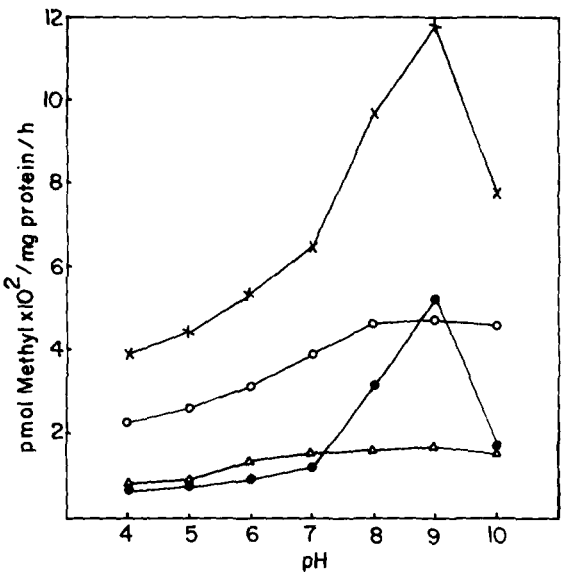


Fig. 3. Effect of pH on phospholipid methylation in basolateral membrane vesicles. Membranes (1 mg protein) were incubated at 37°C with *S*-adenosyl[*methyl*-^{3}H]methionine (100 μM) in the presence of different buffers as described in Materials and Methods. Buffers used were, 50 mM sodium acetate pH 5 and 6, 50 mM Tris acetate, pH 7, 8 and 9 and 50 mM sodium borate, pH 10. After 2 h the reaction was terminated by the addition of 3 ml of chloroform/methanol/2 M HCl (6:3:1, v/v). The reaction products were extracted and separated by thin-layer chromatography as described in Materials and Methods. Values are expressed as pmol [^{3}H]methyl group incorporated into phospholipids per mg protein. ×——×, Total phospholipids; O——O, phosphatidylmonomethylethanolamine; △——△, phosphatidyldimethylethanolamine; ●——●, phosphatidylcholine.

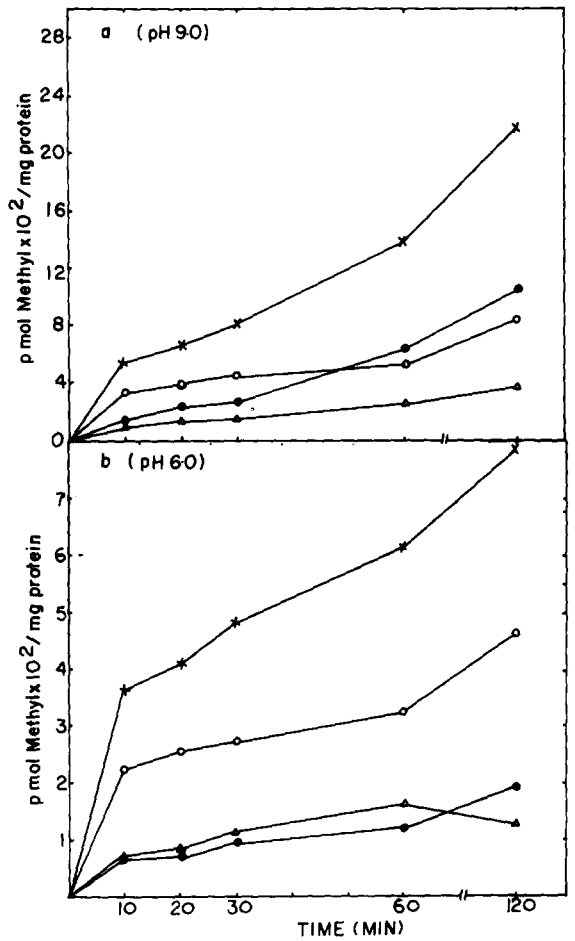
cubated at 4°C for various periods of time (30 min–2 h) with trinitrobenzenesulfonic acid (TNBS), a probe to modify amino groups of proteins and phospholipids, followed by extraction and analysis of phospholipids, the results showed that up to 80% of phosphatidylethanolamine was chemically modified (Fig. 1). The reaction was virtually complete after 2 h and a maximum of 85% of the total membrane phosphatidylethanolamine was trinitrophenylated. To ensure that there was sufficient TNBS to react with all available PE, membranes were incubated with double the concentration of TNBS. The maximum amount of PE that could be modified was 85%, while at 37°C > 95% of PE was modified (data not shown).

Phospholipase C treatment of basolateral membranes at 37°C revealed that 80–85% of total phosphatidylethanolamine, 40% of phosphatidylcholine and 35% of phosphatidylserine were hydrolyzed (Fig. 2). Treatment of basal lateral membrane vesicle preparation with sphingomyelinase at 37°C resulted in the hydrolysis of 20% of total sphingomyelin (data not shown).

As shown in Table I, most of the total cholesterol pool in basal lateral membranes was present as free cholesterol. When intact basal lateral membranes were treated with cholesterol oxidase, 40% of the free cholesterol pool in the membrane was accessible to oxidation (data not shown).

Phospholipid methylation in basal lateral membranes

Basal lateral membrane preparation was incubated with *S*-adenosyl[*methyl*-^{3}H]methionine at different pH values ranging from 5 to 10, to



examine the extent of incorporation of [^{3}H]methyl groups into phospholipids. As shown in Fig. 3, there was optimum incorporation of [^{3}H]methyl groups into phosphatidylethanolamine at pH 9.0. The K_m value of *S*-adenosylmethionine at pH 6 and pH 9.0 was similar i.e. 100 μM as has been previously observed [6] for brush border membrane phospholipid methyltransferases. The amount of [^{3}H]methyl group incorporated into phosphatidylethanolamine was not affected by Mg^{2+} (4 mM) or EGTA (1 mM) at either pH 6 or pH 9 (data not shown). As shown in Fig. 4B, at pH 6.0 there was greater incorporation of [^{3}H]methyl groups into phosphatidyl-*N*-monomethylethanolamine than into phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine. At pH 9.0, there was more incorporation of

Fig. 4. Time course of phospholipid methylation at pH 6.0 and pH 9.0. Basolateral membrane vesicles (approx. 1 mg protein) were incubated with *S*-adenosyl[*methyl*-^{3}H]methionine (100 μM) in the presence of either (a) 50 mM Tris-acetate buffer (pH 9.0) or (b) 50 mM sodium acetate (pH 6.0). At the indicated time, the reaction was terminated, phospholipids was 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.

TABLE II

EFFECT OF EXOGENOUSLY ADDED PHOSPHOLIPIDS ON [^{3}H]METHYL GROUP INCORPORATION IN VARIOUS METHYL DERIVATIVES OF PHOSPHATIDYLETHANOLAMINE

Basolateral membrane vesicles (approx. 1 mg protein) were pre-incubated for 10 min with 700 μM each of phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PME) and phosphatidyldimethylethanolamine (PDE). These phospholipids were suspended in 50 mM Tris-acetate, pH 7.4 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 1 h by the addition of *S*-adenosyl[*methyl*-^{3}H]methionine (100 μM) either at pH 6.0 or pH 9.0 and the reaction was terminated by addition of 3 ml of chloroform/methanol/2 M HCl (6:3:1, v/v). Phospholipids were extracted and separated. The results are mean ± S.D. of four experiments.

	Addition	pmol[H_3]methyl incorporated/mg protein per h			
		Total incorporation	PME	PDE	PC
pH 6.0	None	652 ± 30	337 ± 18	149 ± 9	165 ± 11
	PE (700 μM)	693 ± 31	381 ± 15	127 ± 8	184 ± 13
pH 9.0	None	1124 ± 60	445 ± 52	213 ± 31	465 ± 37
	PME (700 μM)	1868 ± 62	525 ± 35	536 ± 53	833 ± 63
	PDE (700 μM)	1599 ± 45	492 ± 34	227 ± 29	876 ± 71

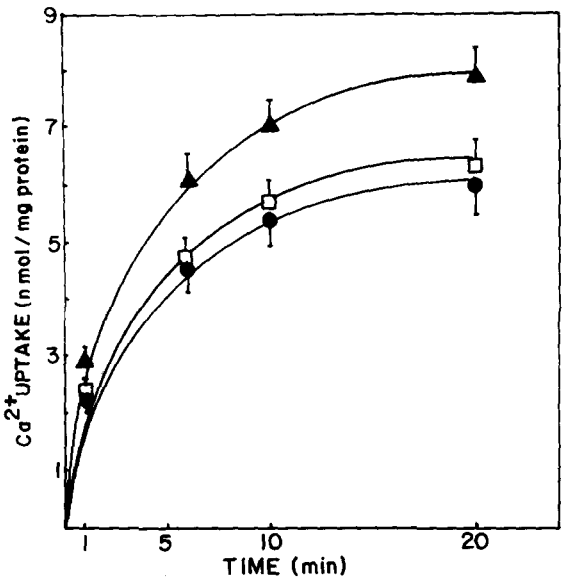


Fig. 5. Effect of phospholipid methylation on ATP-dependent Ca^{2+} uptake in basolateral membranes. Uptake of Ca^{2+} was measured in basolateral membranes in the presence of Tris-ATP (5 mM) as described in Materials and Methods. The uptake of calcium represents difference of uptake in the presence and absence of ATP. ●———●, Membranes; ▲———▲, membranes preincubated with *S*-adenosylmethionine (100 μM), pH 6.0, for 60 min; □———□, membranes preincubated with *S*-adenosylhomocysteine (500 μM) and *S*-adenosylmethionine (100 μM) for 60 min. Results are presented as the mean ± S.D. of four experiments run in duplicate.

[^{3}H]methyl groups into phosphatidylcholine and phosphatidyl-*N*-monomethylethanolamine compared to phosphatidyl-*N*,*N*-dimethylethanolamine (Fig. 4A).

Addition of exogenous phosphatidylethanolamine did not enhance the incorporation of [^{3}H]methyl groups into phospholipids. However, addition of PME (700 μM) resulted in 1.8–2.0-fold stimulation in the incorporation of [^{3}H]methyl groups into total phospholipids as well as in phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine fractions (Table II). Similarly addition of exogenous PDE (700 μM) increased (1.4- to 1.5-fold) the incorporation of [^{3}H]methyl groups into total phospholipids and phosphatidylcholine.

Effect of methylation of phospholipids on (Ca^{2+} + Mg^{2+})-ATPase activity in basolateral membrane vesicles

Since basolateral membranes are endowed with

TABLE III

INHIBITION OF ATP-DEPENDENT Ca^{2+} UPTAKE IN BASOLATERAL MEMBRANE VESICLES

The incubation medium contained 140 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes/Tris (pH 7.4), 50 μM $^{45}CaCl_2$ ($8 \cdot 10^6$ cpm/ml) and 5 mM Tris ATP. Ca^{2+} uptake was determined as described in Materials and Methods. The results are mean ± S.D. of three experiments.

Addition	Ca^{2+} uptake (nmol/mg protein/3 min)	% inhibition
None	3.5 ± 0.1	-
Vanadate (50 μM)	0.1 ± 0.02	97
Lanthanum (0.1 mM)	1.4 ± 0.09	60
Oubain (1 mM)	3.4 ± 0.12	0
Oligomycin (2 μg/ml)	3.22 ± 0.15	8

(Ca^{2+} + Mg^{2+})-ATPase activity which mediate the flux of Ca^{2+} it was of interest to determine whether phospholipid methylation affected (Ca^{2+} + Mg^{2+})-ATPase activity. (Ca^{2+} + Mg^{2+})-ATPase activity in phospholipid methylated basolateral membrane was stimulated 12–15% compared to control from 6.48 ± 0.11 to 7.38 ± 0.15 μmol P_i/mg protein per h. This stimulation in (Ca^{2+} + Mg^{2+})-ATPase activity was insensitive to

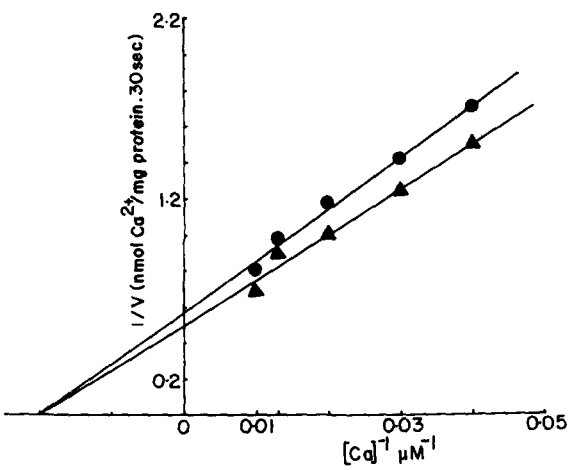


Fig. 6. Kinetics of ATP-dependent calcium uptake in phospholipid methylated basolateral membranes. The uptake of calcium was measured as described in the legend to Fig. 5. Initial rates of uptake were determined at 30 s. The uptake of calcium represents difference between calcium uptake in the presence and absence of ATP. ●———●, Control; ▲———▲, methylated.

oligomycin. Furthermore it was observed that addition of exogenous PME (700 μM) and PDE (700 μM) to basolateral membranes also resulted in stimulation of (Ca^{2+} + Mg^{2+})-ATPase by 10–15% from 6.48 ± 0.11 to 7.42 ± 0.10 and 7.5 ± 0.20 μmol P_i/mg protein per h, respectively).

Effect of phospholipid methylation on ATP-induced uptake of calcium

Since phospholipid methylation moderately stimulated the (Ca^{2+} + Mg^{2+})-ATPase hydrolysis of ATP, its effect on uptake of Ca^{2+} in basolateral membrane was studied. As shown in Fig. 5, the basolateral membrane vesicles exhibited ATP dependent uptake of calcium as has been demonstrated in basolateral membranes isolated from rat kidney cortex [26] and intestine [8]. In order to establish whether the ATP-dependent uptake of calcium was not due to mitochondrial contamination or microsomes several inhibitors were employed. ATP-dependent uptake of calcium at 3 min ([Ca] = 50 μM) was not inhibited by 1 mM ouabain

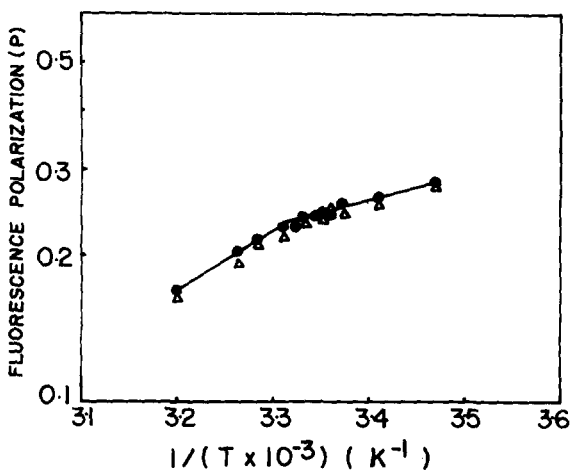


Fig. 7. Temperature dependence of fluorescence polarization of diphenylhexatriene in basolateral membranes. Basolateral membranes or phospholipid methylated membranes (50 μg protein) were incubated with 2 mM 1,6-diphenyl-1,3,5-hexatriene in 300 mM mannitol containing 10 mM Hepes-Tris (pH 7.4) buffer for 45 min. Fluorescence polarization was measured in Perkin-Elmer MPF-4 Spectrofluorometer equipped with thermostatically controlled cuvette holder. The temperature of the cuvette was regulated using a Lauda-K-2 thermostatically controlled bath. Fluorescence polarization *P* value was calculated as described [22]. ●———●, Basolateral membranes; ▲———▲, phospholipid methylated basolateral membranes.

while 0.1 mM La^{3+} decreased uptake by 60% (Table III). Oligomycin (2 μg/ml) decreased the uptake of calcium by 8–10%. At 50 μM vanadate, calcium uptake was completely inhibited (Table III). Since plasma membrane (Ca^{2+} + Mg^{2+})-ATPase appears to be vanadate sensitive while those of mitochondrial origin are not [27], therefore it appears likely that the calcium transport observed is of plasma membrane origin. Furthermore, it was observed that in phospholipid methylated membranes there was stimulation (12–15%) of ATP-dependent calcium uptake (Fig. 5). Inhibitory effects of above mentioned inhibitors was in the same range as observed in untreated membranes.

To further evaluate the role of phospholipid methylation in stimulation of ATP-dependent calcium uptake, the effect of the methyltransferase inhibitor, *S*-adenosyl-L-homocysteine was examined. As shown in Fig. 5, preincubation of basolateral membranes with *S*-adenosylhomocysteine decreased both phospholipid methylation (85–90%; data not shown) and ATP-dependent uptake of calcium.

To determine whether phospholipid methylation stimulated ATP-dependent uptake of calcium by altering the affinity for Ca^{2+}, kinetic studies were carried out. A Lineweaver-Burk plot of the uptake (Fig. 6) indicates that the stimulation in calcium uptake observed was due to a change in V_{max} but not to change in affinity (K_m) for calcium.

Fluorescence polarization of diphenylhexatriene in basolateral membranes

As shown in Fig. 7, methylation of basolateral membrane phospholipids did not significantly affect the fluorescence polarization *P* value of diphenylhexatriene in the temperature range (15–37°C). It was observed that there was phase separation at 27°C. It is pertinent to mention that phase separation has been reported to occur in mouse-human heterokaryons [28] while no phase transition in the lipid bilayer has been observed for normal and transformed 3T3 cells [29] as well as in malignant lymphoma cells [30].

Discussion

Results presented in this study show that the phospholipid composition of basolateral mem-

branes from hog kidney cortex is similar to that observed in brush border membranes [6]. Studies using phospholipase C treatment show that in basolateral membrane vesicles 80% of total phosphatidylethanolamine compared to 40% of phosphatidylcholine and 35% of phosphatidylserine were hydrolyzed. Treatment of basolateral membranes with trinitrobenzene sulfonic acid at 4°C, an amino group modifying vectorial chemical probe, resulted in the labeling of phosphatidylethanolamine by 80% while the content of phosphatidylcholine remained unchanged. Under these conditions, i.e., at 4°C the reagent presumably does not enter the membrane as that would have resulted in the complete modification of PE. However, when labeling studies were carried out at 37°C all (> 95%) of the phosphatidylethanolamine fraction was trinitrophenylated. Rothman and Kennedy [19] also observed that trinitrobenzene sulfonic acid did not cross the membrane of *Bacillus megatarium* at 3°C while at 37°C it entered the membrane and labelled all of the phosphatidylethanolamine fraction. The impermeant nature of trinitrobenzenesulfonic acid at 4°C compared to at 37°C in trinitrophenylation of amino group of phosphatidylethanolamine has been utilized to determine the turnover rate of phosphatidylethanolamine in human erythrocyte membranes [31,32] and its asymmetrical distribution in membranes of *Mycobacterium phlei* [33].

The determination of ouabain-sensitive ATPase activity in basolateral membranes before and after treatment with deoxycholate/EDTA, which transforms vesicles into sheets, yielded a 20–25% increase in $(Na^+ + K^+)$-ATPase activity indicating that majority (80%) of the basolateral membrane vesicles are oriented inside-out. Similar approach has been used by Del Castillo and Robinson [20] and Kinsella et al. [9] to ascertain the orientation of basolateral membranes from intestine and rat kidney cortex, respectively. Del Castillo and Robinson [20] showed that their membrane preparation contained about 60% inside-out vesicles in contrast to Kinsella et al. [9] who observed that majority [76.5%) of the vesicles had right-side-out orientation. It should be pointed out that although we have utilized the method of Kinsella et al. [9], with an additional step of polytron-homogenization for the preparation of basolateral membranes, our membrane preparation contain majority of inside-out vesicles. It is possible that the polytron-homogenization step, used in our studies, induced the formation of vesicles of opposite polarity.

Since majority (about 80%) of basolateral membrane vesicles are oriented inside-out and about 80% of total phosphatidylethanolamine is accessible to TNBS modification and phospholipase C hydrolysis, these results thus indicate that PE is oriented exclusively on the outer surface of inside-out vesicles. Moreover, about 20% of the sphingomyelin was cleaved by sphingomyelinase; it appears, this must have originated from sphingomyelin present in the external surface of 20% of right side-out vesicles. It would have been ideal to utilize defined orientation vesicles for determination of topology, however, attempts to separate these mixture of vesicles on concanavalin A-Sepharose have failed. We can conclude that phospholipids across the bilayer in basolateral membranes are asymmetrically distributed as has been observed in brush border membranes [6] and several other membranes [34].

Results presented in this study also show that methylation of endogenous phosphatidylethanolamine in basolateral membranes, with *S*-adenosyl[*methyl*-^{3}H]methionine, occurs leading to the formation of phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidylcholine. The extent of [^{3}H]methyl group incorporation into phospholipids was lower at pH 6.0 compared to pH 9.0. In addition methylation at pH 6.0 did not show requirement for Mg^{2+}, as has been demonstrated for phospholipid methyltransferase 1 in red blood cell membranes [2] which exhibits absolute requirement for Mg^{2+}. Since methylation at pH 6.0 results in predominant formation of phosphatidyl-*N*-monomethylamine compared to pH 9.0 wherein the major species was phosphatidylcholine, therefore it appears likely that at least two methyltransferases which catalyze successive methylation of phosphatidylethanolamine to phosphatidylcholine are present in these membranes. Similar values of K_m and optimal pH have been observed for phospholipid methyltransferases in brush border membranes [6]. Thus the pH optimum and K_m for *S*-adenosylmethionine are similar for methyltransferases located in the luminal

and antiluminal membranes of kidney cortex.

Our studies also show that basolateral membrane vesicles exhibit ATP-dependent uptake of Ca^{2+}, which was insensitive to oligomycin (2 μg/ml) indicating the absence of mitochondrial contamination. The uptake of Ca^{2+} occurs as a result of hydrolysis of ATP, mediated by (Ca^{2+} + Mg^{2+})-ATPase oriented externally in inside-out membrane vesicles. A reproducible stimulation (12–15%) of ATP-dependent calcium uptake by phospholipid methylation has been observed for these basolateral membrane preparation and represents the first such observation in an epithelial system. If one adds *S*-adenosylhomocysteine, methyltransferase inhibitor, the stimulation in Ca^{2+}-uptake was abolished. It is pertinent to mention that a 35% increase in rate of Ca^{2+}-uptake has been observed with calmodulin in basolateral membrane preparation isolated from rat small intestine [8]. The possibility that stimulation of ATP-dependent Ca^{2+} uptake by phospholipid methylation was due to mitochondrial contamination is ruled out by the observation that vanadate (50 μM) completely abolished the uptake of calcium consistent with the observations of O'Neal et al. [27] and Nelson and Blaustein [35] for inhibition of (Ca^{2+} + Mg^{2+})-ATPases by vanadate in variety of plasma membranes. O'Neal et al. [27] have shown negligible effect of vanadate on mitochondrial (Ca^{2+}-Mg^{2+})-ATPase. Ouabain (1 mM) did not affect ATP-dependent uptake of calcium indicating that plasma membrane (Na^+ + K^+)-ATPase is not involved in the uptake of calcium.

Since phospholipid methylation increased ATP-dependent uptake of Ca^{2+}, its effect on (Ca^{2+} + Mg^{2+})-ATPase was determined. Studies showed that (Ca^{2+} + Mg^{2+})-ATPase activity was stimulated by 12–15% after methylation. In red blood cell membrane phospholipid methylation [5] has been shown to stimulate (Ca^{2+} + Mg^{2+})-ATPase activity by 35–40%. Moreover, it has been shown that an increase (30%) in membrane fluidity occurs upon phospholipid methylation of red blood cell membrane [1] which presumably alters the physical properties of the membrane resulting in enhancement of (Ca^{2+} + Mg^{2+})-ATPase activity. Our studies utilizing fluorescence polarization of diphenylhexatriene do not show any significant differences in *P* value between control and methylated membranes suggesting that environment of the probe in the lipid bilayer remained unchanged before and after methylation. This may be due to formation of a small amount (0.16%) of phosphatidylmonomethylethanolamine from the total pool of phosphatidylethanolamine present in the membrane resulting in a insignificant change in membrane fluidity although in red blood cell membranes phosphatidylmonomethylethanolamine formation was 0.0012% of total PE present in the membrane which resulted in a significant (30%) change in microviscosity. In our studies, the addition of exogenous phosphatidylmonomethylethanolamine and phosphatidyldimethylethanolamine also resulted in 12–15% stimulation in (Ca^{2+} + Mg^{2+})-ATPase activity. It is pertinent to mention that in sarcoplasmic reticulum Ca^{2+}-ATPase activity has been shown to be inhibited by the addition of dipalmitoylphosphatidylcholine, which also reduces the fluidity of the membrane [36].

The stimulation of ATP-dependent calcium transport, although moderate, by phospholipid methylation, does suggest that this process in vivo could play a role in the regulation of both cytosolic free calcium concentration and transepithelial calcium flux.

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