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Isolation of purified brush-border membranes from rat jejunum containing a Ca^{2+}-independent phospholipase A_2 activity

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A novel phospholipase activity was recognized in intact, rat jejunal brush-border membranes and its effect on membrane lipid composition was evaluated following various incubation protocols. Brush-border membranes were isolated from mucosal scrapings by a combination of existing techniques. A brush-border plus nuclei fraction was first prepared by homogenization and low-speed centrifugation in isotonic mannitol, in the presence of 5 mM EDTA. Brush-border membrane vesicles were isolated from this fraction by homogenization, followed by precipitation of the remaining undesired membranes with 10 mM $CaCl_2$. Membranes were judged to be highly purified by marker enzyme content, protein profile, and electron microscopy. In total lipid extracts, prepared immediately following membrane isolation, the ethanolamine phosphatides were found to be the major phospholipid class, accounting for nearly 45% of the total lipid phosphorus. Storage of the intact membranes, at either room temperature or at −20 °C, but not at −70 °C, resulted in a gradual and progressive hydrolysis of phosphatidylethanolamine to lysophosphatidylethanolamine. Over 60% of the total ethanolamine phospholipid was converted to the lyso form during a 2 week storage period. Incubation of the intact membranes at 37 °C produced a similar effect in one hour. Only small amounts of other glycerophospholipids were degraded under these conditions. Hydrolysis was specific for the *sn*-2 position as more than 80% of the fatty acids in the lysophosphatidylethanolamine were found to be saturated. Substitution of $MgCl_2$ for $CaCl_2$ in the precipitation step did not block the hydrolysis. It was concluded that rat brush-border membranes contain a Ca^{2+}-independent phospholipase A_2 with a high substrate preference for phosphatidylethanolamine. The physiological significance of this enzyme is not known.

Introduction

Various phospholipase activities have been described in the intestinal mucosa (Refs. 1–4, and references therein), but the function, subcellular location, and positional and substrate specificities of most remain uncertain. Specifically, phospholipases in the brush-border membrane of the villus enterocyte have been examined only to a limited extent. Subbaiah and Ganguly [1] provided the first evidence that a phospholipase A_2 was associated with the rat membrane, but conclusive

Abbreviations: EDTA, ethylenediaminetetraacetate (disodium); EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; GLC, gas-liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TLC, thin-layer chromatography.

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documentation was lacking. Enzyme activity was equally distributed between a partially purified brush-border fraction and a 'microsomal' fraction, although the brush border had the higher specific activity. The enzyme was maximally active at pH 8.6 in the presence of deoxycholic acid, and was stimulated only slightly by Ca^{2+}. Phospholipase activity has also been demonstrated in the rabbit brush-border membrane. Hauser et al. [5] reported that membrane vesicles prepared from fresh or frozen tissue by the Ca^{2+}-precipitation technique [6,7] contained high levels of lyso-PC, lyso-PE, and free fatty acids. These levels were presumably the result of a Ca^{2+}-activated phospholipase because substiuttion of Mg^{2+} and EGTA for Ca^{2+} resulted in membranes with low lysophospholipid and free fatty acid levels. These claims could not be confirmed by Aubry et al. [8], who re-examined the composition of the rabbit brush border. Although membrane vesicles obtained with Mg^{2+} or Mg^{2+}-EGTA were less pure than Ca^{2+}-prepared vesicles, similar levels of lysophospholipids and free fatty acids were detected in all three preparations. They concluded that, while Ca^{2+} could potentially activate phospholipase A_2 and promote degradation of endogenous phospholipids, it did so only to a limited extent. Further, Aubry et al. [8] suggested that exposure of membranes to adverse conditions, such as freezing and thawing, could be the reason why high levels of lysophospholipids are sometimes obtained in the Ca^{2+}-prepared vesicles [5]. The present study demonstrates that an intrinsic, Ca^{2+}-independent, phospholipase A_2 activity in the rat jejunal brush-border membrane is responsible for phospholipolysis during membrane storage, incubation, and possibly preparation. A preliminary report has appeared [9].

Materials and Methods

Materials. Glucose oxidase (*Aspergillus niger*, grade 1), peroxidase (horse radish, enzyme immunoassay grade) and ouabain (g-Strophanthin) were purchased from Boehringer Mannheim Canada Ltd. (Dorval, Que.). Hepes, *p*-hydroxybenzoic acid, 4-aminoantipyrine and bovine serum albumin (fatty acid free) were from Sigma Chemical Co. (St. Louis, MO). 4-Nitrophenyl disodium orthophosphate and 4-nitrophenol (spectrophotometric grade) were from BDH Chemicals, Canada Ltd. (Toronto, Ont.). Reagents and molecular weight standards for SDS-polyacylamide gel electrophoresis were from Bio-Rad Laboratories Canada Ltd. (Mississauga, Ont.). Silica gel H (Merck 60 H) was obtained from Terochem Laboratories Ltd. (Mississauga, Ont.). All other chemicals and solvents were of reagent grade or better quality and were obtained from local suppliers.

Animals. Male Wistar rats (Charles River Canada Inc., LaSalle, Que.), weighing 250–300 g, were used. They were allowed free access to laboratory chow and water and were housed under conditions of controlled illumination. All surgical procedures began between 9 and 10 am.

Preparation of brush-border membranes. Rats were anesthetized with diethyl ether and exsanguinated via their abdominal aortae. The small intestine, between the ligament of Trietz and the ileocecal junction, was isolated and divided into thirds. The proximal two-thirds were removed and placed into ice-cold 0.9% NaCl, 2 mM Hepes (pH 7.1) (Buffer I). All manipulations from this point on were carried out at 4°C, on ice or in a cold room. The luminal contents were removed from the gut tubes by flushing, once from each end, with 20 ml of Buffer I. Each tube was tied at one end and everted over a stainless steel microspatula; then the mucosa was scraped off with 5 or 6 passes of Teflon-coated forceps as described by Hoffman and Kuksis [10]. The mucosal scrapings from 2–4 rats, collected in approx. 200 ml of 300 mM mannitol, 5 mM EDTA, 5 mM Hepes (pH 7.1) (Buffer II), were homogenized in a Waring blender, set at low speed (power setting of 50 on a Powerstat), for 35 s. The homogenate was then gently filtered through a single layer of gauze (Nu Gauze®, Johnson and Johnson Inc., Toronto) followed by a double layer of 111 μm pore size polyethylene mesh (Spectromesh PE, Spectrum Medical Industries Ltd., Los Angeles, CA), to remove mucus and fat particles. Following removal of an aliquot for enzyme assay, the homogenate was centrifuged at $1500 \times g$ for 5 min in 50 ml polyethylene centrifuge tubes. The resulting pellets were washed twice by homogenization in 80 ml of Buffer II, with 7 strokes of a motor-driven

Potter-Elvehjem homogenizer fitted with a Teflon pestle (Thomas, B size, A.H. Thomas Co. Philadelphia, PA), and centrifugation at $1500 \times g$ for 5 min. An aliquot of the combined supernatants was kept for enzyme assay, while the remainder was saved for further fractionation (mitochondria, microsomes or cytosol preparation). The final washed, soft pellets were used for brush-border membrane preparation, essentially by the Ca^{2+}-preparation technique [6,7]. Briefly, the pellets were homogenized in 100 ml of 50 mM mannitol, 2 mM Hepes (pH 7.1) (Buffer III) in a Waring blender, set at full speed (power setting of 120 on a Powerstat), for 2 min. Following the addition of 1 M $CaCl_2$, to a final concentration of 10 mM, and a further homogenization of 15 s, the resulting homogenate was stirred slowly for 20 min on ice. Extraneous material was removed by centrifugation at $2500 \times g$ for 10 min (Sorvall SS34 rotor). Membrane vesicles were pelletted from the resulting supernatant by centrifugation at $27\,000 \times g$ for 30 min (Sorvall SS34). They were then washed once in 40 ml of Buffer III, and finally suspended in 5–10 ml Buffer III. In some instances, $MgCl_2$ was used in place of $CaCl_2$ [17].

Enzyme assays. Sucrase activity was assayed using 0.06 M sucrose, in a final volume of 1 ml [11]. The reaction was terminated after 15 min by adding 2 ml of glucose assay reagent, prepared in 1 M Tris-HCl (pH 7.0) to inhibit sucrase [12]. Liberated glucose was assayed using glucose oxidase and peroxidase, in the presence of 4-aminoantipyrine and *p*-hydroxybenzoate (Worthington Diagnostics, Statzyme Glucose 500 nm, Milipore Corporation, Toronto). Alkaline [13,14] and acid [14,15] phosphatases were estimated by measuring the hydrolysis of 4-nitrophenyl phosphate. Succinate dehydrogenase [16] and ouabain-sensitive, K^+-dependent phosphatase activity [17] were assayed according to established procedures. All assays were completed on the day of membrane preparation.

Storage and incubation conditions. Freshly prepared vesicles, suspended at a concentration of 1–2 mg protein/ml in Buffer III, were stored under several experimental conditions. Membranes were quick-frozen in a dry ice-methanol bath and stored at −20 or −70 °C. Membranes incubated at 23 °C were left at room temperature and those assessed at 37 °C were shaken in a rotory water bath. In some instances 0.1 M EDTA (pH 7.1) was added, to a final concentration of 10 mM, prior to freezing or incubation. Experiments were terminated by performing total lipid extractions.

Lipid extraction. Brush-border membrane lipids were extracted by a modification of the method of Folch et al. [18]. Membranes, suspended in Buffer III, were added to at least 5 volumes of methanol in a test tube. Following mixing, chloroform was added to give a final chloroform to methanol ratio of 2 : 1 (v/v). This mixture was vortexed and partitioned into an upper and lower phase by adding 0.9% NaCl, to give a final aqueous to organic solvent ratio of 1 : 5 (v/v). After vortexing well and allowing the phases to separate, the lower phase was removed and passed through anhydrous sodium sulphate into a round bottom flask. The remaining upper phase was then re-extracted once with a volume of 'synthetic' lower phase (chloroform/methanol/0.58% NaCl, 86 : 14 : 1, by v/v) equal to that of the original lower phase. The resulting second lower phase was combined with the first and concentrated to dryness in vacuo. The lipid residue was then transferred to a 7 ml vial with chloroform/methanol (2 : 1, v/v) and stored at −20 °C for further use.

Lipid analyses. Phospholipid classes were resolved by TLC on silica gel H plates prepared with 1 mM EDTA [19]. The plates were developed in chloroform/methanol/acetic acid/water (50 : 35 : 8 : 4, v/v) (modified from Ref. 20) and the resolved lipids were located by spraying with 2′,7′-dichlorofluorescein in methanol [21], or with the acid molybdate reagent of Dittmer and Lester [22] prior to phosphorus assay. The above TLC system effectively separated lyso-PC and lyso-PE from other major phospholipids, but failed to resolve PS and PI into individual bands. In some cases the combined PS/PI band was assayed for total phosphorus content. In other instances the PS/PI mixture was eluted from the silica gel [23] and resolved into separate components by TLC on silica gel H plates prepared with 3% aqueous magnesium acetate [24]. For this purpose the plates were developed in chloroform/methanol/7% NH_4OH (65 : 35 : 6.5, v/v). Phospholipids were quantitated by phosphorus determination [25] di-

rectly on the silica gel scrapings. Fatty acid methyl esters were prepared from the individual phospholipid bands by transmethylation, using 6% H_2SO_4 in methanol [21], in the presence of the silica gel. The methyl esters were separated, identified, and quantitated by GLC [26]. Cholesterol and free fatty acids were also quantitated. A total lipid extract, corresponding to a known amount of protein, was mixed with an internal standard (tridecanoylglycerol) and resolved into neutral and polar lipids using the acidic TLC system. The neutral lipid fraction, which migrated with the solvent front, was eluted from the gel [23], silylated, and chromatographed on a non-polar GLC column, as described by Myher and Kuksis [27].

Other methods. Proteins were determined, following precipitation with deoxycholate and trichloroacetic acid, using the Peterson adaptation [28] of the Lowry method [29]. Bovine serum albumin was used as the protein standard. SDS-polyacrylamide gel electrophoresis was performed under reducing conditions on 10% polyacrylamide gels by the procedure of Laemmli [30]. Samples for electrophoresis were prepared by precipitation as described above. The precipitates were washed once with ice-cold acetone, the residues being dissolved directly in sample buffer. Samples for electron microscopy were pelletted in 400 μl polyethylene tubes by centrifugation at 15 000 $\times$ g for 15 min in an Eppendorf microcentrifuge (Brinkman Instruments Ltd., Toronto). The resulting pellet was fixed with glutaraldehyde and processed for electron microscopy using standard techniques.

Results

Membrane characterization

In the present study, initial homogenization of the mucosal scrapings was done in an isotonic mannitol buffer containing 5 mM EDTA (Buffer II). Homogenization conditions were chosen that effectively disrupted the cells but left the brush-border region intact. Low-speed (1500 $\times$ g) centrifugation of this homogenate yielded a combined supernatant fraction containing more than 74% of the total protein (Table I), and a pellet fraction containing more than 75% of the sucrase activity. When this pellet fraction was used as a starting material for brush-border membrane preparation, most of the remaining protein was aggregated in the presence of 10 mM $CaCl_2$ and pelletted by centrifugation at 2500 $\times$ g. Approximately 50% of the total sucrase activity, but less than 2% of the total protein, was recovered in the final brush-border membrane pellet.

Based on the specific activity of sucrase (a brush-border marker), the membranes were purified 28.5-fold when compared with the initial homogenate (Table II). Contamination by other subcellular organelles was very low. Succinate dehydrogenase (a mitochondrial marker), and K^+-stimulated phosphatase (a basolateral membrane marker), were significantly reduced in the final preparation. There was a small increase in the acid phosphatase content of the final membranes.

The protein profile of the purified brush-border membrane (Fig. 1, lanes 6 and 7) showed the characteristic prominent bands due to the sucrase-isomaltase complex and also a distinct band due to actin [7,31]. The sucrase-isomaltase bands, which had been barely discernible in the total homogenate (lane 1), were visible in the 1500 $\times$ g pellet (lane 3), and were the major bands of the final preparation.

The membranes were found to be vesicular in structure with a heterogenous size distribution, but otherwise homogenous in appearance, when

TABLE I

DISTRIBUTION OF SUCRASE ACTIVITY AND TOTAL PROTEIN DURING BRUSH-BORDER MEMBRANE PREPARATION

Membranes were purified and aliquots from each step were assayed for sucrase activity and proteins as described in Materials and Methods. Results are expressed as the percentage of total activity/protein in each fraction, relative to that in the original homogenate (set at 100%), and are the mean $\pm$ S.D. for five different membrane preparations.

Fraction	Relative percentage	
	sucrase activity	protein
Combined 1500 $\times$ g supernatant	13.1 $\pm$ 4.3	74.2 $\pm$ 7.0
Final 1500 $\times$ g pellet	76.5 $\pm$ 8.5	15.2 $\pm$ 2.9
Ca^{2+}-aggregated, 2500 $\times$ g pellet	19.9 $\pm$ 3.2	11.0 $\pm$ 1.7
27 000 $\times$ g supernatant	1.8 $\pm$ 0.7	0.6 $\pm$ 0.5
27 000 $\times$ g pellet, final membranes	49.6 $\pm$ 6.3	1.7 $\pm$ 0.3

TABLE II

MARKER ENZYME CONTENT OF PURIFIED BRUSH-BORDER MEMBRANES

Results are enzyme specific activities and are expressed as μmol/mg protein per min, except for succinate dehydrogenase and K^+-stimulated phosphatase, which are expressed as ΔA_{420} per mg and nmol/mg protein per min, respectively. Values are mean ± S.D. of determinations from at least four different membrane preparations.

	Homogenate (Ho)	Brush-border membrane (BBM)	Ratio BBM/Ho
Sucrase	0.068 ± 0.01	1.94 ± 0.056	28.5
Alkaline phosphatase [a]	1.23 ± 0.14	17.8 ± 1.59	14.5
Succinate dehydrogenase	0.134 ± 0.01	0.009 ± 0.007	0.1
K^+-stimulated phosphatase	5.32 ± 0.20	2.90 ± 1.67	0.5
Acid phosphatase	0.03 ± 0.003	0.06 ± 0.023	2.0

[a] In order to prevent enzyme inactivation by chelation with zinc, the homogenate value for alkaline phosphatase was obtained from mucosal scrapings prepared in the absence of EDTA.

examined by transmission electron microscopy (Fig. 2). They appeared to be right-side out, with a 'fuzzy' external surface and a fibrous interior, and had an average diameter of 100 nm. Some vesicles were long and thin in shape, similar to the intact microvilli which were originally present on the cell. There were no signs of membranes which could be attributed to other subcellular fractions.

Lipid composition

Results presented in Table III show the lipid class composition of the purified membranes. In total lipid extracts of freshly isolated membranes, cholesterol was the major neutral lipid, accounting for 74% of the total on a molar basis. Free fatty acids (11%), plant sterols (13%), and monoacylglycerols (1%) were also present. Higher acylglycerols and cholesterol esters were not detected. Glycosphingolipids were also found but were not quantitated as they were incompletely extracted by the Folch procedure [32]. Phospholipids were a major component of the brush-border membrane (265 nmol/mg protein), giving a phospholipid to cholesterol molar ratio of 1.2. The phospholipid distribution (Table IV) indicated that ethanolamine (44.3%) and choline (31.3%) phosphatides were the major components, with the lysophospholipids making significant contributions (11.2% of total phosphorus).

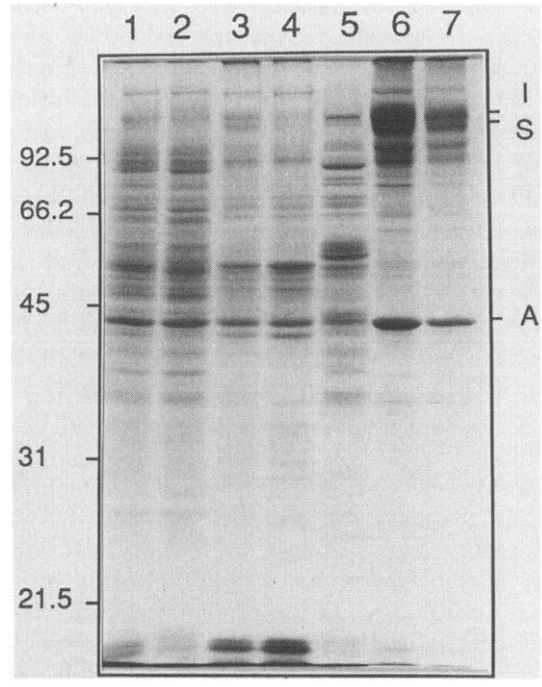

Fig. 1. SDS-polyacrylamide gel electrophoresis of the various fractions obtained during membrane preparation. Electrophoresis performed as described in Materials and Methods. Bands visualized with Coomassie blue R-250. 100 μg of protein applied to each lane, except lane 7, which received 50 μg. The mobilities of molecular weight standards ($M_r \times 10^{-3}$) are indicated down the left margin. Lane 1: total homogenate; lane 2: combined 1500 × *g* supernatant; lane 3: final 1500 × *g* pellet; lane 4: 2500 × *g* pellet; lane 5: 27000 × *g* supernatant; lanes 6 and 7: final brush-border membranes. I: isomaltase-rich band; S: sucrase-rich band; A: actin-rich band.

Phospholipolysis during storage and incubation

There was a gradual and progressive accumulation of lyso-PE upon storage at room temperature or at −20 °C (Table V). None of the other phospholipids were similarly hydrolyzed during storage. There was a small increase in the lyso-PC content following 2 weeks at −20 °C; during this period less than 15% of the total PC was hydrolyzed. Over 60% of the total ethanolamine phospholipid was present as lyso-PE following 2 weeks of storage at −20 °; there was no detectable in-

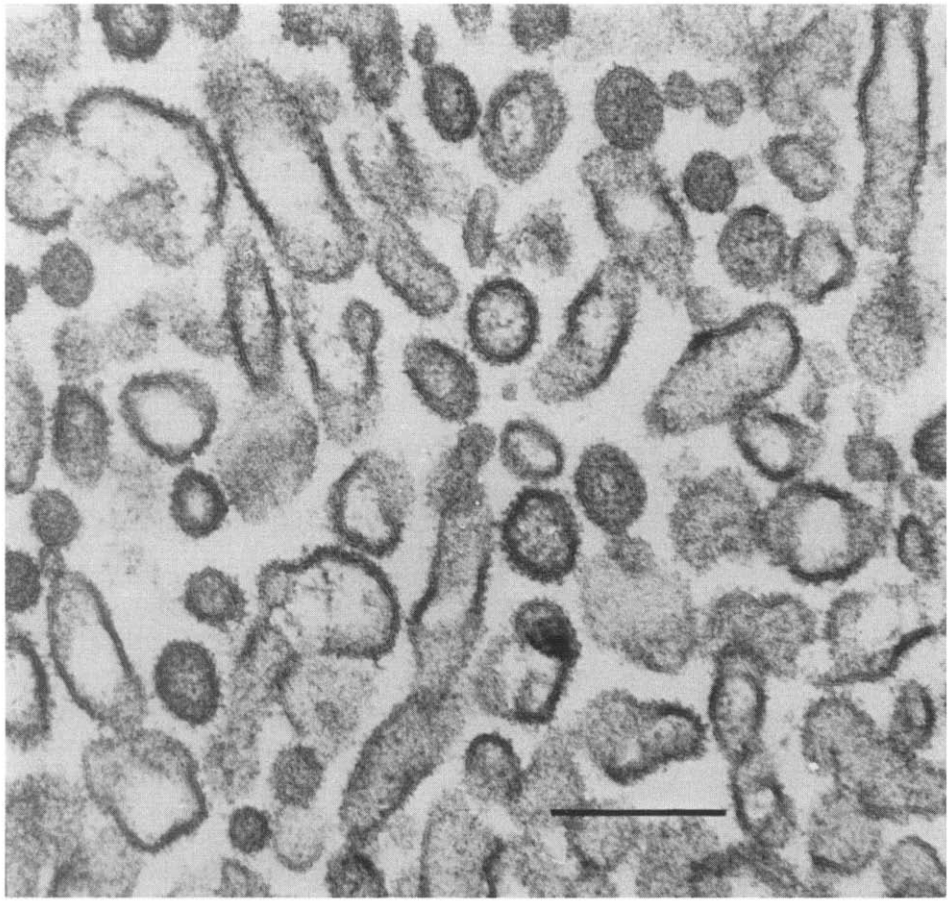

Fig. 2. Transmission electron micrograph of the final membrane preparation. Membranes were fixed as a pellet and prepared for electron microscopy by standard techniques. Magnification: ×75 240, reduced in reproduction. Bar equals 250 nm.

TABLE III

LIPID COMPOSITION OF PURIFIED BRUSH-BORDER MEMBRANES

Lipids were extracted immediately following membrane isolation and analyzed as described in Materials and Methods. Values represent the mean ± S.D. of analyses done on at least four different membrane preparations.

Lipid class	μg/mg protein
Cholesterol	84.5 ± 1.8
Total sterols [a]	97.8 ± 2.5
Monoacylglycerols	1.2 ± 0.02
Free fatty acids	11.4 ± 2.4
Lipid phosphorus	8.2 ± 1.3

[a] Cholesterol and other sterols, primarily the plant sterols campesterol and β-sitosterol.

TABLE IV

PHOSPHOLIPID DISTRIBUTION OF PURIFIED BRUSH-BORDER MEMBRANES

Total lipid extracts were prepared immediately following membrane isolation. Phospholipids were separated by TLC and quantitated by phosphorus content, as described in Materials and Methods. Results are expressed as % of total lipid phosphorus, and are the mean ± S.D. of analyses done on eight different membrane preparations.

Phopsholipid	% of total
Lysophosphatidylcholine	4.3 ± 1.8
Sphingomyelin	7.0 ± 0.8
Phosphatidylcholine	26.8 ± 4.2
Lysophosphatidylethanolamine	6.9 ± 0.9
Phosphatidylserine	11.2 ± 1.1
Phosphatidylinositol	6.4 ± 0.7
Phosphatidylethanolamine	37.4 ± 4.3

TABLE V

EFFECT OF STORAGE OR INCUBATION ON BRUSH-BORDER MEMBRANE LYSOPHOSPHATIDYL-ETHANOLAMINE CONTENT

Membranes, suspended in Buffer III, were subjected to various conditions of storage and incubation as described in Materials and Methods. Total lipid extracts were then prepared and phospholipids were separated and quantitated as described in Table IV. Results are expressed as the ratio of lyso-PE to total ethanolamine phospholipid, and are the mean ± S.D. (for n = 3–20) or the mean ± range/2 (for $n = 2$) of (n) different membrane preparations.

Treatment	Percentage lyso-PE / (PE + lyso-PE)	n
None, extracted fresh	14.1 ± 2.5	20
1.5 d at −20 °C	32.7 ± 0.5	3
5–6 d at −20 °C	45.1 ± 2.7	3
5 d at −20 °C, Mg^{2+}-prepared BBM	43.8 ± 1.0	2
2 wk at −20 °C	62.1 ± 6.7	5
2 wk at −70 °C	13.8 ± 2.3	5
1.5 d at 23 °C	34.7 ± 5.0	3
1 h at 37 °C	29.5 ± 3.9	4
1 h at 37 °C + 10 mM EDTA	12.8 ± 2.5	2
6 d at −20 °C + 10 mM EDTA	12.1 ± 0.4	2
Boil 3 min; then 6 d at −20 °C	12.9 ± 0.6	2

crease in lyso-PE following a similar storage at −70 °C. The extent of hydrolysis following 1.5 days of storage at 23 °C was not significantly different from that observed at −20 °C. In contrast, a similar level of hydrolysis was observed following only 1 h of incubation at 37 °C. Heating the membranes, for 3 min in a boiling water bath, abolished any lyso-PE increase during subsequent storage. Adding 10 mM EDTA to the storage or incubation buffer blocked phospholipase activity. Activity was also partially inhibited by storage in the presence of 5 mM $CaCl_2$ (not shown). Membranes prepared with $MgCl_2$ displayed phospholipase activity during storage, which was equal to that of Ca^{2+}-prepared membranes. These results indicated that the PE hydrolysis did not require exogenous Ca^{2+} ions, and suggested that the EDTA inhibition was due to an as yet unexplained membrane, or substrate, effect, rather than to chelation of an essential metal cofactor. In support of this, we have shown that an EDTA-resistant, Ca^{2+}-independent phospholipase A_2 can be solubilized from the brush-border membrane with bile salt detergents (Pind, S. and Kuksis, A., unpublished results). There was no evidence for the presence of a second, Ca^{2+}-dependent enzyme in the solubilized preparation. This also eliminated the possibility that the Ca^{2+}-dependent, luminal phospholipase A_2 was merely adsorbed non-specifically to the membrane during homogeniza-

TABLE VI

FATTY ACID COMPOSITION OF THE BRUSH-BORDER MEMBRANE PHOSPHOLIPIDS

Fatty acid methyl esters were prepared from TLC purified phospholipids and analyzed by GLC as described in Materials and Methods. The values for lyso-PE were obtained from membranes which had been stored at −20 °C for 5 d. All the other values were from membranes extracted immediately following isolation. The results are expressed as the average of duplicate determinations. Values less than 0.1% have been omitted. Sph, sphingomyelin; DMA, dimethylacetal.

Fatty acid	Composition (mole%)					
	Sph	PC	PS	PI	PE	lyso-PE
14:0	0.9	0.6	0.1	0.2	0.1	0.3
15:0	0.9	0.8	0.2	0.3	0.1	0.2
16:0 DMA					0.6	0.6
16:0	75.5	26.4	2.4	13.6	7.3	15.3
16:1 ($n-7$, $n-9$)	0.1	0.9	0.4	0.2	0.3	0.1
17:0	1.1	0.6	0.6	0.9	0.7	1.5
18:0 DMA					1.6	1.5
18:1($n-9$, $n-7$) DMA					0.3	0.9
18:0	5.1	16.8	42.2	26.3	31.1	62.2
18:1($n-9$,$n-7$)	1.7	9.7	16.8	7.6	10.8	9.9
18:2($n-6$)	0.7	29.0	19.1	11.0	17.8	1.9
20:0 DMA					0.9	0.9
20:0	3.3	0.5	1.2	4.0	0.5	0.9
18:3($n-3$)		0.2	0.2	0.1	0.2	
20:1($n-9$)		0.4	0.4	0.1	0.5	0.5
21:0	3.7	0.8	0.1	0.6	0.2	0.2
20:2($n-6$)		0.4	0.5	0.4	0.3	0.3
20:3($n-6$)		0.7	2.5	2.1	1.1	0.1
22:0	2.8	0.5	1.1	3.0	0.5	0.6
20:4($n-6$)		8.3	6.7	23.5	15.9	0.9
22:1($n-9$)		0.2	0.3	0.2	0.1	0.3
23:0	1.1	0.3	0.2	0.5	0.1	0.1
20:5($n-3$)		1.4	0.6	1.1	1.9	0.1
24:0	1.6	0.3	0.3	1.2	0.2	0.3
22:4($n-6$)			0.5	1.3	1.3	
24:1($n-9$)	1.5	0.3	0.3			
22:5($n-6$)			0.2		0.2	
22:5($n-3$)		0.2	0.8	0.6	1.5	0.1
22:6($n-3$)		0.6	2.2	1.2	3.9	0.3

tion. In any event, washing the final membranes in Buffer III containing 150 mM NaCl, prior to storage, had no effect on the subsequent phospholipolysis. Phospholipase activity due to bacterial growth during the storage period was excluded by storage in the presence of 0.1% sodium azide. In those samples that had a high value of lyso-PE, there was a corresponding decrease in PE and a corresponding increase in free fatty acids.

The fatty acid composition of lyso-PE differed greatly from that of PE (Table VI). Lyso-PE contained more than 80% saturated acids and virtually no polyunsaturated acids, which were present in the parent PE. This indicated that the *sn*-2 position of PE had been cleaved to yield *sn*-1-acyl-lyso-PE, a product known to contain saturated fatty acids [21]. The other membrane phospholipids each had a distinctive fatty acid profile, different from both PE and lyso-PE. Dimethylacetals of 16, 18, and 20 carbons were present in PE and lyso-PE, denoting the presence of ethanolamine plasmalogens. The dimethylacetal content in the lyso-PE was not increased to the same extent as that of the saturated fatty acids, suggesting that the plasmalogen PE was not hydrolyzed as readily as the diacyl-PE.

Discussion

Membrane isolation

In most procedures for brush-border membrane purification, contaminating membranes are precipitated from mucosal homogenates with either 10 mM $CaCl_2$ or $MgCl_2$ and discarded (Refs. 5–7; for review see Ref. 31). Mg^{2+}-prepared membranes have been reported to be both heterogenous [33] and less pure [8,34] than those prepared with Ca^{2+}. Our initial attempts at applying these techniques to rat mucosal scrapings yielded vesicles with less than acceptable purity, possibly due to the large amount of mucus present in rat intestine [35]. We eventually settled on the purification scheme described in the present report, the advantages of which are two-fold. First, membrane vesicles were obtained in high purity and yield and, second, other subcellular fractions could be obtained from the same total homogenate.

Miller and Crane [36] originally showed that in the presence of 5 mM EDTA the intact brush border is stable to osmotic and mechanical forces, and is easily isolated by low-speed centrifugation. Furthermore, following gentle homogenization in an isotonic sucrose buffer, the brush border sediments with nuclei [11,37], whereas with more vigorous homogenization conditions, more than 90% sediments with mitochondria and microsomes [37]. In the present scheme, the stability of the brush border in Buffer II provided a means of removing most of the contaminating cellular organelles before attempting the Ca^{2+}-precipitation step. This method resulted in the isolation of a white, membrane pellet with sucrase activity enriched 25–30-fold and recovered in 45–50% yield. Although purification factors ranging from 10 to 31 and recoveries of 20 to 50% have been reported [5–8,34,38–40], in most cases the combination of high purity and high yield was not achieved. The final sucrase specific activity of 1.94 was higher than that reported for most preparations of rat [34,38] or rabbit [5,7] membranes. In one other preparation [41], rat sucrase activity has been purified 100-fold with a recovery of 20% by treatment with thiocyanate ions. This treatment removes most of the extrinsic proteins, including cytoskeletal proteins, which are trapped in the interior of most vesicle preparations. Alkaline phosphatase was purified to a lower extent than sucrase in the present preparation, consistent with the belief [33,42,43] that alkaline phosphatase occurs in membranes other than the brush border. Acid phosphatase activity in the final preparation may indicate a low level of lysosomal contamination. However, this enzyme also occurs in several enterocyte membrane populations [43].

Lipid composition

The lipid composition of the present preparation agrees qualitatively with several earlier reports for both rat [5,38,44–46] and rabbit [5,8] brush-border membranes. Quantitatively similar values to the present estimates of cholesterol content have been reported [38,45,46], but no distinction was made between cholesterol and plant sterols. The phospholipid content is higher than that in many previous reports [8,34,38,44–46], but similar to the value obtained by Hauser et al. [5]. The phospholipid distribution agrees with the earlier claims [5,8,45] that PE is the major phos-

pholipid class of the brush-border membrane. This constitutes a characteristic difference between this membrane and the total mucosa [44], or the basolateral membrane [46], in which PC is the predominant phospholipid. The fatty acid composition of the individual phospholipid classes can be compared only qualitatively with earlier reports of total lipid, or total phospholipid, fatty acids [45,47]. Previously, plasmalogens were reported to be absent from the rat brush border [45], but have been found, together with alkylacylglycerophospholipids, in rabbit membranes [5]. In the present study, plasmalogens were detected in both PE and lyso-PE by the presence of dimethylacetals following acid catalyzed transmethylation. The free fatty acid content of the present membranes was slightly lower than that reported by Forstner et al. [45] and Aubry et al. [8] and much lower than that reported by Hauser et al. [5] and Yakymyshyn et al. [34]. High free fatty acid levels would have been expected if extensive phospholipid breakdown had occurred during membrane preparation [5].

Evidence for an intrinsic phospholipase A_2

The present report demonstrates that highly purified brush-border membrane vesicles contain a novel phospholipase A_2, which reproducibly causes preferential PE hydrolysis during membrane storage, incubation, and possibly during preparation. How this membrane-bound enzyme functions, in vivo, and its mode of regulation are not known. Our results indicated that the enzyme was Ca^{2+}-independent. Although most phospholipases A_2 studied to date require Ca^{2+} as a specific cofactor [48], the presence of Ca^{2+}-independent enzymes has been recently established in both heart [49] and lung [50]. The possibility remains that small amounts of tightly bound, endogenous Ca^{2+} ions were not removed by Buffer II and were sufficient to stimulate activity in the Mg^{2+}-prepared vesicles (Table V). It is also possible that Mg^{2+} stimulates the enzyme as well as Ca^{2+} does. Both of these possibilities seem unlikely in view of our demonstration of a Ca^{2+}- and Mg^{2+}-independent phospholipase A_2 following membrane solubilization. These results, as well as further characterization of the enzyme, will be presented elsewhere.

The hydrolysis of phospholipid during membrane storage at $-20\,^{\circ}C$, is in agreement with previous reports on low temperature degradation of phospholipids in other cell and tissue types [21,51,52]. Such extensive hydrolysis at $-20\,^{\circ}C$ suggests that the enzyme is in close contact with its substrate in its natural environment. Catalysis may be occurring only on substrate present in the same membrane vesicle as the enzyme, as was shown for mitochondrial phospholipase A_2 [53]. Greater enzyme activity at $37\,^{\circ}C$ than at lower temperatures indicates that the membrane phospholipids were more readily hydrolyzed above their transition temperature, which is approx. 25–26 °C [54,55].

The preferential hydrolysis of PE by the membrane associated enzyme is more difficult to rationalize. This specificity may reflect a relative abundance of PE in proximity to the enzyme, but it appears not to be due to sidedness of the substrate in the membrane, as both PC and PE exist largely in the inner bilayer [56]. Barsukov et al. [56] have recently demonstrated that prolonged incubation of rabbit brush-border membrane vesicles also leads to a preferential hydrolysis of PE to lyso-PE. Although it has been pointed out that intracellular phospholipases are often much more active with PE than with PC as substrate [48], the basis of this discrimination is not known. Exogenous PE is more readily hydrolyzed by mitochondrial phospholipase A_2 because PE can adopt non-bilayer lipid phases, and associate with the membrane prior to hydrolysis, whereas PC cannot [57]. The importance of non-bilayer lipid phases for brush-border membrane PE hydrolysis is not clear.

Following the submission of this work Diagne et al. [58] reported that the guinea pig intestinal brush-border membrane contains a Ca^{2+}-independent phospholipase A_2 activity towards bile salt dispersed PC. The relationship between this enzyme and that described in the present report remains to be established.

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References

1 Subbaiah, P.V. and Ganguly, J. (1970) Biochem. J. 118, 233–239
2 Takagi, R. and Sasaki, T. (1979) J. Biochem. 85, 29–39
3 Mansbach, C.M.II, Pieroni, G. and Verger, R. (1982) J. Clin. Invest. 69, 368–376
4 Senegas-Balas, F., Balas, D., Verger, R., De Caro, A., Figarella, C., Ferrato, F. Lechene, P., Bertrand, C. and Ribet, A. (1984) Histochemistry 81, 581–584
5 Hauser, H., Howell, K., Dawson, R.M.C. and Bowyer, D.E. (1980) Biochim. Biophys. Acta 602, 567–577
6 Schmitz, J., Preiser, H., Maestracci, D., Gosh, B.K., Cerda, J.J. and Crane, R.K. (1973) Biochim. Biophys. Acta 323, 98–112
7 Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M. and Semenza, G. (1978) Biochim. Biophys. Acta 506, 136–154
8 Aubry, H., Merrill, A.R. and Proulx, P. (1986) Biochim. Biophys. Acta 856, 610–614
9 Pind, S. and Kuksis, A. (1985) Programme Proc. Annu. Meet. Can. Fed. Biol. Soc. 28, Abstr. PA-175
10 Hoffman, A.G.D. and Kuksis, A. (1979) Can J. Physiol. Pharmacol. 57, 832–842
11 Hubscher, G., West, G.R. and Brindley, D.N. (1965) Biochem. J. 97, 629–642
12 Dahlqvist, A. (1968) Anal. Biochem. 22, 99–107
13 Weiser, M.M. (1973) J. Biol. Chem. 248, 2536–2541
14 Hubscher, G. and West, G.R. (1965) Nature 205, 799–800
15 Murer, H., Ammann, E., Biber, J. and Hopfer, U. (1976) Biochim. Biophys. Acta 433, 509–519
16 Pennington, R.J. (1961) Biochem. J. 80, 649–654
17 Colas, B. and Maroux, S. (1980) Biochim. Biophys. Acta 600, 406–420
18 Folch, J., Lees, M. and Sloane Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509
19 Allan, D. and Cockcroft, S. (1982) J. Lipid Res. 23, 1373–1374
20 Skipski, V.P., Peterson, R.F. and Barclay, M. (1964) Biochem. J. 90, 374–378
21 Christie, W.W. (1982) Lipid Analysis, 2nd Edn., Pergamon Press, Oxford
22 Dittmer, J.C. and Lester, R.L. (1964) J. Lipid Res. 5, 126–127
23 Arvidson, G.A.E. (1968) Eur. J. Biochem. 4, 478–486
24 Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496
25 Nelson, G.J. (1972) in Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism (Nelson, G.J., ed.), pp. 25–73, Wiley-Interscience, New York
26 Myher, J.J. and Kuksis, A. (1982) Can. J. Biochem. 60, 638–650
27 Myher, J.J. and Kuksis, A. (1984) J. Biochem. Biophys. Methods 10, 13–23
28 Peterson, G.L. (1977) Anal. Biochem. 83, 346–356
29 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
30 Laemmli, U.K. (1970) Nature 227, 680–685
31 Semenza, G., Kessler, M., Hosang, M., Weber, J. and Schmidt, U. (1984) Biochim. Biophys. Acta 779, 343–379
32 Hakomori, S. and Siddiqui, B. (1974) Methods Enzymol. 32, 345–367
33 Stieger, B. and Murer, H. (1983) Eur. J. Biochem. 135, 95–101
34 Yakymyshyn, L., Walker, K. and Thomson, A.B.R. (1982) Biochim. Biophys. Acta 690, 269–281
35 Hulsmann, W.L., Van den Berg, J.W.O. and De Jonge, H.R. (1974) Methods Enzymol. 32, 665–673
36 Miller, D. and Crane, R.K. (1961) Biochim. Biophys. Acta 52, 293–298
37 Carnie, J.A. and Porteous, J.W. (1962) Biochem. J. 85, 620–629
38 Forstner, G.G., Sabesin, S.M. and Isselbacher, K.J. (1968) Biochem. J. 106, 381–390
39 Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25–32
40 Selhub, J. and Rosenberg, I.H. (1981) J. Biol. Chem. 256, 4489–4493
41 Hopfer, U., Crowe, T.D. and Tandler, B. (1983) Anal. Biochem. 131, 447–452
42 Mircheff, A.K. and Wright, E.M. (1976) J. Membrane Biol. 28, 309–333
43 Mircheff, A.K., Ahnen, D.J., Islam, A., Santiago, N.A. and Gray, G.M. (1985) J. Membrane Biol. 83, 95–107
44 Child, P. and Kuksis, A. (1983) J. Lipid Res. 24, 552–565
45 Forstner, G.G., Tanaka, K. and Isselbacher, K.J. (1968) Biochem. J. 109, 51–59
46 Chapelle, S. and Gilles-Baillien, M. (1983) Biochim. Biophys. Acta 753, 269–271
47 Brasitus, T.A., Yeh, K.-Y., Holt, P.R. and Schachter, D. (1984) Biochim. Biophys. Acta 778, 341–348
48 Van den Bosch, H. (1982) in Phospholipids (Hawthorne, J.N. and Ansell, G.B., eds.), pp. 313–357, Elsevier Biomedical Press, Amsterdam
49 Wolf, R.A. and Gross, R.W. (1985) J. Biol. Chem. 260, 7295–7303
50 Nijssen, J.G., Roosenboom, C.F.P. and Van den Bosch, H. (1986) Biochim. Biophys. Acta 876, 611–618
51 Mock, T., Pelletier, M.P.J., Man, R.Y.K. and Choy, P.C. (1984) Anal. Biochem. 137, 277–281
52 Shaikh, N.A. (1986) in The Heart and Cardiovascular System (Fozzard, H.A., Haber, E., Jennings, R.B., Katz, A.M. and Morgan, H.E., eds.), Vol. 1, pp. 289–302, Raven Press, New York
53 De Winter, J.M., Lenting, H.B.M., Neys, F.W. and Van den Bosch, H. (1987) Biochim. Biophys. Acta 917, 169–177
54 Schachter, D. and Shinitzky, M. (1977) J. Clin. Invest. 59, 536–548
55 Mutsch, B., Gains, N., and Hauser, H. (1983) Biochemistry 22, 6326–6333
56 Barsukov, L.I., Bergelson, L.D., Spiess, M., Hauser, H. and Semenza, G. (1986) Biochim. Biophys. Acta 862, 87–99
57 Lenting, H.B.M., Neys, F.W. and Van den Bosch, H. (1987) Biochim. Biophys. Acta 917, 178–185
58 Diagne, A., Mitjavila, S., Fauvel, J., Chap, H. and Douste-Blazy, L. (1987) Lipids 22, 33–40