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Effects of phospholipids on sphingomyelin hydrolysis induced by intestinal alkaline sphingomyelinase: An in vitro study

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Digestion of dietary sphingomyelin (SM) is catalyzed by intestinal alkaline sphingomyelinase (SMase) and may have important implications in colonic tumorigenesis. Previous studies demonstrated that the digestion and absorption of dietary SM was slow and incomplete and that the colon was exposed to SM and its hydrolytic products including ceramide. In the present work, we studied the influences of glycerophospholipids and hydrolytic products of phosphatidylcholine (PC; i.e., lyso-PC, fatty acid, diacylglycerol, and phosphorylcholine) on SM hydrolysis induced by purified rat intestinal alkaline SMase in the presence of 10 mM taurocholate. It was found that various phospholipids including PC, phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and phosphatidic acid (PA) inhibit alkaline SMase activity in a dose-dependent manner, with the degree of inhibition being in the order PA > PS > PI > PC > PE. Similar inhibition was also seen in a buffer of pH 7.4, which is close to the physiologic pH in the middle of the small intestine. When the effects of hydrolytic products of PC were studied, lyso-PC, oleic acid, and 1,2-dioleoyl glycerol also inhibited alkaline SMase activity, whereas phosphorylcholine enhanced SMase activity. However, in the absence of bile salt, acid phospholipids including PA, PS, and PI mildly stimulated alkaline SMase activity whereas PC and PE had no effect. It is concluded that in the presence of bile salts, glycerophospholipids and their hydrolytic products inhibit intestinal alkaline SMase activity. This may contribute to the slow rate of SM digestion in the upper small intestine. (J. Nutr. Biochem. 11:192–197, 2000) © *Elsevier Science Inc. 2000. All rights reserved.*

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Introduction

Sphingomyelin (SM) is not only a constituent of cell membranes but also a dietary component. In milk and most dairy products, SM is the major phospholipid, the amount being equal to or higher than that of phosphatidylcholine (PC).[1–3] SM is also present to a considerable extent in eggs, meat, and fish.[4] Recent studies have indicated that digestion of SM may have important implications in colonic tumorigenesis.[5] Administration of SM has been shown to reduce the number of aberrant colonic crypt foci and to decrease the proportion of carcinomas to adenomas induced by 1,2-dimethylhydrazine.[6,7] A significant decrease in sphingomyelinase (SMase) activity has been recently demonstrated to be associated with human colonic adenoma, carcinoma, and adenomatous polyposis.[8,9]

SM is hydrolyzed by SMase and at least three types of SMase have been identified based on their optimal pH values. Acid SMase is a lysosomal enzyme with an optimal pH of 4.5, and neutral SMase is a membrane-bound enzyme with an optimal pH of 7.5.[10] Both acid and neutral SMases are common intracellular enzymes and it is unlikely that their role in SM digestion in the intestinal lumen are important. Nilsson[11] identified in both rat intestinal tract and human intestinal content a unique SMase, which was a brush-border enzyme and preferred an alkaline pH of 9.0.

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The intestinal alkaline SMase is present in both the intestinal mucosa and lumen, with the peak activity localized in the distal part of jejunum.[12,13] Bile and bile salt have been shown to be able to dissociate the enzyme from intestinal mucosa into the lumen.[14] Animal studies have demonstrated that digestion of dietary SM occurs mainly in the middle and lower parts of the small intestine where alkaline SMase activity is high, indicating an important role of the enzyme in SM digestion.[15]

Hydrolysis of dietary SM in the intestinal tract is characterized as a slow and incomplete process. Three hours after ingestion of 1 μmol sphingosine-labeled SM, 22 to 42% of the radioactivity could be found in the intestinal content, of which 75 to 85% was nonhydrolyzed SM.[16] Twenty-four hours after feeding rats 5 μg ^{14}C-steroyl-SM, 10 to 15% of the radioactivity still could be found in feces as intact SM.[15] This finding was also described by Schmelz et al.[17] in an in situ study. The factors that limit the rate of digestion of SM have not been characterized. Obviously, the amount of the enzyme available and luminal factors may be important. In the present study, we investigated the role of glycerophospholipids on SM hydrolysis using purified rat intestinal alkaline SMase. We found that phospholipids inhibited the catalytic activity of alkaline SMase, which may contribute to the delay in hydrolysis of SM in the intestinal tract.

Materials and methods

Materials

Rat intestinal alkaline SMase was purified from rat intestinal mucosa as described elsewhere.[18] SM was purified from bovine milk as previously described[19] and was provided by Lena Nyberg at the Swedish Dairies' Association. The purified SM was then labeled with ^{14}C-choline (^{14}C-SM) according to the method of Stoffel[20] and the specific activity was 56 μCi/mg. Taurocholate (TC), dioleoyl PC, phosphatidylinositol (PI; from soybean), phosphatidylethanolamine (PE; from pig liver), phosphatidylserine (PS; from bovine brain), lysophosphatidylcholine (LPC; from soybean, contains primarily C18 unsaturated fatty acids), oleic acid, and 1,2-dioleoyl glycerol, were purchased from Sigma Chemical Co. (St. Louis, MO USA).

Methods

Experimental design. Two sets of experiments were performed. In the first series of experiments, we determined the SM hydrolysis induced by intestinal alkaline SMase in the presence and absence of different concentrations of glycerophospholipids including PC, PE, PS, PI, and phosphatidic acid (PA). Studies were performed at pH values of 9.0 and 7.4. The former is the optimal pH of alkaline SMase[11] and the latter is the average pH of the middle small intestine in humans.[21] In the second series of experiments, we studied the effects of hydrolytic products of PC generated by the action of phospholipase A2 and phospholipase C on intestinal alkaline SMase activity, because PC is the most abundant form of phospholipid in the intestinal lumen. SM hydrolysis was therefore determined in the presence of oleic acid, LPC, phosphorylcholine, and 1,2-dioleoyl glycerol at the same molar concentrations as PC. Finally, the roles played by phospholipids in alkaline SMase activity in the absence of bile salts were determined.

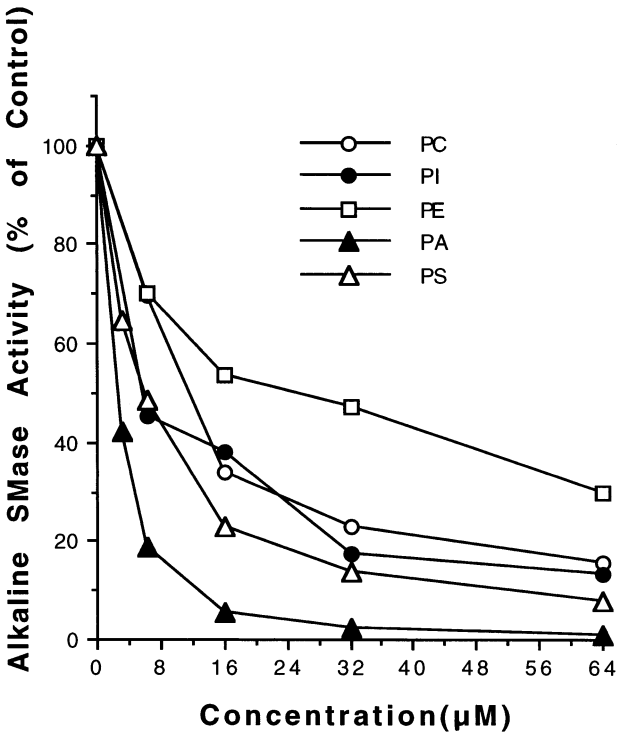

Figure 1 Effects of phospholipids on intestinal alkaline sphingomyelinase (SMase) activity. Different amounts of phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidic acid (PA) and phosphatidylserine (PS) dissolved in chloroform:methanol (2:1) were added to test tubes and dried under nitrogen. Alkaline assay buffer (pH 9.0) was added followed by sonication. Alkaline SMase and ^{14}C-choline-labeled sphingomyelin were added thereafter. The alkaline SMase activity was determined and activity in the absence of any phospholipids was taken as 100%. Similar results were obtained from two additional experiments.

Preparation of phospholipid, oleic acid, and 1,2-dioleoyl glycerol solutions. All phospholipids including PC, PE, PI, PS, PA, LPC, and 1,2-dioleoyl glycerol and oleic acid were dissolved in chloroform:methanol (2:1) as 1 mM stock solution and stored at −20°C. Phosphorylcholine was dissolved in 0.15 M NaCl in a concentration of 1 mM as a stock. Under the experiment, different amounts of the water-insoluble lipid solutions tested were added in the test tubes and dried under nitrogen. The assay buffer, alkaline SMase, and substrate SM were added thereafter. Phosphorylcholine was directly added in the assay buffer. The hydrolysis of SM was determined as described below.

Assay SM hydrolysis by intestinal alkaline SMase. In each test tube with the lipids tested, 75 μL of assay buffer containing 30 mM Tris, 0.15 M NaCl, 2 mM ethylenediamine tetraacetic acid (EDTA), and 10 mM TC, pH 9.0, was added followed by sonication for 12 sec. Intestinal alkaline SMase (0.1 μg) in 20 mM Tris buffer, pH 8.2, was then added in a volume of 5 μL. The reaction was started by addition of ^{14}C-SM (10,000 dpm) in 20 μL 0.15 M NaCl containing the same concentrations of bile salt. After incubation at 37°C for 30 min, the reaction was terminated by adding 0.4 mL chloroform:methanol (2:1). Phase separation was obtained by centrifugation at 10,000 rpm for 3 sec. An aliquot of the upper phase containing the released phosphorylcholine was taken and the radioactivity was determined by liquid scintillation counting. The procedure of SMase assay described above was based on the method of Gatt[22] with modifications as described by

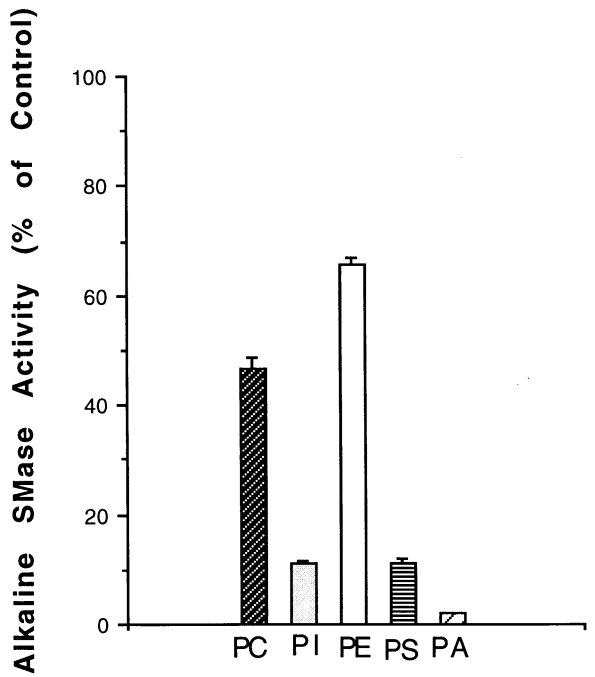

Figure 2 Effects of phospholipids on alkaline sphingomyelinase (SMase) activity at pH 7.4. Different phospholipids at 16 μM were incubated with alkaline SMase and ^{14}C-sphingomyelin (SM) in 30 mM Tris-HCl, containing 0.15 M NaCl, 2 mM ethylenediamine tetraacetic acid, and 10 mM taurocholate, pH 7.4. The hydrolysis of SM was determined. The activities of alkaline SMase in the absence of phospholipids were taken as 100%. PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid.

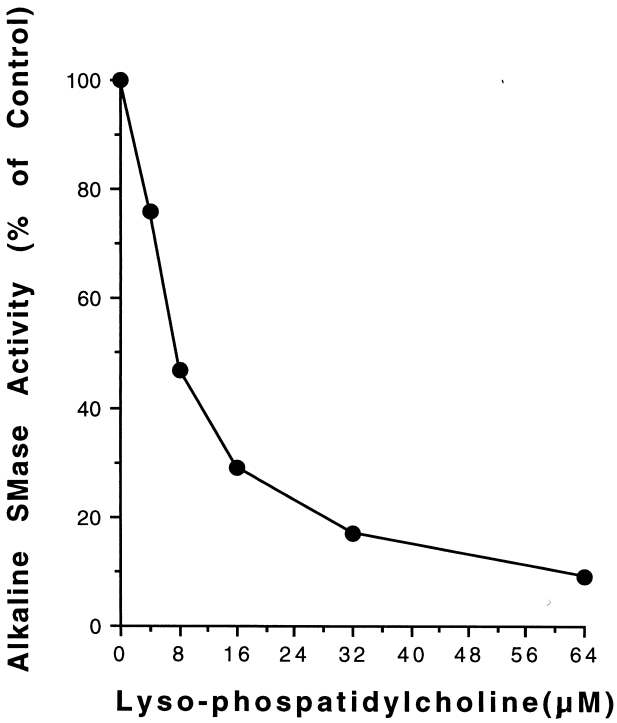

Figure 3 Effect of lyso-phosphatidylcholine (lyso-PC) on alkaline sphingomyelinase (SMase) activity. Lyso-PC was added to the test tubes and dried under nitrogen. Alkaline assay buffer (pH 9.0), alkaline SMase, and ^{14}C-sphingomyelin were then added. The activity of alkaline SMase was determined and the activity in the absence of lyso-PC was taken as 100%. Similar results were obtained from another two determinations.

Duan and Nilsson.[18] Similar experiments were performed as above with a 30 mM Tris buffer containing 0.15 M NaCl, 2 mM EDTA, and 6 mM TC, pH 7.4. When the role of phospholipids in SM hydrolysis in the absence of bile salts were studied, different amounts of PC, PE, PI, and PS were added to the test tubes and dried as described. Seventy-five microliters of 30 mM Tris buffer containing 0.15 M NaCl and 2 mM EDTA, pH 9.0, was added, followed by addition of alkaline SMase and ^{14}C-SM. The assay procedure thereafter was the same as described above.

Results

Effects of phospholipids on the activity of alkaline SMase

The effects of various phospholipids on the activity of rat intestinal alkaline SMase at the optimal pH (9.0) are shown in *Figure 1*. All phospholipids tested inhibited alkaline SMase in a dose-dependent manner. PA exerted the strongest inhibition, followed by PS, PI, and PC. The least effective phospholipid was PE. The molar ratio of phospholipids to SM at 50% inhibition of alkaline SMase for PE, PC, PI, PS, and PA were approximately 294, 128, 102, 64, and 32, respectively. Whether the inhibitory effect of phospholipids also occurs in the pH of the intestinal lumen was studied. As shown in *Figure 2*, all phospholipids tested at 16 μM also inhibited SM hydrolysis induced by alkaline SMase at pH 7.4. The inhibitions were more pronounced for acid phospholipids and the order of inhibition was largely the same as that at pH 9.0.

Effects of hydrolytic products of PC produced by phospholipase A2 on alkaline SMase activity

PC and PE are hydrolyzed by phospholipase A2 in the intestine to generate lysophospholipid and fatty acids.[23] The effects of the hydrolytic products of PC on intestinal alkaline SMase were studied. As shown in *Figure 3*, LPC also reduced the alkaline SMase activity dose-dependently. We then examined the effect of oleic acid, which was the fatty acid released from the type of PC used in this experiment. As shown in *Figure 4*, oleic acid up to a concentration of 8 μM did not influence alkaline SMase activity. However, 16 to 64 μM of oleic acid inhibited alkaline SMase activity dose-dependently.

Effects of hydrolytic products of PC produced by phospholipase C on alkaline SMase activity

Phospholipase C hydrolyzes PC to diacylglycerol and phosphorylcholine. The roles of these products were also studied. As shown in *Figure 5*, 1,2-dioleoyl glycerol inhibited intestinal alkaline SMase activity and the inhibition was less potent than that of either PC or LPC. On the contrary,

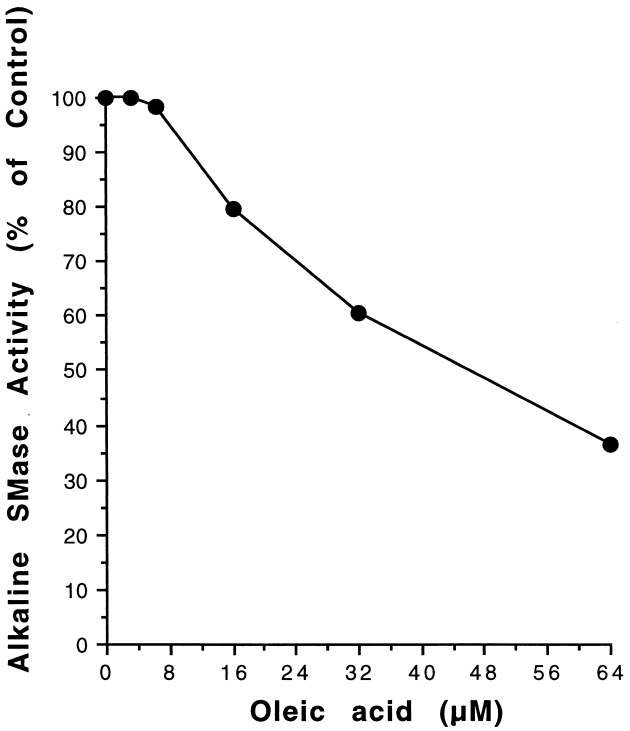

Figure 4 Effects of oleic acid on alkaline sphingomyelinase (SMase) activity. Oleic acid dissolved in chloroform:methanol (2:1) was added to the test tubes and dried under nitrogen. The alkaline assay buffer (pH 9.0) containing alkaline SMase and ^{14}C-sphingomyelin (SM) was added thereafter. The SM hydrolysis in the presence of oleic acid was assayed. Activity in the absence of oleic acid was taken as 100%. Similar results were obtained from two additional experiments.

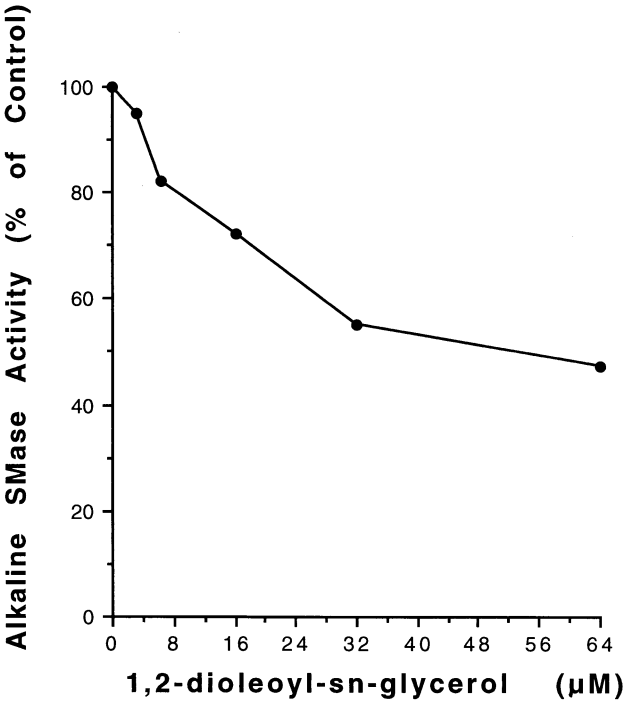

Figure 5 Effects of dioleoyl glycerol on alkaline sphingomyelinase (SMase) activity. 1,2-Dioleoyl glycerol dissolved in chloroform:methanol (2:1) was added to the test tubes and dried under nitrogen. The alkaline assay buffer (pH 9.0) containing alkaline SMase and ^{14}C-sphingomyelin (SM) were added thereafter. The SM hydrolysis was determined and the enzyme activity in the absence of 1,2-dioleoyl glycerol was taken as 100%. Similar results were obtained from two additional experiments.

phosphorylcholine dose-dependently enhanced the alkaline SMase activity (*Figure 6*).

Effect of phospholipids on alkaline SMase activity in the absence of bile salt

The intestinal alkaline SMase is bile-salt dependent and TC was the most effective phospholipid to activate alkaline SMase.[12] In the absence of bile salt, the addition of PS and PI increased the alkaline SMase activity and the high activity was maintained up to the concentration of 64 μM (*Figure 7*). The maximal effect is approximately 20% of that induced by 10 mM TC. The addition of PA at a low concentration also enhanced alkaline SMase activity, although to a smaller extent than PI and PS, followed by a rapid decline at higher concentration. However, the addition of PC and PE had little effect on alkaline SMase activity.

Discussion

Sphingolipids and glycerophospholipids are two major phospholipid classes that are present in cells as membrane constituents and in the intestinal tract as dietary components. Digestion of phospholipids, particularly PC, has been well studied whereas the digestion of SM has been ignored for about 25 years since the first study.[11] It was not until recently that the intestinal digestion of sphingolipids became an area of investigation.[15,17] However, the possible influence of phospholipids on the hydrolysis of sphingolipids by intestinal SMase has not been studied. In the present study, we demonstrated that various phospholipids and their hydrolytic products produced by phospholipase A2 and phospholipase C inhibit the hydrolysis of SM caused by intestinal alkaline SMase in vitro.

The mechanism by which phospholipids inhibit alkaline SMase may be related to the formation of mixed micelles of TC and phospholipids. Diacylglycerol, fatty acid, and virtually all the phospholipids tested significantly inhibited alkaline SMase activity in the presence of optimal concentrations of TC. The inhibition by phospholipids depended on their polar head groups. The inhibition was in the order of PA > PS > PI > PC > PE, indicating that the acidity of the phospholipids or their negative charges of phospholipids were also important. The inhibition occurred not only at the optimal pH of alkaline SMase, but also at the pH of middle small intestine,[21] indicating that such inhibitions may be physiologically relevant.

In the intestinal tract, the major phospholipid is PC. Dietary PC is derived mainly from milk and meat. The amounts of PC and SM in milk and most dairy products are largely equal.[1–3] In meat, PC is approximately 7 to 40 times higher than SM depending on the type of meat.[4] In the intestinal tract, bile delivers a large amount of PC to the intestinal tract, the PC:SM ratio in bile being as high as 240.[24] PE is the second most abundant phospholipid in the

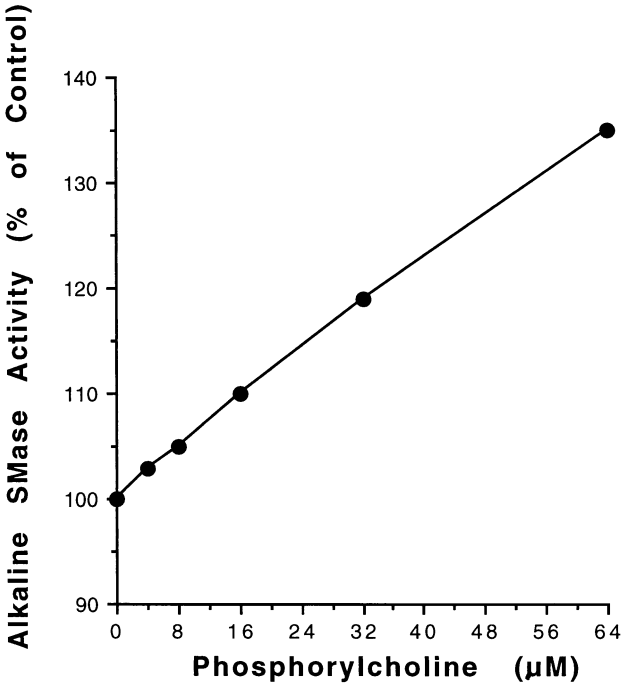

Figure 6 Effects of phosphorylcholine on alkaline sphingomyelinase (SMase) activity. Alkaline SMase was first added to the test tubes. Phosphorylcholine dissolved in 0.15 M NaCl was added to the alkaline assay buffer containing ^{14}C-sphingomyelin (SM). The buffer was then transferred to the test tubes and SM hydrolysis determined. SMase activity in the absence of phosphorylcholine was taken as 100%. Similar results were obtained from two additional experiments.

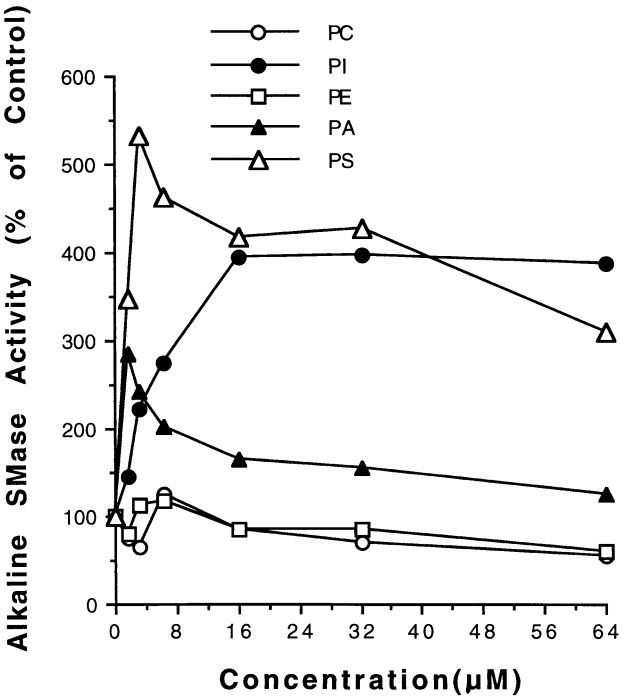

Figure 7 Effects of phospholipids on alkaline sphingomyelinase (SMase) in the absence of bile salts. Different amounts of phospholipids were added to the test tubes and dried under nitrogen. Intestinal alkaline SMase and ^{14}C-sphingomyelin (SM) were then added in the buffer containing 30 mM Tris, 0.15 M NaCl, and 2 mM ethylenediamine tetraacetic acid, pH 9.0. The hydrolysis of SM in the presence of different concentrations of phospholipids were determined. PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PA, phosphatidic acid; PS, phosphatidylserine. The activities in the absence of any phospholipid were taken as 100%. Similar results were obtained from two additional experiments.

diet and in bile. The level of PE in meat is approximately 3 to 10 times higher than that of SM.[4] Other phospholipids such as PI and PS occur in the diet in small amounts, but in most dietary products their levels are higher than that of SM. Therefore, in the intestinal tract, the total amount of glycerophospholipids is much higher than that of SM. These phospholipids may function as inhibitors of SM hydrolysis induced by alkaline SMase. The efficient digestion of SM may thus be delayed until most of the phospholipids have been hydrolyzed by phospholipases and their products such as fatty acids, diacylglycerols, and lysophospholipids have been absorbed. This might be one reason why hydrolysis of SM occurs mainly at the distal part of the jejunum[15] and why nonhydrolyzed SM is often found in the colon and feces even when fed in small amounts.[15,16] The effects of other types of lipids, particularly triacylglycerol, on SM digestion is unknown. Hydrolysis of triacylglycerol generates a high amount of fatty acids and diacylglycerols, which may also inhibit SM digestion in the intestine.

We also found a stimulatory effect of phosphorylcholine on SM hydrolysis caused by alkaline SMase. This might be important for dairy SM digestion in infants, because in human milk the level of phosphorylcholine is more than four times higher than that of SM.[3] The effects of head groups other than PC on SM hydrolysis were not studied, because under physiologic conditions, PC is the most abundant form of phospholipid in the gut.

The role of the pancreas in SM digestion was previously considered not to be important, because it does not contain and secrete SMases.[11,12] This concept needs to be reconsidered. Because PC is the major species of phospholipid in the gut, and hydrolysis of PC as well as PE is performed mainly by pancreatic phospholipase A2,[23] the pancreas may enhance the role of intestinal alkaline SMase by clearing of PC and PE. Whether pancreatic insufficiency may also cause problems in digestion of SM might be an interesting topic for further investigation.

Finally, we found that in the absence of bile salt, the addition of acid phospholipids such as PS, PA, and PI at certain concentrations enhanced alkaline SMase activity. However, the stimulatory actions were less than 20% of the effect of 10 mM TC. Previous studies have shown that bile salts, particularly TC, up to the critical micelle concentrations stimulated alkaline SMase activity.[25] The stimulatory effects of phospholipids may be related to their amphiphilic properties, leading to the formation of vesicles. Why only acid phospholipids increase alkaline SMase activity is not clear. Our finding is also reminiscent of previous studies showing that some acid phospholipids enhanced neutral SMase activity in the presence of Triton X-100.[26,27] Whether the mechanisms involved are similar requires further investigation.

Acknowledgment

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