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Solubilization and assay of phospholipase A_2 activity from rat jejunal brush-border membranes

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The phospholipase activity of rat jejunal brush-border membranes was examined in the presence of several solubilizing agents, by measuring the hydrolysis of endogenous membrane phospholipids, as well as the hydrolysis of exogenous, radiolabelled substrates. Enzyme activity was highly stimulated by dispersion in 1% solutions of bile salts, or in a synthetic, bile-salt derivative, 3-[(3-cholamidopropyl)dimethylammonio]propanesulphonate (CHAPS). Under these conditions the endogenous membrane phospholipids were largely degraded to free fatty acids and water-soluble phosphate. In the presence of 1% CHAPS, hydrolysis of exogenous phosphatidylcholine was shown to be due to an initial phospholipase A_2-type attack followed by a subsequent lysophospholipase-type attack. These activities co-purified with the brush-border membrane. Maximal phospholipase A_2 hydrolysis occurred at an alkaline pH of 8–11, with bile-salt detergents present at greater than their critical micellar concentrations. Hydrolysis was completely divalent-ion independent. Phospholipase A_2 activity was not stimulated by 50% diethyl ether or ethanol, or in the presence of 1% solutions of Triton X-100, Zwittergent 3–12, sodium dodecyl sulphate, or *n*-octylglucoside. Stimulation of phospholipase activity by detergents was not related to their effectiveness at solubilizing the membrane proteins. When assayed individually phosphatidylcholine and lysophosphatidylcholine were each hydrolyzed (at the *sn*-2 and *sn*-1 positions, respectively) at a rate of approximately 125 nmol/mg protein per min. When assayed together, the two substrates appeared to compete for the same active site over a wide range of concentrations. It was concluded that the brush-border membrane contains an integral membrane protein with phospholipase A_2 and lysophospholipase activities, which is specifically stimulated by bile salts and bile salt-like detergents.

Introduction

Although the small intestinal mucosa has long been known to contain both phospholipase A_2 and lysophospholipase activities [1–6], a detailed characterization of these enzymes has yet to be achieved. The possibility that phospholipase A_2 activity is present in brush-border membranes was

Abbreviations: BCA, bicinchoninic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-propanesulphonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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first suggested by the work of Subbaiah and Ganguly [1] with partially purified preparations from rat intestinal mucosa. An apparently Ca^{2+}-dependent phospholipase activity in brush-border membranes of rabbit small intestine was later implicated by Hauser et al. [7], but this observation has been questioned by Aubry et al. [8]. In addition, guinea pig brush-border membranes have recently been shown, by Diagne et al. [6], to exhibit a Ca^{2+}-independent phospholipase A_2 activity towards bile-salt dispersed phosphatidylcholine (PC). We have previously shown [9–11] that purified brush-border membranes from rat jejunum contain an intrinsic, Ca^{2+}-independent phospholipase A_2 with a substrate preference for phosphatidylethanolamine (PE). In the absence of added lipids or detergents, the enzyme hydrolyzes endogenous PE during both storage at $-20\,^{\circ}C$ and incubation at $37\,^{\circ}C$. The relationship between this enzyme activity [9–11] and those reported by others [1,6,7] is not known, although they may represent the same enzyme.

In order to further characterize the rat brush-border phospholipase(s), we investigated the effect of different detergents on enzyme activity, and on solubilization of the total membrane proteins. It is shown that solubilization by bile salts resulted in a marked stimulation and loss of specificity of phospholipase A_2 activity, as well as a subsequent lysophospholipase attack. A membrane-bound phospholipase was responsible for both of these activities. A preliminary report has appeared [10].

Materials and Methods

Materials

Triton X-100 (purified for membrane research), *n*-octylglucoside, Hepes, and CHAPS were purchased from Boehringer Mannheim Canada Ltd. (Dorval, Quebec). Cholic, deoxycholic, and taurocholic acids (sodium salts, greater than 96% purity), and Zwittergent™ 3-12 were from Calbiochem-Behring Corp. (San Diego, CA). Fluorescamine (Fluram®) and BCA protein assay reagent were obtained from Pierce Chemical Co. (Rockford, IL), Egg yolk phosphatidylcholine (type III-E, approx. 99%), heptadecanoic acid, snake venom (*Crotalus atrox*), and Bicine were from Sigma Chemical Co. (St. Louis, MO). 1-Palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerol-3-phosphorylcholine (57 mCi/mmol) and 1,2-di-[1-^{14}C]palmitoyl-*sn*-glycerol-3-phosphorylcholine (117 mCi/mmol) were purchased from Amersham Canada Ltd. (Oakville, Ontario). Aquasol was purchased from New England Nuclear/Dupont Canada Inc. (Lachine, Quebec). All other chemicals and solvents were of reagent grade or better quality and were obtained from local suppliers [7].

Preparation of radiolabelled lysoPC

1,2-Di-[1-^{14}C]palmitoyl-*sn*-glycerol-3-phosphorylcholine was diluted with egg yolk PC and digested to yield 1-acyl-*sn*-glycerol-3-phosphorylcholine. For this purpose the mixture was incubated with snake venom phospholipase A_2 using a 10-fold scaled-down version of the assay described by Smith and Kuksis [12]. The incubation was carried out for 4 h in 17.5 mM Tris-HCl (pH 7.4)/1 mM $CaCl_2$. The resulting lysoPC, radiolabelled in the *sn*-1 position, was separated from free fatty acids by TLC and recovered from the silica gel using procedures described earlier [11]. Unlabelled 1-acyl-lysoPC was prepared in an analogous fashion by omitting the labelled PC.

Brush-border membrane preparation

Membrane vesicles were purified from rat jejunal scrapings as previously described [11]. The final preparations were typically 25–30-fold enriched in sucrase-specific activity (a brush-border marker) when compared with the crude mucosal scrapings. Membranes were suspended, at a concentration of 1–2 mg protein/ml, in 50 mM mannitol/2 mM Hepes (pH 7.1), and stored on ice prior to assay. Proteins were estimated as previously described [11] or by using the BCA assay [13].

Mass assay of phospholipase activity

Brush-border phospholipase activity was assayed on a substrate mass basis, by measuring the hydrolysis of endogenous membrane phospholipids in the presence of various detergents or organic solvents. For this purpose the detergents were dissolved in the 50 mM mannitol buffer (pH 7.1) to a final concentration of 2% (w/v). A 1 ml aliquot of the membrane vesicles was mixed with an equal volume of detergent solution, or organic

solvent, and incubated at 37 °C for 60 min. Incubations were stopped by adding 10 ml chloroform/methanol (2 : 1, v/v), vortexing, and preparing Folch total lipid extracts as previously described [11]. Extracts were assayed for phosphorus, either prior to, or following, fractionation into phospholipid classes by TLC [11]. Extracts were also assayed for ethanolamine phosphatide content. For this purpose the total lipids were reacted with fluorescamine as described by Schmid et al. [14]. Fluorescamine-PE and -lysoPE adducts were resolved from each other and from unreacted fluorescamine by TLC on silica gel H plates prepared with 3% magnesium acetate. Plates were developed in chloroform/methanol/28% NH_4OH/water (60 : 40 : 5 : 2.5, by vol.) and were visualised by exposure to ultraviolet light. Bands corresponding to fluorescamine-PE and -lysoPE were eluted from the gel and quantitated by fluorescence intensity [14]. Measurements were done on an Aminco-Bowman spectrophotofluorometer (American Instrument Co. Inc., Silver Spring, MD) with excitation at 395 nm and emission measured at 468 nm. Standard curves prepared with pure PE demonstrated that fluorescence intensity was linear between 0.5 and 50 nmol PE.

Radioactive substrate assays of phospholipase activities

Brush-border phospholipase A_2 activity was determined by measuring the release of [1-^{14}C]oleate from exogenous 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerol-3-phosphorylcholine. Except where otherwise noted, the complete incubation mixture (total volume 1 ml) contained: 45 μmol Hepes (pH 8.0); 10 mg CHAPS (1% final concentration); 340 nmol egg yolk PC containing 50 000–80 000 cpm labelled PC; and 0.1 ml membrane suspension. The incubations were performed for 15 min at 37 °C on a Dubnoff shaker. Brush-border lysophospholipase activity was determined by measuring the release of [1-^{14}C]palmitate from the 1-acyl-*sn*-glycerol-3-phosphorylcholine prepared above. Assays were conducted both in the absence [15] and presence of detergent. The complete incubation mixture (total volume 1 ml) contained: 45 μmol Hepes (pH 8.0); 300 nmol 1-acyl-*sn*-glycerol-3-phosphorylcholine (containing approximately 40 000 cpm); 0.1 ml membrane suspension; and either 10 mg CHAPS (1% final concentration) or no detergent. Incubations were carried out for 15 min at 37 °C. All assay reactions were stopped by adding 5 ml isopropanol/heptane/1 N H_2SO_4 (40 : 10 : 1, by vol.) and vortexing well. Free fatty acids were extracted into heptane by performing a modified Dole extraction, as described by Van den Bosch and Aarsman [16]. The heptane phase was passed through a Pasteur pipet containing silicic acid and collected directly into a scintillation vial. The silicic acid was washed with 1 ml diethyl ether and the combined eluates were mixed with 10 ml Aquasol and quantitated by liquid scintillation counting. Unless otherwise noted the amount of added membrane protein was adjusted so that a maximum of 20% of the radioactive substrate was hydrolyzed during the assay period. All assays were done in duplicate. In some instances PE replaced PC as substrate for the phospholipase A_2 assay. For this purpose the radiolabelled PC was converted into PE, by incubation in the presence of ethanolamine and phospholipase D, as described by Eibl and Kovatchev [17]. In other instances the free fatty acids formed during the assays were analyzed and quantitated by GLC. A known amount of heptadecanoic acid was added to each tube during the Dole extraction. The heptane phase and diethyl ether wash were collected into a 7 ml vial and the solvents were removed under N_2 at 40 °C. The dried free fatty acids were transmethylated and analyzed by GLC [11] and quantitated by comparison to the added internal standard. Phospholipid substrates were quantitated by phosphorus assay [11] and were stored in organic solvents at −20 °C. Prior to use aliquots of the substrates were dried under N_2 in the tubes used for incubation and were then resuspended in the appropriate assay buffer.

Solubilization studies

Several agents were tested for their ability to solubilize brush-border membrane protein and phospholipase A_2 activity. Detergents (2%, w/v), KCl (2 M), and EDTA (0.02 M) were dissolved in 50 mM Hepes (pH 8.0). A 0.5 ml aliquot of the membrane vesicles (containing 0.6–0.9 mg protein) was mixed with an equal volume of detergent, or salt, solution and vortexed well. Follow-

ing incubation for 15 min at 4°C, the mixtures were centrifuged at 15000 × *g* for 30 min in an Eppendorf microcentrifuge (Brinkman Instruments Ltd., Toronto). Aliquots of the resulting supernatants were assayed for phospholipase activity, using the radioactive substrate assay, and for protein, using the BCA assay.

Results

Hydrolysis of endogenous substrates

The ability of various solubilizing reagents to stimulate the enzymatic hydrolysis of brush-border membrane phospholipids was investigated. Initially, the hydrolysis of endogenous PE to lysoPE was recognized on a mass basis (Table I). Total lipid extracts from freshly isolated membranes contain 14–15% of the total ethanolamine phospholipid in the lyso form (Ref. 11, and Table I). The table shows that incubation in membrane vesicles for 60 min at 37°C nearly doubled the lysoPE content, but decreased the recovery of total ethanolamine phosphatide by 16.5%. Assuming linear reaction kinetics, this extent of PE hydrolysis would represent a rate of less than 1 nmol/mg protein per min. Incubation in 50% diethyl ether or ethanol, or in the presence of 1% Triton X-100, Zwittergent 3-12, or *n*-octylglucoside gave results which were similar to those obtained for the incubation control. In contrast, incubation in 1% deoxycholate or 1% CHAPS increased the proportion of lysoPE to approximately 60% of the total, but also significantly decreased the recovery of total ethanolamine phosphatide. Under identical assay conditions, the effect of four of the detergents on the recovery of total phospholipid phosphorus paralleled that observed for the PE (Table II). Incubation with deoxycholate and CHAPS decreased the phosphorus recovery in the Folch organic phase by 56% and 75%, respectively, while *n*-octylglucoside or Triton X-100 reduced the recovery by less than 10%. For both CHAPS and deoxycholate loss of phosphorus in the organic phase was compensated for by a quantitative increase in phosphorus present in the water phase of the biphasic Folch extraction (data not shown). Analysis of the total lipid extracts following a 60 min incubation in deoxycholate or CHAPS showed that there was a proportional decrease in the ethanolamine, choline, serine, and inositol phosphatides; the major lipid classes remaining were lysoPE, lysoPC and sphingomyelin, with a concomitant increase in the

TABLE I

HYDROLYSIS OF ENDOGENOUS BRUSH-BORDER MEMBRANE PHOSPHATIDYLETHANOLAMINE IN THE PRESENCE OF DETERGENTS OR ORGANIC SOLVENTS

Membranes, suspended in 50 mM mannitol buffer (pH 7.1), were mixed with detergent solution or organic solvent and incubated as described for the mass assay of phospholipase activity. The relative amounts of PE and lysoPE were determined by phosphorus assay following TLC separation, and also by ethanolamine assay. Results represent the % of total ethanolamine phospholipid present as lysoPE, as well as the recovery of total PE plus lysoPE following assay. Results are the averages of two to four determinations.

Treatment	60 min at 37°C	lysoPE (%)	(PE + lysoPE)
None, extracted fresh	–	14.6	100%
None	+	27.2	83.5
50% diethyl ether	+	22.3	80.9
50% ethanol	+	28.0	82.0
1% Triton x-100	+	18.4	84.3
1% Zwittergent 3-12	+	28.6	82.4
1% *n*-octylglucoside	+	31.1	89.9
1% deoxycholate	+	60.4	31.4
1% CHAPS	+	59.5	26.7

TABLE II

RECOVERY OF BRUSH-BORDER MEMBRANE TOTAL LIPID PHOSPHORUS FOLLOWING INCUBATION IN DETERGENTS

Membranes were incubated as described in Table I and Materials and Methods. Following incubation total lipid extracts were prepared and assayed for phosphorus. Results are expressed relative to the amount of lipid phosphorus present before assay, and represent the mean ± S.D. (for $n = 5$) or mean ± range/2 (for $n = 2$) for determinations from (n) different membrane preparations.

Treatment	60 min at 37°C	Total lipid phosphorus	(n)
None, extracted fresh	–	100%	
1% Triton X-100	+	91.9 ± 1.4	2
1% *n*-octylglucoside	+	92.3 ± 3.5	2
1% deoxycholate	+	43.9 ± 10.2	5
1% CHAPS	+	25.2 ± 5.6	5

content of both saturated and unsaturated free fatty acids. Reducing the time of incubation in deoxycholate or CHAPS to 5 min did not result in higher phospholipid recoveries. Even after this short incubation period, little or no diacyl phospholipid could be recovered. These results suggested that in the presence of the detergents one or more phospholipases were stimulated, resulting in the hydrolysis of both fatty acid positions of the phospholipid molecules.

Hydrolysis of exogenous substrates

In order to examine the phospholipase activation further, specific radioactive substrate assays were employed. Our preliminary experiments, based on GLC analyses of released fatty acids, demonstrated that the brush-border membranes possessed both phospholipase A_2 and lysophospholipase activities, the properties of which are described below.

Fig. 1 shows the dependence of the phospholipase A_2 activity on the concentration of exogenous substrate and total protein in the incubation medium, as well as on the time and pH of the reaction. The yield of free fatty acids was dependent upon the amount of PC added, but tended to level off at about 200 nmol phospholipid (panel A). Panel B shows that under conditions of maxi-

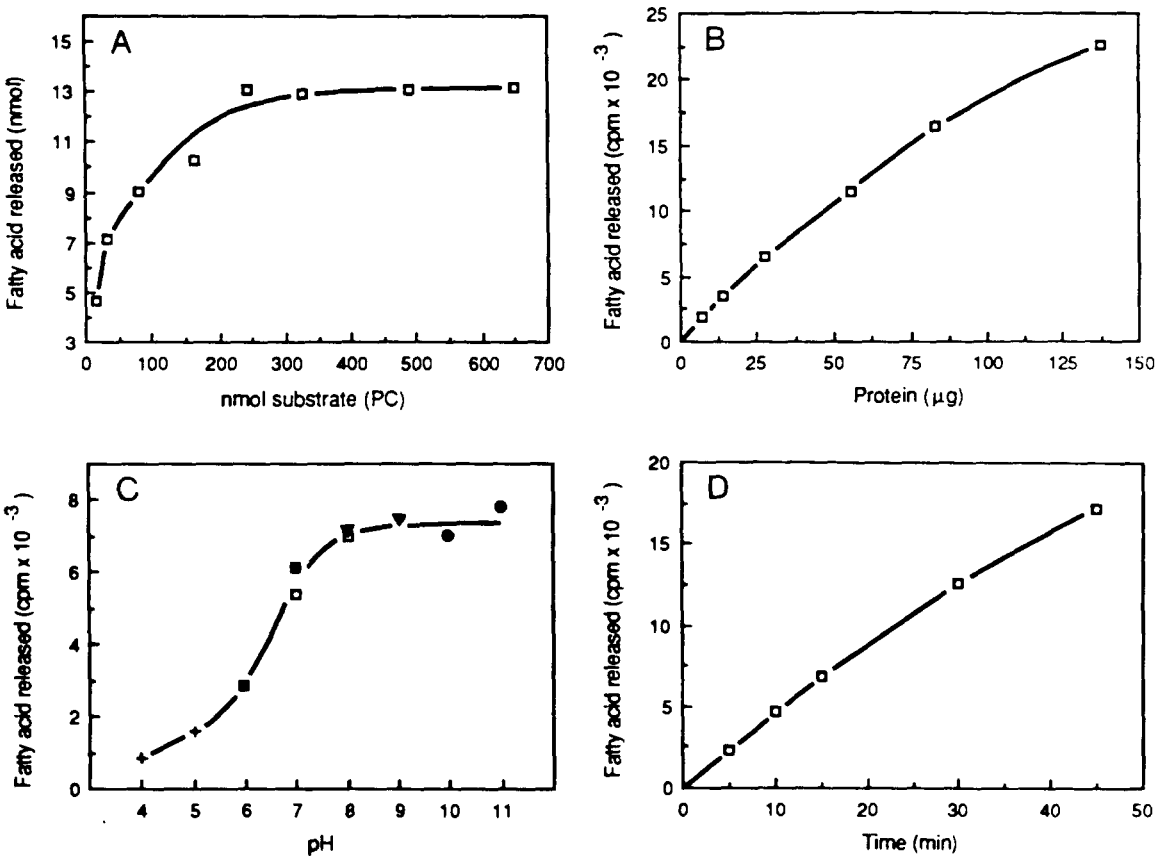

Fig. 1. Optimization of reaction conditions for phospholipase A_2 activity towards 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerol-3-phosphorylcholine. A, dependence on substrate concentration; B, dependence on protein; C, dependence on pH; D, dependence on time. Assays performed in the presence of 1% CHAPS as described in Materials and Methods, except where noted: all incubations, except A, had 340 nmol substrate; all, except B, had 40 μg membrane protein added; all, except C, were done at pH 8.0; all, except D, were done for 15 min. The pH values in C were set at room temperature in 50 mM concentrations of the following buffers: sodium acetate (+), imidazole (■), Hepes (□), Bicine (▼) and sodium carbonate (●). All values were corrected for the amount of background hydrolysis that occurred at each pH.

TABLE III

HYDROLYSIS OF EXOGENOUS, *sn*-2-[1-^{14}C]OLEATE-LABELLED PHOSPHATIDYLCHOLINE BY BRUSH-BORDER MEMBRANES

Membranes were incubated with labelled PC as described for the radioactive substrate assay of phospholipase A_2 activity, except that detergents or solvents were substituted for CHAPS as indicated. Results represent averages of a minimum of two determinations. Hydrolysis is expressed relative to that obtained in 1% CHAPS (set at 100%).

Treatment	Relative hydrolysis (%)
No detergent	0.9
1% CHAPS, 0 °C	1.1
50% diethyl ether	1.1
50% ethanol	2.4
1% SDS	0.0
1% Triton X-100	4.5
1% Zwittergent 3-12	1.6
1% *n*-octylglucoside	8.3
1% deoxycholate	66.8
1% CHAPS	100.0
1% taurocholate	149.6
1% cholate	120.4
1% CHAPS/5 mM EDTA	102.4
1% CHAPS/5 mM EGTA	100.7
1% CHAPS/5 mM $CaCl_2$	100.6
1% CHAPS/5 mM $MgCl_2$	102.3

mal substrate concentrations (340 μM) there was a linear increase in the yield of free fatty acids for up to about 50 μg of added membrane protein, at which point the production began to level off (50 μg of brush-border membrane protein would add approximately 13 nmol membrane phospholipid [11]). Enzyme activity was maximal in the pH range 8–11 (panel C). On the basis of these results, and the results shown below concerning detergents, the standard reaction conditions described in Materials and Methods were chosen. Panel D shows that under these conditions an essentially linear increase in product formation was obtained for reaction times up to 30 min. GLC analysis of the free fatty acids released after a 30 min incubation with the egg yolk PC showed that approximately 85% of the released fatty acids were unsaturated, indicating that there was indeed an initial attack on the *sn*-2 position (see below).

Table III shows the effect of various agents on the hydrolysis of labelled PC. Very little hydrolysis occurred in the absence of detergent, at 0 °C, or in the presence of 50% diethyl ether, 50% ethanol, or 1% SDS. Results similar to those seen in the mass assays were observed for the other detergents. Zwittergent 3-12, Triton X-100, and *n*-octylglucoside all resulted in low levels of hydrolysis. In contrast, CHAPS, deoxycholate, taurocholate and cholate all produced much higher levels of enzyme activity, resulting in high yields of released free fatty acids. Although all these detergents yielded high activities, variations in the total amount of product formed suggest that subtle changes in the steroid side chain and hydroxyl functional groups influence enzyme activity. Table III also shows that enzyme activity was independent of divalent cations, confirming our previous observations [11]. Approximately equal rates of hydrolysis were obtained in the presence of Ca^{2+} or EDTA. Essentially identical results were observed when Mg^{2+} or EGTA were included in the assays.

Fig. 2 shows the effect of detergent concentration on the phospholipase A_2 activity. When the concentration of substrate and protein remained constant, the amount of hydrolysis was found to be dependent on the concentration of added detergent. Half-maximal enzyme activity occurred at detergent concentrations which coincided with their critical micellar concentration (0.04–0.08% for deoxycholate [18], and 0.49% for CHAPS [19]). Although both of these detergents stimulated hy-

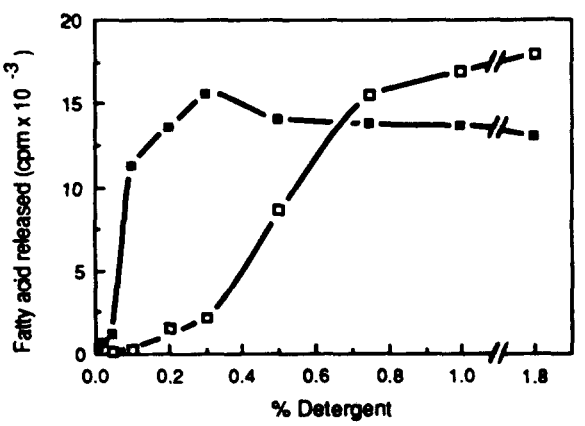

Fig. 2. Dependence of detergent concentration on phospholipase A_2 activity. Assays performed as described in Materials and Methods, except that the detergent concentration was varied as shown. ■, deoxycholate; □, CHAPS.

drolysis, they displayed curves of different shapes, with deoxycholate giving a much steeper rise in activity around its critical micellar concentration. Further, deoxycholate concentrations above 0.3% began to slowly inhibit product formation, while CHAPS continued to stimulate activity to a concentration of at least 1.8%. The amount of hydrolysis produced in 1% CHAPS was slightly greater than that in 1% deoxycholate, in agreement with the results presented in Table III. For Triton X-100, Zwittergent 3-12, and *n*-octylglucoside, detergents that did not stimulate activity at a 1% concentration (Table III), activity was measured at several points from 1% down to their respective critical micellar concentrations. Activities did increase 0.5–2-fold as the concentration approached the critical micellar concentration, but these activities were still less than 10–15% of that observed in 1% CHAPS.

Table IV shows that phospholipase A_2 activity, as measured with the standard assay conditions, was enriched 21-fold and recovered in 45.4% yield during brush-border membrane purification. These values closely parallel the enrichment and recovery seen for the marker enzyme sucrase [11]. The specific activity of brush-border phospholipase A_2 activity is at least 126-fold greater than that observed for incubation of the intact membranes in the absence of detergent (Table I), and several orders of magnitude greater than that observed during storage at $-20\,^{\circ}C$ [11]. Substituting PE for PC as the substrate in this assay resulted in a specific activity which was 112.5 ± 2.2 (mean $\pm$ S.D., $n = 3$) percent of that observed for PC. This value indicates that the 4–5-fold preference for PE hydrolysis seen in the intact membranes [11] had been abolished in the presence of detergent. Table IV also shows that the lysophospholipase activity of the intestinal preparations was dependent on the type of assay conditions employed. When assayed using the phospholipase A_2 assay conditions (+detergent, 1% CHAPS) lysophospholipase specific activity in the total mucosal homogenate was similar to the phospholipase A_2 specific activity. In addition, this lysophospholipase activity was purified and recovered in the brush-border membrane in an identical fashion to the phospholipase A_2 activity. When assayed in the absence of detergents, lysophospholipase activity was approximately 5-fold higher in the total homogenate and 2-fold lower in the brush border than that seen in the presence of 1% CHAPS.

Substrate competition

Table V and Fig. 3 show the interaction of phospholipase A_2 and lysophospholipase activities when presented with substrate. When membranes were incubated with labelled PC, increasing amounts of label were released with time. Quantitation of the released mass of fatty acids by GLC gave parallel increases, except that at each time point the value was larger than that measured by radioactivity (Table V). Such a discrepancy is reasonable, since quantitation of radioactivity released measures only phospholipase A_2 activity, whereas quantitation of total fatty acids released measures both phospholipase A_2 and lysophospholipase activities. The percentage of unsaturated fatty acids (16 : 1, 18 : 1, 18 : 2, 20 : 4) released fell

TABLE IV

PHOSPHOLIPASE ACTIVITY AND YIELD DURING PURIFICATION OF BRUSH-BORDER MEMBRANES

Phospholipase activity was assayed using exogenous substrates as described in Materials and Methods. Values are the mean $\pm$ S.D. of determinations from four to seven different membrane preparations. Ho, homogenate, BBM, brush-border membrane.

Enzyme assay	nmol/mg protein per min		Ratio BBM/Ho	Yield of activity in BBM (%)
	Ho	BBM		
Phospholipase A_2	6.3 ± 1.9	125.6 ± 23.7	20.7 ± 3.7	45.4 ± 4.2
Lysophospholipase (+detergent)	5.5 ± 1.8	121.1 ± 32.9	22.7 ± 3.9	47.2 ± 5.0
Lysophospholipase (−detergent)	27.6 ± 9.7	61.1 ± 12.3	2.5 ± 0.7	5.9 ± 2.7

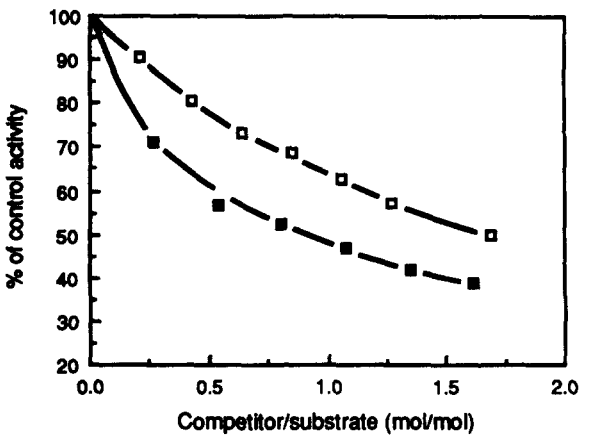

Fig. 3. Competition between PC and lysoPC for hydrolysis. Assays were done in the presence of 1% CHAPS. The standard amount of labelled substrate for the phospholipase A_2 and lysophospholipase assays were mixed with several concentrations of the other, unlabelled substrate as competitor. Values are the percentages of activity remaining, relative to those in the absence of competitor. □, Labelled PC + unlabelled lysoPC; ■, labelled lysoPC + unlabelled PC.

from 87.6% at 5 min to 80.0% at 60 min of assay, and further decreased to 56.7% when an equimolar amount of lysoPC was added to the PC. The initial site of PC hydrolysis must therefore be the *sn*-2 position as the majority of egg yolk unsaturated fatty acids are found in this position [20]. As incubation time and hence the amount of lysoPC produced increased, or when exogenous lysoPC was added, the recovery of unsaturated acids dropped reflecting an increased lysophospholipase hydrolysis of the largely saturated acids of the lysoPC. GLC analysis of the fatty acid composition of the lysoPC generated during a 15 min incubation with PC showed that 84.2% of the fatty acids were saturated (16 : 0, 18 : 0). Furthermore, the fatty acid distribution closely paralleled that reported [20] for the *sn*-1 position of egg yolk PC (data not shown). Addition of an equimolar amount of cold lysoPC to the labelled PC substrate decreased the amount of [^{14}C]oleate released from the *sn*-2 position by almost 40% (Table V) when compared to the corresponding 15 min time point as a control. However, the recovery of total fatty acids (GLC analysis) was unchanged from the control value. Since it was shown above that individually assayed PC and lysoPC were hydrolyzed at equal rates, the present result suggests that PC and lysoPC are competing for the same active site, without inhibiting total enzyme activity. Fig. 3 shows that the two substrates compete over a wide range of concentrations. Addition of increasing amounts of unlabelled lysoPC to a constant level of labelled PC progressively decreased the release of [^{14}C]oleate from the PC. A similar effect was seen when unlabelled PC was added to labelled lysoPC. These effects were not completely equal, however, for at any given competitor/substrate ratio cold PC reduced lysoPC hydrolysis more than unlabelled lysoPC reduced PC hydrolysis. This result suggests that, when the two substrates were combined, PC was the more effective competitor for the active site. Differences in the activities of the enzyme with the two different substrates or variations in the types of micellar structures present under the two assay conditions may explain this effect.

TABLE V

QUANTITATION AND ANALYSIS OF FATTY ACIDS RELEASED DURING PHOSPHOLIPASE ASSAYS

Incubations were carried out in quadruplicate with 340 nmol labelled PC (± 340 nmol unlabelled lysoPC) using the standard phospholipase A_2 assay conditions. Reactions were stopped after various time periods and duplicate tubes were quantitated by liquid scintillation counting or by GLC analysis. Results are the means of the duplicates. FAME, fatty acid methyl esters. % Unsaturated FAME, nmol unsaturated FAME/nmol total FAME (×100).

Substrate	Length of assay (min)	Fatty acid released (nmol ^{14}C)	Fatty acid released (nmol FAME)	% Unsaturated FAME
PC	5	14.0	17.7	87.6
PC	15	40.1	45.8	86.2
PC	30	78.3	92.5	84.7
PC	60	140.5	178.7	80.0
PC + lysoPC (equimolar)	15	25.4	46.4	56.7

Solubilization of membrane proteins

The ability of the bile salt detergents to stimulate enzyme activity was not related to their ability to solubilize membrane protein (Table VI). Although Triton X-100, Zwittergent 3-12, and *n*-oc-

TABLE VI

SOLUBILIZATION OF BRUSH-BORDER MEMBRANE PROTEIN

Values represent the percentage of total protein solubilized by the various treatments, following procedures described in Materials and Methods, and are the mean ± range/2 for duplicate measurements made on two different membrane preparations.

Treatment	% protein solubilized
Control, no detergent	4.3 ± 0.3
1 M KCl	18.1 ± 2.4
10 mM EDTA	10.6 ± 2.1
1% Triton X-100	76.0 ± 6.1
1% Zwittergent 3-12	76.8 ± 3.6
1% *n*-octylglucoside	76.6 ± 3.4
1% deoxycholate	98.4 ± 1.6
1% CHAPS	63.5 ± 4.8
1% taurocholate	24.8 ± 1.6
1% cholate	28.4 ± 3.6

tylglucoside failed to stimulate activity, the table shows that all three of these detergents solubilized more than 75% of the total membrane protein. Approximately 18% of the total protein was solubilized by treatment with a high concentration of salt (1 M KCl), whereas the bile salts solubilized from 25% (cholate) to 98% (deoxycholate) of the protein. When small aliquots of the solubilized proteins were assayed using the standard assay conditions (1% CHAPS), less than 10% of the phospholipase A_2 activity was found to be solubilized by the control, KCl, or EDTA treatments; in contrast, each of the detergents solubilized a minimum of 50% of the enzyme activity. Lysophospholipase activity was solubilized in parallel to phospholipase A_2 activity by the various treatments. These results indicate that this phospholipase must be regarded as an integral membrane protein.

Discussion

Using mass and radioactivity assays we have demonstrated that different detergents affect brush-border membrane phospholipase activity in a markedly different fashion. Bile salts and the synthetic, cholic acid derivative, CHAPS, stimulated phospholipase activity towards both endogenous, membrane phospholipids and exogenous phospholipid substrates. Hydrolysis was not stimulated by a number of organic solvents and non-ionic detergents that are stimulatory for other phospholipases [20–22]. Stimulation was not due to the solubilization of membrane protein, but was related to the ability to activate the phospholipase A_2 hydrolysis, the initial step in the deacylation pathway. Modification of the physico-chemical state of the substrate by detergents is known to exert a dramatic influence on reactions involving lipids. It has been shown that this effect can, in some instances, completely change the apparent substrate specificity of an enzyme (reviewed in Refs. 20 and 21). Such phenomena may explain why the previously observed PE-specific phospholipase A_2 activity [11] is lost upon solubilization with bile-salt detergents. Apparently, by forming mixed bile-salt micelles, other phospholipid substrates are presented to the active site of the enzyme in a way that results in effective hydrolysis. The ratio of detergent to total phospholipid was approximately 30 : 1 in the endogenous substrate assay and 50 : 1 in the exogenous substrate assay. Although only a small portion of the total phospholipids were lysophospholipids, it should be noted that the observed effects may reflect a combined interaction between the detergent properties of the lysophospholipids and those of the bile-salt detergents.

The component phospholipase activities of the brush-border membrane, phospholipase A_2 and lysophospholipase, had similar specific activities in 1% CHAPS, co-purified during membrane preparation, and were solubilized in a similar fashion from the membranes. Lysophospholipase activity of the brush-border membranes was unusual in that it was stimulated by incubation in 1% CHAPS. Most lysophospholipases are inhibited by a wide variety of different detergents [21,22]. In agreement with this, lysophospholipase activity in the total mucosal homogenate was 5-fold inhibited by CHAPS (Table IV), presumably due to detergent effects on the soluble intestinal lysophospholipase [5]. Furthermore, the substrates, PC and lysoPC, competed with each other for hydrolysis without affecting the total enzyme activity. For these reasons, we propose that a single enzyme is responsible for both activities.

Previous studies on most phospholipases B have shown combined phospholipase A_1 and lysophospholipase activities [20,21,23]. The enzyme from *Penicillium notatum* [24–26] is similar to the brush-border enzyme in attacking diacyl phospholipid initially at the *sn*-2 position. The ratio of lysoPC to PC hydrolysis by the *P. notatum* enzyme, like other phospholipases B [20,21], is also affected by the presence or absence of detergents [25]. The fungal enzyme, however, cleaves both fatty acid chains from phospholipids without a lysophospholipid accumulation [24,25]. The brush-border enzyme appeared to cleave the *sn*-2 position and then release the lysophospholipid, which became diluted in the substrate pool and had to compete with that pool for hydrolysis of the *sn*-1 position. The brush-border enzyme, therefore, does not behave like any of the known phospholipases B and its exact classification must await enzyme purification.

When assayed in the presence of excess PC the predominant phospholipase activity in the brush-border membrane is of the A_2 type. The purification and recovery factors (Table IV) suggest that the brush-border is the only location of phospholipase A_2 activity in the villus cell. Although previous investigations carried out with different intestinal preparations and assay systems may not be strictly comparable, this activity appears to be the same as the A_2 activity ascribed to the brush-border membrane by Subbaiah and Ganguly [1] and Diagne et al. [6]. Common features between these activities include the membrane location, Ca^{2+} independence, pH optima, and stimulation by bile salts. The present activity also shares many similarities with the A_2 activities described by Takagi and Sasaki [2] and Bonnefis et al. [3,4] from experiments conducted with homogenates of the intestinal mucosa.

The physiological significance of an enzyme with phospholipase A_2 and lysophospholipase activities in the intestinal brush-border membrane is not known. Activation of such an enzyme by bile salts may be important for the hydrolysis of dietary phospholipids [1,6]. Thus, this enzyme may assist pancreatic phospholipases in this role and may hydrolyze phospholipids in bile-salt micelles which reach the luminal surface of the brush border. Identification and localization of the enzyme within the brush border would help substantiate or disprove such a possibility.

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