Intestinal Phospholipase, a Novel Enzyme

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ABSTRACT We evaluated phospholipase activity in the intestine of rats and other species. Phospholipase activity was assayed by a surface barostat technique or an egg yolk titration system. Mucosal activity was found only by the surface barostat technique with phosphatidylglycerol as substrate; it was not found with phosphatidylcholine as substrate in assays by either technique. In gut luminal fluid activity was found when both phosphatidylcholine and phosphatidylglycerol were used as substrate in assays by the surface barostat technique, and phosphatidylcholine as substrate yielded activity in egg yolk titration. In rats in which pancreatic juice had been diverted, mucosal and gut luminal phospholipase activity was greater than in controls, thus demonstrating that enzyme activity was not due to pancreatic phospholipase. Bacterial origin of phospholipase activity was excluded in that phospholipase activity was found in germ-free rats; gastric and salivary gland origins were excluded in that continued phospholipase activity was found in rats with gastric fistula. The physiological importance of the enzyme was established by the finding that rats with pancreatic fistula absorbed 111 μmol of phosphatidylcholine and that controls absorbed 119 μmol of a 135-μmol load. Activity was found to be three times greater in the distal than in the proximal intestine; in cryptal cells it was 10 times greater than in villus tip cells. 65% of the activity in the gut lumen was tightly bound to particulate matter. We propose that intestinal phospholipase may be important in gut bacterial control, in the digestion of vegetable matter (phosphatidylglycerol is a major phospholipid in both plants and bacteria), and in the digestion of phospholipids in the gut lumen.

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INTRODUCTION

Phospholipids are considered to be essential structural components of cellular and intracellular membranes. It is well established that these phospholipids undergo constant metabolic turnover, suggesting the presence of intracellular phospholipases (1). Indeed, phospholipases have been demonstrated in several tissues of various species of animals (1). Although the phospholipases of exocrine secretions, e.g., those of the pancreas (for a review, see de Haas et al. [2]) or of snake venoms (3), have been studied extensively, the physiological functions of intracellular phospholipases are only beginning to be understood (1).

Beginning in 1931, workers in several laboratories (4–16), using endogenous phospholipids, exogenous phosphatidylcholine (PC),[1] or phosphatidylethanolamine (PE) as substrates, have described phospholipases from the intestinal mucosa. Unfortunately, these investigations were carried out with crude intestinal preparations, and contamination by enzymes from other origins (e.g., the pancreas) cannot be ruled out. Five years ago we reinvestigated this question, using monomolecular films of didodecanoylphosphatidylglycerol ($diC_{12}PG$) as a phospholipase substrate. Recently, using pig intestinal mucosa, we purified an acid-stable, low-molecular-weight (16,200) phospholipase A that hydrolyzes phosphatidylglycerol (PG) at least 500 times more rapidly than PC (R. Verger et al., manuscript in preparation). We consider this narrow enzymatic substrate specificity, in association with other molecular properties, to be proof of the existence of a new intestinal enzyme different from the well-known pancreatic phospholipase A_2. In the present paper, we report evidence of this intestinal phospholipase in rats and compare its activity in rats with its activity in several other species, including man.

[1] *Abbreviations used in this paper*: $diC_{12}PG$, didodecanoylphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine, PG, phosphatidylglycerol.

METHODS

Animal procedures

Nonfasting male Sprague Dawley rats weighing 200–400 g were routinely used. The animals were killed by a blow to the head, and the intestines were quickly removed. The intestines were rinsed in 0.15 M NaCl (4°C), divided into quarters from pylorus to cecum, and kept in 0.15 M NaCl at 4°C until scraped. Where indicated, the luminal washings were saved. Two gnotobiotic rats obtained from Centre de Recherche et d'Elevage des Oncins, St. Germain sur l'Arbresle, France, were treated in a similar manner. The lack of an intestinal microflora was confirmed by culturing cecal contents for 48 h under aerobic conditions at 37°C. In one of the two rats more extensive bacteriological studies were performed. Three samples from the colon were placed in Schaedler broth with 0.025% sodium polyanthenol sulfanate and vitamin K_3 to test for anaerobic growth. Every 48 h for 8 d, samples were placed in "chocolat Pointelex" broth and triptocase-soy, 5% sheep-blood broth. No growth was observed under these conditions. Two colonic samples were placed in brain-heart broth with 0.025% sodium polyanthenol sulfanate to test for aerobic growth. No growth was observed.

Rats in which pancreatic juice was diverted were operated on under ether anesthesia. A tight ligature was placed at the junction of the common bile duct and the duodenum. The common bile duct was cannulated above the ligature, allowing bile to be diverted to the exterior. The animals were placed in a restraining cage and given alimentation fluid (Trophysan or Trophysan Glucidique 50, Laboratoire Egic, Amilly, France) to drink ad lib. for 48 h. This ensured the removal of residual pancreatic enzymes, some of which are known to closely adhere to the intestinal mucosa (17, 18). After the 48-h period, the rats were killed, and the intestines were removed as described above. Unoperated control rats were maintained similarly and given the same diet.

Rats in which gastric and pancreatic-biliary secretions were diverted were operated on as described above, except that, in addition, the duodenum was transsected and catheters were placed retrograde into the stomach and antegrade into the proximal duodenum. Both catheters were secured with ligatures and the abdomen was closed. The gastric and common duct catheters were allowed to drain. Trophysan was administered through the duodenal catheter at a rate of 60 ml/d by a constant-infusion pump (LKB-Produkter AB, Stockholm). Because rats operated on in this manner did not tolerate the surgery as well as the rats with the lesser operation, they were killed at 24 h.

The common bile duct of rats in which PC absorption was measured was cannulated, and the common duct was ligated either at its junction with the duodenum (pancreatic juice-diverted rats) or 2 cm proximal to the duodenum (control rats). Three pairs of rats were infused with Vivonex HN (Eaton Laboratories, Norwich, N. Y.) at a rate of 4.5 ml h^{-1} for 2 d. An infusion of 0.5 μCi [^{14}C-Me]PC (Amersham Corp, Arlington Heights, Ill.) in 10 mM PC, 20 mM taurocholate (Sigma Chemical Co., St. Louis, Mo.), and Tris buffer (pH 7.0) (Sigma Chemical Co.) in 0.15 M NaCl was given at a rate of 4.5 ml h^{-1} for 3 h. The rats were given an overdose of pentobarbitol intraperitoneally, and the gastric contents and cecal contents were separately removed. The intestine was flushed with 0.15 M NaCl (15 ml), and the contents were collected.

Pig, sheep, and ox intestines were obtained from the local slaughter house. Dog intestine was obtained through the courtesy of Dr. Verine, INSERM, Marseille, France. The dog had received an ileal Thiery-Vella fistula 8 mo previously. At death, samples of duodenum, fistula, and distal ileum were obtained. Cat intestine was obtained from Dr. M. Gonella, Institut de Neurophysiologie, Centre National de la Recherche Scientifique, Marseille, France. Two samples of histologically normal distal duodenal mucosa were obtained through the courtesy of Dr. C. Figarella, Institut National de la Santé et de la Recherche Médicale, Marseille, France.

Enzyme samples

The intestine was opened on a glass plate on ice, cleaned, and scraped as described previously (19). The resulting mucosa was prepared for analysis by two different procedures. In the first, the mucosa was delipidated by homogenizing sequentially with 2 vol of acetone twice, 3 vol of 9:1 chloroform-butanol thrice, and 2 vol of ethyl ether twice. The resulting dry powder was extracted in 100 vol of 1 N HCl. The suspension was continuously stirred with a magnetic stirrer in a cold room for 1 h. A clear supernatant was produced by centrifugation at 20,000 *g* for 20 min in a Sorvall RC 2B at 4°C (DuPont Instruments-Sorvall, DuPont Co., Norwalk, Conn.). In the second, 3 vol of 1 N HCl was added, and the mucosa was homogenized. A clear to slightly turbid supernatant was produced by centrifugation in a table-top centrifuge with conical plastic centrifuge tubes (Beckman Instruments, Inc., Palo Alto, Calif.). This method was also employed when rat organ surveys were carried out and when species other than rat were investigated.

To obtain a particulate and a supernatant fraction, other samples of the homogenate were centrifuged first. The particulate fraction was homogenized in 3 vol of 1 N HCl, and the supernatant fraction was mixed vol:vol with 1 N HCl. In other studies, the particulate fraction was homogenized twice in 0.15 M NaCl and finally with 3 vol of 1 N HCl.

When phospholipase activity was determined in the intestinal luminal fluid, the collected intestinal content was homogenized in 0.15 M NaCl in a Teflon-glass homogenizer in the cold room.

To localize enzyme content in intestinal cells as it changes along the villus, the method of Weiser (20), as modified by Gratecos et al. (21), was used to differentially release cells from the villus. Aliquots of released cells were collected by centrifugation and separated into two parts. One sample was frozen for subsequent determination of alkaline phosphatase assay, and the other was homogenized with 3 vol of 1 N HCl for phospholipase assay.

Enzyme assays

Phospholipase. All assays, unless otherwise specified, were performed with a three-compartment zero-order trough as previously described (22). The water used was first deionized and then distilled from alkaline permanganate in an all-glass still. To this water was added Tris-base, $CaCl_2$, and EDTA at the respective concentrations of 10, 100, and 1 mM and adjusted to pH 8.0 with HCl. This buffer was added to the trough, and the surface was cleansed five times with a pasteur pipette connected to a water aspirator. diC_{12}PG (a generous gift of Dr. J. F. Tocanne, Toulouse, France) was added to the surface in a methanol-chloroform (1:5) solution with a 1-ml all-glass syringe to the required surface pressure. Because the surface pressure for optimal enzyme activity was found to be ~20 dyn cm^{-1}, this pressure was used in all experiments unless otherwise indicated. The temperature

of all experiments was 23°±0.5°C, which was maintained in a thermostatically controlled metallic box. After a 2-min control period that ensured the absence of spontaneous hydrolysis of substrate, enzyme (25–200 μl) was injected into the stirred subphase of the reaction compartment, and the rate of hydrolysis of substrate at constant surface pressure was recorded. For calculating the reaction rate, only the initial linear portion of the curve was used. Reaction rates were stable for at least 5 min. After the lipid covering approximately one-third of the surface of the reservoir had been hydrolyzed, the communication between the first and second reaction compartments was interruped by putting a small Teflon bar across the communication channel. The solution from the first reaction compartment was quickly aspirated, and, after a 2-min control period that ensured that enzyme from the first reaction compartment did not contaminate the second, the procedure was repeated for the second compartment and finally for the third compartment.

Surface pressure (π) was measured by the Whilhelmy plate method with a thin platinum plate attached to a Prolabo tensionmeter (Paris, France). The system was automated in our laboratory by M. Bidaud. As hydrolysis in the reaction compartment occurred, product desorbed from the surface and surface pressure fell. Surface pressure was maintained with a surface barostat as described (23). The rate of movement of the barrier was taken as the rate of activity of the enzyme. This value could be converted into nanomoles of substrate hydrolyzed per minute by the following considerations:

First, to determine the area occupied by a single $diC_{12}PG$ molecule, a π per area curve was constructed with the buffer routinely employed. A known amount of $diC_{12}PG$ was added to the surface of the water. Surface pressure was measured as surface area was contracted until film collapse occurred (46 dyn cm^{-1}). This determination was performed in triplicate, as was the automated analysis for phosphorus in a measured quantity of the $diC_{12}PG$ sample (24). The results agreed closely with those previously presented by Tocanne et al. (25). At each surface pressure, moles of substrate hydrolyzed per minute per milligram of protein was then calculated:

$$\frac{\text{width of trough (cm)} \times \text{rate of movement of barrier (cm min}^{-1}) \times \text{molecules/cm}^2}{\text{Avogadro's number } (6.02 \times 10^{23}) \times \text{mg of protein}}. \quad (1)$$

Because the area of the reaction chamber was 121 cm^2, the results of Eq. 1 were divided by 121 to yield the reaction rate per square centimeter of reaction surface.

Where utilized, one arbitrary unit of activity is defined by the hydrolysis of the lipid occupying 4 cm^2 of surface area of the reservoir compartment.

It should be noted that, unlike the case in bulk assays, only a small proportion of the enzyme added is actually at the surface of the reaction compartment. It is only this surface-adsorbed enzyme that is active. Preliminary evidence, obtained with ^{125}I-labeled purified enzyme from the intestinal lumen,[2] indicates that only 7.5% of the enzyme added could actually be localized to the surface at 20 dyn cm^{-1} by the techniques previously described (22). Therefore, the specific activities from monolayer studies quoted in this paper are probably underestimated by a factor of 13.3.

Enzyme activity was also determined by the egg yolk assay of de Haas et al. (26). Although this assay is optimized with regard to Ca^{2+} and bile acid concentration for porcine pancreatic phospholipase, the concentrations of these assay constituents were found to be optimal for the rat as well. The assays were performed with a Radiometer TTT 60 Titrator (Copenhagen, Denmark) and pH stated to 8.00.

To determine the hydrolytic site specificity of the mucosal enzyme, mucosa (villus tips discarded) from the distal one-half intestine from a rat in which the pancreatic juice had been diverted for 48 h was delipidated, and the enzyme was extracted with 20 vol (wt/vol) 1 N HCl. 0.1–0.2 ml of enzyme was incubated for 5 and 20 min at 37°C with 0.48–0.58 ml of 0.15 M NaCl, 6 μmol of $CaCl_2$, [*sn*-1-^3H, *sn*-2-^{14}C]PG (^3H, 3.0×10^4 cpm and ^{14}C, 9.4×10^3 cpm), and 40 μmol of Tris-HCl in a total volume of 1.1 ml. The incubation mixture was titrated to pH 7.84 with 1 N NaOH. At the end of the incubation, the lipids were extracted, and the radioactivity of the fatty acids released was differentially measured after thin-layer chromatography of the lipid extract (silica gel G layers: hexanes/ether/methanol/acetic acid, 80:20:9:2, vol/vol). Incubations were also performed with phospholipase A_2 from Russell viper venom (Sigma Chemical Co.) and without enzyme addition to test for spontaneous release of fatty acid.

Alkaline phosphatase. Alkaline phosphatase was used to identify the level of released intestinal villus cells. The assay used was that described by Louvard et al. (27) and was thermostated to 37°C. Absorbance at 410 nm was followed in a Unicam SP 1700 Spectrometer equipped with a Unicam AR 25 Recorder (Philips Science et Industrie, Paris).

Chymotrypsin. Chymotrypsin was used to test the efficacy of the exclusion of pancreatic juice from the intestine and was assayed according to the method of Louvard and Puigserver (28). Acetyl-L-tyrosine ethyl ester was used as substrate.

Chemical analysis

Protein was measured by the method of Lowry et al. (29). Lipids were extracted by the method of Folch et al. (30). PC and lyso-PC were isolated by thin-layer chromatography (31) and quantitated (32).

Radioactivity

A Packard model 3390 Liquid Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) was used to determine the radioactivity of appropriate samples.

Chemicals

PC was isolated from eggs as previously described (31). [^3H,^{14}C]PG was synthesized from *sn*-1-[^3H]palmitoyl, *sn*-2-[^{14}C]linoleoyl PE by transesterifying the PE with glycerol (33). The PE, a generous gift of Dr. Mosley Waite, Bowman Gray School of Medicine, Winston-Salem, N. C., was reacted with 80 μmol of sodium acetate, 40 μmol of $CaCl_2$, 20% vol:vol glycerol, and 0.1 ml of phospholipase D in a final volume of 0.6 ml. 0.5 ml of ethyl ether, washed three times with water, was added, and the reaction mixture was placed on a Vortex mixer (Scientific Industries, Springfield, Mass.) at room temperature for 20 min. The reaction was stopped by adding 0.1 ml of 1 N HCl, and the lipids were extracted with ethyl ether/ethanol 4:1 (vol/vol). The ether phase was

[2] Verger, R., G. Pieroni, C. Mansbach, and F. Ferrato. Submitted for publication.

aspirated and dried under N_2, and PG was isolated by thin-layer chromatography (34). Phospholipase D was partially purified (through stage 3) from Savoy cabbage (35).

RESULTS

Because no mucosal phospholipolytic activity was demonstrable by the classic egg yolk assay, PG was investigated and found to be a suitable substrate. However, PG precipitates from solution at the concentrations of Ca^{2+} required for optimum enzyme activity. Therefore, the surface barostat technique had to be employed.

As shown in Table I, there is high phospholipase specific activity present in intestinal mucosa. In contrast to previous reports of enzymes associated with the digestion of dietary substrates (36), phospholipase specific activity in intestinal mucosa was found to increase with increasing distance from the pylorus.

With $diC_{12}PG$ as substrate, phospholipase activity was found in the intestinal luminal contents, 117±3 nmol min^{-1} mg $protein^{-1}$. This value is close to the average mucosal specific activity and suggests that the luminal activity could have been secreted by the intestinal mucosa. With $diC_{12}PG$ as substrate, the total amount of activity present in the intestinal contents was also found to be quite large, 6.2±1.1 μmol min^{-1}. It was also of interest to determine whether the enzyme was bound to particulate matter in the intestinal content. 65% of the activity was found to be bound to easily centrifugable particulate contents. Only 5% of this activity could be removed by resuspending the sediment in 0.15 M NaCl. Thus, the binding of phospholipase activity to luminal particulate matter was assumed to be tight.

The activity in the intestinal lumen could well have come from pancreatic phospholipase A_2. This possibility was enhanced by the finding that, unlike the phospholipase activity extractable from the mucosa, the luminal enzyme was active in the egg yolk assay (440 nmol min^{-1} mg $protein^{-1}$). In addition to the pancreas, however, several other possible sources for the activity in the intestinal lumen and in the mucosa were considered. The enzyme could have come from the gastric mucosa (see below) or from bacteria within the intestinal lumen. These possibilities were ruled out as follows.

TABLE I
Specific Activity of Intestinal Phospholipase in Rat Mucosa

	Gut segment*			
	1	2	3	4
Specific activity‡	62±9	118±22	143±26	185±33
Univariate *P* values§	0.0092	0.2365	0.6082	
Multivariate *P* value‖	$P < 0.0078$			

* Each quarter of the intestine, pylorus to cecum.
‡ Nanomoles per minute per milligram of protein.
§ The univariate *P* values compare each quarter of the intestine with segment 4.
‖ The multivariate *P* value tests the null hypothesis that enzyme activity from each gut segment is derived from a single population. Performed on an IBM 1130 Computer (IBM Corp., Armonk, N. Y.)

A pancreatic derivation of the enzyme was considered the most likely alternative. To rule out this possibility, three rats had their pancreatic juice diverted from the intestine. Chymotryptic activity in the intestinal fluid averaged only 0.63±0.32% of the activity observed in controls. By contrast, the total phospholipase activity in the intestinal lumen, as judged by hydrolysis of $diC_{12}PG$, was 7.8±1.4 μmol min^{-1} compared with 6.2±1.1 μmol min^{-1} in normal rats. The specific activity with egg yolk as substrate was 600 nmol min^{-1} mg $protein^{-1}$ (one determination) compared with 440 nmol min^{-1} mg $protein^{-1}$ in normal rats. 65% of the activity with $diC_{12}PG$ as substrate was bound to particulate matter, as was seen in the intact rat. Enzyme activity was also determined in the intestinal mucosa of two pancreatic juice-diverted rats. The averaged phospholipase specific activity ($diC_{12}PG$ as substrate) in the intestinal mucosa in each quarter of the intestine was greater than in normal rats 199, 350, 432, and 538 nmol min^{-1} mg $protein^{-1}$, respectively. Thus, it is highly unlikely that the phospholipase activity in the mucosa or in the intestinal lumen was of pancreatic origin.

Next, it was considered possible that the phospholipase activity was of bacterial origin. However, the two germ-free rats demonstrated phospholipolysis ($diC_{12}PG$ as substrate) in the mucosa of each quarter of the intestine (245, 414, 441, and 518 nmol min^{-1}, respectively) at the same level as normal rats (220, 329, 363, and 407 nmol min^{-1}, respectively). However, the total amount of activity in the intestinal lumen with $diC_{12}PG$ as substrate was somewhat reduced in these rats (1.4 μmol min^{-1}) as compared with controls (6.2±1.1 μmol min^{-1}), although the specific activity remained high, 407 nmol min^{-1} mg $protein^{-1}$ compared with 117 nmol min^{-1} mg $protein^{-1}$ in normal rats. The reduction in total activity was assumed to be due to reduced content of particulate matter in the intestinal lumen of these gnotobiotic rats. The intestinal contents of one gnotobiotic rat were tested at a surface pressure of 10 dyn cm^{-1} in the surface barostat with $diC_{12}PC$ as substrate. The activity, 382 nmol min^{-1} mg $protein^{-1}$, was similar to that found in normal rats by the egg yolk assay (440 nmol min^{-1} mg $protein^{-1}$). These data suggest that the phospholipase

activity observed in normal rats was not of bacterial origin.

Lastly, it was theorized that the phospholipase activity could be of gastric or salivary gland origin. To rule out this possibility, two rats with gastric and duodenal fistulae and pancreatic juice diversion were studied. Pancreatic juice was successfully diverted, as judged by chymotryptic activity that was 1% of control values, despite the shortened length of time of fistulation (25 h). Specific activity ($diC_{12}PG$ as substrate) in the intestinal lumen remained high, 84 nmol min^{-1} mg $protein^{-1}$ compared with 117 μmol min^{-1} mg $protein^{-1}$ in normal rats, despite diversion of gastric, salivary gland, and pancreatic secretions from the intestinal tract.

Taken together, these data suggest that the phospholipase activity observed in the intestinal lumen and in the intestinal mucosa was of intestinal mucosal origin. The data do not establish that they are the same enzyme, because the luminal enzyme differs significantly from the mucosal enzyme in that it is active in the egg yolk assay, whereas the mucosal enzyme is not.

Because luminal phospholipase activity continued high, despite pancreatic juice diversion, we considered it important to test whether pancreatic juice-diverted rats could absorb physiologically important amounts of PC. 7.8 mM PC containing [^{14}C Me]PC was infused for 3 h at 4.5 ml h^{-1}. Because <1% of the counts per minute infused were found in the stomach and cecum, the percent PC absorbed was calculated on the basis of the number of counts per minute infused as compared with the number of counts per minute recovered from the lumen in the lyso-PC and PC fractions. By this method, 84±4 and 87±5% of the infused PC was absorbed in the control and pancreatic juice-diverted rats, respectively. Thus, as judged by infusate PC specific activity, control rats absorbed 88 μmol and diverted rats 92 μmol of PC during the 3-h infusion period. These data were confirmed when the mass of PC infused was compared with the mass of PC and lyso-PC remaining in the intestinal lumen at the end of the infusion. When the amount absorbed was calculated on the basis of mass, 86±5 and 91±6 μmol of PC were absorbed in the control and pancreatic duct-diverted rats, respectively. The percent of counts per minute recovered from the intestinal lumen present as lyso-PC was also the same in both groups, 29±0.5 and 30±3% in the control and pancreatic juice-diverted groups, respectively. In summary, the pancreatic juice-diverted rat was able to normally hydrolyze and absorb PC, despite the lack of pancreatic phospholipase.

We next studied the possibility of a villus site of origin of the phospholipase activity. As shown in Fig. 1, cells were successively fractionally released from villus tip to crypt, as judged by the progressive reduction in alkaline phosphatase activity, as previously described (20, 21). The specific activity of alkaline phosphatase was 42 times higher in villus tip cells than in cryptal cells in the proximal intestine and 81 times higher than in cryptal cells in the distal intestine. By contrast, as shown in the figure, the specific activity of intestinal phospholipase was significantly increased in cryptal cells vs. mature villus tip cells. This suggests that the synthesis of intestinal phospholipase is 7–12 times more active in cryptal cells as compared with villus tip cells. Because of this finding, we considered it possible that the phospholipase activity originated in Paneth cell granules. This possibility was ruled out by the finding that there was no phospholipase activity in a preparation of Paneth cell granules (kindly donated by Dr. D. Balas, Toulouse, France).

Because phospholipases hydrolyze various parts of phospholipids, we considered it important to establish which ester bond was attacked by the mucosal enzyme. Enzyme was prepared from a rat in which the pancreatic juice had been diverted for 48 h and incubated with *sn*-1-[^{3}H]palmitoyl, *sn*-2-[^{14}C]linoleoyl PG. The $^{3}H/^{14}C$ ratio of released fatty acids was 6.6:1, which should be compared with a ratio of 0.3:1 ob-

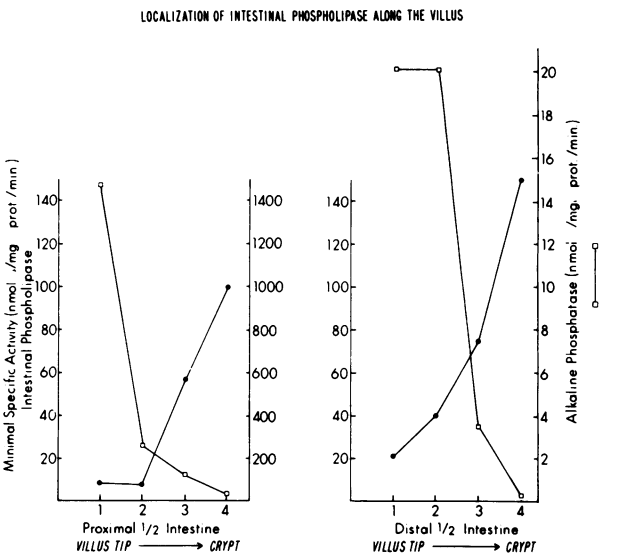

FIGURE 1 The distribution of intestinal phospholipase activity along the crypt-villus complex from the proximal (left) and distal (right) one-half intestine. Cells were progressively released from the villus as described in Methods. The released cells were combined into four separate fractions as indicated on the abscissa. Phospholipolytic activity ($diC_{12}PG$ as substrate) was determined on 1 N HCl extracts (Methods) and is shown by reference to the left ordinate in both panels. Alkaline phosphatase activity was used to confirm the depth of the released cells and is shown by reference to the right ordinate of both panels. Alkaline phosphatase activity was considerably reduced in the distal one-half intestine as compared with the proximal one-half, in agreement with Kwong et al. (69). Prot., protein.

TABLE II
*Survey of Various Organs in Rat for Phospholipase Activity**

Organ	Specific activity
	nmol min^{-1} mg prot^{-1}
Intestine, mucosa‡	127
Stomach, mucosa	23
Colon, mucosa	21
Serum	9
Lung	0
Kidney	0
Liver	0
Heart	0
Muscle, striated	0
Adipose tissue	0
Spleen	0

* The substrate utilized was diC_{12}PG, assayed at a surface pressure of 20 dyn cm^{-1}.
‡ Average value.

tained with Russell viper venom phospholipase A_2. Because the $^3H/^{14}C$ ratio of the original PG was 3.2:1, this indicates considerable (12-fold) enrichment of the released fatty acids by ^{14}C as expected for the venom phospholipase A_2 and a modest (twofold) enrichment of ^3H by intestinal phospholipase. The results establish that the muscosal enzyme is a phospholipase A, because of its release of fatty acids. The results further suggest that the enzyme is of the A_1 type because of its preferential release of the fatty acid esterified at the *sn*-1 position of PG. However, we emphasize that the preparation used was impure and that final assignment of the enzyme's substrate specificity awaits similar studies with pure enzyme. 6% of the ^3H and no ^{14}C were released spontaneously from PG incubated without enzyme.

Although phospholipase activity has been demonstrated in the intestine with diC_{12}PG as substrate, phospholipases have also been reported in many other tissues. For this reason, a tissue survey was undertaken in the rat to determine the uniqueness of this enzymatic activity. As can be seen in Table II, serum, gastric mucosa, and colonic mucosa demonstrated activity. Pancreas was not assayed, since purified porcine pancreatic phospholipase A_2 is quite active against C_{12}PG.[3] Plasma activity was 75% less than that of serum, which was not due to heparin, because the addition of heparin to serum did not reduce its activity (10 nmol min^{-1} ml^{-1} serum vs. 9.44 nmol min^{-1} ml^{-1} upon the addition of heparin 1:5). The location of activity was further investigated by bringing the serum to a density of 1.21 with KBr and centrifuging it at 45,000 rpm in a SW 50.1 rotor (Beckman Instruments Co., Inc.) for 24 h. The resulting serum was fractionated by piercing the bottom of the tubes. 96% of the activity was recovered, of which 59% was in the heaviest fraction. No activity was found in the lipoprotein-containing fractions at the top of the tube. The presence of activity in serum was peculiar to rat, since it was not found in pig or human samples.

A variety of species was assayed for activity in the intestine, and the results are presented in Table III. Activity was low or nonexistent in carnivorous species, whereas activity was present in omnivorous and herbivorous species.

DISCUSSION

Tissue surveys for phospholipase have consistently revealed enzymatic activity in the intestine (4–16). However, it is doubtful that the enzyme described in the present report is the same as those that have been described by past investigations. There are three reasons for this. Firstly and most importantly, the enzyme isolated from the intestinal mucosa described herein is not active against PC as substrate, neither in the egg yolk assay nor when injected beneath a monolayer of diC_{12}PC at different surface pressures (10–25 dyn/cm). Secondly, the level of activity we observed when diC_{12}PG was used as substrate is considerably greater than previously reported phospholipase activities as determined by other assay systems. Lastly, in two stud-

TABLE III
*Intestinal Mucosal Phospholipase: Activity in Various Animal Species**

Animal species	Duodenum	Ileum
	nmol min^{-1} mg protein^{-1}	
Rat (n = 5)	62±11‡	185±33‡
Pig (n = 2	67	153
Dog (n = 1)§	14	0
Cat (n = 2)	NA‖	0
Man (n = 2)**	670	NA‖
Sheep (n = 4)	80±13	110±77
Ox (n = 1)	140	150

* diC_{12}PG was used as substrate at a surface pressure of 20 dyn cm^{-1}.
‡ Mean±SEM for $n > 4$ or average when n = 2.
‖ Not available.
** Duodenal biopsies obtained for diagnostic purposes were histologically normal.
§ No activity was found in the Thiery-Vella loop (made from ileum) or in secretions from the loop. The activity reported in the table is from ileum distal to the loop.

[3] Pieroni, G., C. Mansbach, F. Ferrato, and R. Verger. Submitted for publication.

ies (7, 8) the phospholipase was acid precipitable, whereas the enzyme presented here is clearly acid soluble.

In general, phospholipases can be divided into two groups on the basis of specific activity. The phospholipases of the first group, whose specific activity is in the range of 1×10^{-9} mol min^{-1} mg $protein^{-1}$, are often membrane bound and do not always require Ca^{2+} for activity (37–50). The second group of phospholipases, with specific activities that exceed 1×10^{-6} mol min^{-1} mg $protein^{-1}$, require Ca^{2+} for activity, are of low molecular weight (~14,000), and are soluble and acid-stable proteins that are secreted from their cell of origin (51–58). The enzyme described in the present study, when partially purified from porcine intestinal mucosa, is small, has a specific activity of 25×10^{-6} mol min^{-1} mg $protein^{-1}$, requires Ca^{2+} for activity, and is acid stable, clearly placing it in the group of phospholipases that are secreted.

The total activity in the intestinal lumen of the pancreatic juice-diverted rat, 37 μmol PC hydrolyzed min^{-1} as determined by the egg yolk assay, clearly is enough to hydrolyze all the PC delivered to the intestine each day (59). Thus, rats should be able to, and have been demonstrated in this study to, absorb physiologically important amounts of PC, despite the absence of pancreatic juice in the intestine. The relevance of this data assumes that PC must be hydrolyzed before its absorption (60). This intestinal enzyme might also explain the persistance of phospholipase activity (PC as substrate) in the lumen of the distal intestine at a level similar to that found in the proximal intestine, in contrast to other pancreatic enzymes (61). Indeed, Arnesjo et al. (61) suggested the possibility of a phospholipase other than pancreatic phospholipase to explain their findings.

What kind of a physiologic role could be envisaged for such an enzyme? In several cell systems, most notably the platelet system, phospholipase activity is related to providing arachidonic acid for prostaglandin synthesis (1). Clearly, prostaglandins have effects on the intestine, and a local site of its synthesis is reasonable to expect. However, the quantity of enzyme present in the tissue, 16 μg/g wet wt, is considerably greater than would be expected for this purpose alone.

Another possibility is a digestive function. Clearly, this would be in addition to pancreatic phospholipase A_2. However, like most proteins, the pancreatic phospholipase is subject to degradation by the proteolytic enzymes within the intestinal lumen (62). Therefore, during digestive periods, PC or PG not hydrolyzed by pancreatic phospholipase A_2 could be hydrolyzed by the secreted intestinal enzyme, and, during interdigestive periods, when pancreatic phospholipase A_2 concentrations would be expected to be low, PC entering the lumen could be digested by intestinal phospholipase. Considerable amounts of PC enter the lumen from sloughed cells during all hours of the day. Approximately 8 μmol of PC could be expected to enter the intestinal lumen via this route each day in the rat, assuming a cell $t_{1/2}$ of 48 h (32). This PC would otherwise be lost to the organism.

Lastly, an intestinal phospholipase active against PG suggests two prime substrates, bacteria and plants, since these are high in PG content. In terms of bacteria, potential hydrolysis could occur either intraluminally or intracellularly. If one considers intraluminal hydrolysis of bacteria first, it is obvious that the small bowel is not sterile, and, therefore, by definition, the enzyme is not completely capable on its own of hydrolyzing all living bacteria. As shown in Table III, carnivores have low intestinal phospholipase activity compared with herbivores and omnivores. Since the bacterial population in the carnivore's intestine is lower than that in plant-eating animals (63), intestinal phospholipase could be related to the number of bacteria free in the intestinal lumen. By contrast, luminal bacteria provide a great part of the nutrition of herbivores. However, the pancreatic phospholipase of horse, ox, and sheep is less "penetrating" than that of the pig, that is, it is not able to hydrolyze substrate at a surface pressure as high as that of the porcine enzyme (64, 65). Therefore, in the case of the herbivora, intestinal phospholipase may play an important digestive role, since its penetrability[4] could enable bacterial digestion. The higher enzyme levels noted in the herbivora also suggest a role for the enzyme in digesting plant PG, but this is unexplored.

The bacteria digested by the intestinal phospholipase does not necessarily have to be within the intestinal lumen. Some bacteria characteristically invade the intestinal mucosa as part of their pathologic process. Clearly, this enzyme could be involved in host defense mechanisms. When bacteria invade the intestinal mucosa from the lamina propria, such as has been postulated to occur in Whipple's disease (66), the bacteria in the mucosal cells have been noted to be undergoing degenerative changes.

Although it is not conclusively proven that the luminal enzyme is the same as the intestinal one, it is highly likely, because sources other than the intestinal mucosa (stomach, salivary gland, bacteria, and pancreas) have been eliminated. Final proof would require in vitro conversion of the mucosal enzyme that lacks activity against PC to the luminal enzyme that hydrolyzes PC. Thus, the mucosal enzyme is viewed as a zymogen in terms of its substrate specificity. However, the analogy with the pancreas is not strict, since treatment of the mucosal enzyme with trypsin, which

[4] Pieroni, G., C. Mansbach, F. Ferrato, and R. Verger. Manuscript in preparation.

activates pancreatic prophospholipase A_2 (67), causes loss of activity against PG with no appearance of activity against PC. In any event, trypsin would not be expected to be the activator in the case of the mucosal enzyme, because the pancreatic juice-diverted rat, with no luminal tryptic activity, retains its ability to hydrolyze PC.

Comparison of the mucosal enzyme reported here and a phospholipase A_1 isolated from the pancreas (68) suggest that they likely are different enzymes. The intestinal mucosal enzyme requires Ca^{2+} for activity, whereas the pancreatic enzyme works even in the presence of EDTA. Furthermore, the evidence that the mucosal enzyme is a phospholipase A_1 further distinguishes it from the major phospholipase secreted by the pancreas, which is a phospholipase A_2 (65, 67).

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