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Identification of Bisphosphatidic Acid and Its Plasmalogen Analogues in the Phospholipids of a Marine Bacterium

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A relatively nonpolar unidentified phospholipid (phospholipid X), isolated from the gram-negative marine bacterium MB 45, was characterized both chromatographically and by chemical analysis. Phospholipid X was shown to be an acidic phospholipid without vicinal hydroxyl, free-amino, or amide groups. The presence of *O*-alkenyl groups was indicated by a positive reaction for plasmalogen. Mild alkaline methanolysis of phospholipid X yielded only glycerophosphorylglycerol as the derivative. Acetolysis produced only diacylglycerol monoacetate. Cleavage of *O*-alkenyl chains by methanolic hydrochloride resulted in the formation of three lyso derivatives. It was estimated that 18.2% of phospholipid X was plasmalogen. From these data, together with chromatographic comparisons with standards, infrared spectra, a molecular weight estimation, and the determination of the glycerol-phosphate-acyl ester ratio, it was concluded that phospholipid X was bisphosphatidic acid mixed with its plasmalogen analogues.

In the analyses of the phospholipids of several marine bacterial isolates, several unidentified phospholipids were detected (9). One unidentified phospholipid (PX) was relatively nonpolar and found in almost all the isolates examined. The general occurrence of PX suggested that it might have an important function in the membranes of marine bacteria. The marine isolate MB 45 was chosen as a source of this phospholipid because of its comparatively simple lipid composition and the absence of cardiolipin, which, also being a relatively nonpolar lipid, could interfere with the purification of PX. Analysis of the deacylated derivative of PX and its chromatographic properties indicated that PX is an acylated analogue of phosphatidylglycerol (PG).

At least two phospholipid structures fit the characteristics of PX. One structure is acylphosphatidylglycerol (lyso-bisphosphatidic acid) which has been found in gram-negative bacteria, (7, 14), animal (5, 6) and plant (8) tissues, drug-induced lipidosis (26), and Niemann-Pick disease (18). Another possible identity of PX was the completely acylated derivative of PG, bisphosphatidic acid (BPA). This lipid has not been definitely identified in natural sources although BPA-like structures have been reported in fish lipids (13).

The chromatographic and chemical analysis reported in this study identified PX as BPA mixed with plasmalogen analogues. The increasing number of studies identifying acylated derivatives of PG in biological sources and also their appearance as a result of disease suggest that these lipids may have important effects on membrane function and structure.

MATERIALS AND METHODS

Origin and growth conditions of the marine bacterium MB 45. MB 45 is a gram-negative, nonfermentative, rod-shaped organism which is motile by means of a polar flagellum. It is a member of a collection of marine bacterial isolates which were selected on the basis of their inability to grow without the addition of seawater (22).

Cultures of MB 45 were grown in a medium containing 10 g of Trypticase (BBL), 6 g of tris-(hydroxymethyl)aminomethane (Tris) base (Sigma Chemical Co., St. Louis, Mo.), 1.0 ml of 0.3% ferrous sulfate in 2.0% HCl, brought up to 1 liter with artificial seawater. Artificial seawater was prepared as described (9). The medium was adjusted to pH 7.4 with HCl and sterilized. Cultures were grown at room temperature for 48 h in two, 5-liter fermentors (New Brunswick Scientific Co., New Brunswick, N. J.) each containing 3.25 liters of medium and 1 to 2 ml of antifoam A emulsion (Sigma Chemical Co.). Cells were harvested by centrifugation at 4 C and washed once with cold artificial seawater.

Lipid extraction. Total lipid was extracted according to the method of Bligh and Dyer (4) and stored as described (9).

Chromatography of phospholipids. Total lipid samples were separated on Whatman SG81 silica gel-loaded paper developed ascending in one direction

with solvent systems based on those of Wuthier (25). Solvent A is an acid solvent consisting of chloroform, methanol, 2,6-dimethyl-4-heptanone, acetic acid, and water, 90:30:60:40:8 (vol/vol). Solvent B, a basic solvent, consisted of chloroform, methanol, 2,6-dimethyl-4-heptanone, pyridine, and 0.5 M ammonium chloride buffer (pH 10.4), 60:50:50:70:16 (vol/vol).

Two-dimensional ascending chromatograms were run with solvent A in the first direction and solvent C consisting of chloroform, methanol, 2,6-dimethyl-4-heptanone, pyridine, 0.5 M ammonium chloride buffer (pH 10.4), 60:20:60:70:10 (vol/vol), in the second direction. Purification of individual phospholipids was accomplished by applying a continous heavy streak of total lipid on sheets of silica gel-loaded paper and developing ascending for 7 to 8 h in solvent D which was similar to solvent B except the molarity of the ammonium chloride buffer was increased from 0.5 M to 2.6 M. This solvent was modified to provide better preparative separation. PG and phosphatidylethanolamine were also purified using either solvent A or B. Test strips were cut from the edge of dried chromatograms and stained with rhodamine 6G to localize the lipid bands (12). The corresponding bands on the unstained chromatograms were cut out and the phospholipids were eluted from the paper with chloroform-methanol (1 + 2, vol/vol). Two-dimensional chromatography was used to confirm the purity of the isolated phospholipids. A yield of 8.3 mg of PX was obtained.

Tests performed directly on chromatograms. Lipids were stained with 0.0012% rhodamine 6G (12). Acidic phospholipids appeared blue or purple and neutral phosphatides, tri-, di-, and monoglycerides and free fatty acids stained yellow or orange under ultraviolet illumination. Free amino groups were detected by the ninhydrin test (12); phosphate-containing lipids by an ammonium molybdate reagent (2); vicinal hydroxyl groups by the periodate test (23); amide groups using a Clorox-benzidine spray (20); and plasmalogens by spraying chromatograms with 0.4% dinitrophenylhydrazine in 2 N HCl (Reitsema spray) (15).

Samples of ^{32}P-labeled lipid extracts were separated by two-dimensional chromatography. The phospholipids were labeled by growing MB 45 in ASW-Trypticase medium to which [^{32}P]orthophosphate had been added. Autoradiograms were made on washed and stained chromatograms using Dupont Cronex X-ray safety film (E. I. Dupont De Nemours and Co., Wilmington, Del.).

Infrared spectroscopy of phospholipids. The infrared absorption spectra of phospholipids were obtained using a Perkin-Elmer model 457 grating infrared spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.). A gravimetrically determined sample of phospholipid (5 mg) dissolved in a small volume of $CHCl_3$ was pipetted onto the surface of a single, chloroform-washed, sodium chloride disk. The solvent was evaporated and the sample was scanned for absorption in the 3- to 16-μm wavelength range.

Mild alkaline methanolysis. Deacylated derivatives of phospholipids were produced by mild alkaline methanolysis as described by White and Frerman (24). Water-soluble derivatives were chromatographed ascending on Whatman no. 1 paper previously washed with 2 N HCl. Solvent systems used were solvent E, phenol, water, acetic acid, ethanol, 50:22:3:3 (vol/vol) (10), and solvent F, ethanol, 1 M ammonium acetate (pH 7.5), 65:35 (vol/vol) (7).

Acetolysis. Acetylated phospholipid derivatives were produced by acetolysis according to the method of Renkonen (17). The derivatives were dissolved in chloroform and ascending chromatographs prepared on Whatman SG81 paper in solvent G, hexane, 2,6-dimethyl-4-heptanone, 100:10 (vol/vol), and solvent H, petroleum ether, diethyl ether, 100:8 (vol/vol). Lipid spots were detected with rhodamine 6G. Standards, which were treated identically and processed simultaneously with PX, were 1-monopalmitate (Serdary Research Lab., London, Ontario, Canada), tripalmatin (Supelco, Inc., Bellefonte, Pa.), and PG (isolated from MB 45).

Cleavage of O-alk-1-enyl chains of plasmalogens. The selective removal of alk-1-enyl chains from plasmalogens of PX and estimations of the amount of the plasmalogen analogues present were accomplished by modification of the method of Singh et al. (19). PX, labeled with ^{32}P and purified as described for unlabeled lipid, was spotted on Silica Gel G thin-layer plates (Analtech, Inc., Newark, Del.) and developed two dimensionally. Solvent I, chloroform, methanol, 2.6 M ammonium chloride buffer (pH 10.4), 70:17:2 (vol/vol), was used for the first dimension to separate PX from any remaining impurities. After dying, the plate was masked with paper to expose only the chromatographed lipid strip, sprayed with 12% HCl in methanol, dried, and exposed to ammonia vapors. Solvent H was also used for the second dimension development to separate lyso derivatives of PX. Autoradiograms were prepared, the corresponding labeled spots on the plates were scraped into planchets, and the amount of radioactivity was determined.

Quantitative tests. Glycerol was determined by the method of Renkonen (16). DL-α-Glycerophosphate dissolved in methanol was used to prepare a standard curve. An absorbance of 0.37 at 570 nm in a Spectronic 20 corresponded to 0.5 μmol of glycerol.

Total phospholipid phosphate was determined as described by Ames and Dubin (1). DL-α-Glycerophosphate was used to prepare a standard curve. An absorbance of 0.45 at 820 nm on a Spectronic 20 corresponded to 0.05 μmol of phosphate.

The amount of acyl ester was determined by the methods of Snyder and Stephens (21). Tripalmitin (Supelco, Inc., Bellefonte, Pa.) was used to prepare a standard curve. PG (purified from MB 45) was used as a control and quantitated simultaneously with PX.

RESULTS

An autoradiogram of a two-dimensional chromatogram of ^{32}P-labeled phospholipids of MB 45 is shown in Fig. 1. PX, labeled BPA, constitutes 1.9% of the total phospholipid. PX and another unidentified phospholipid, number 1,

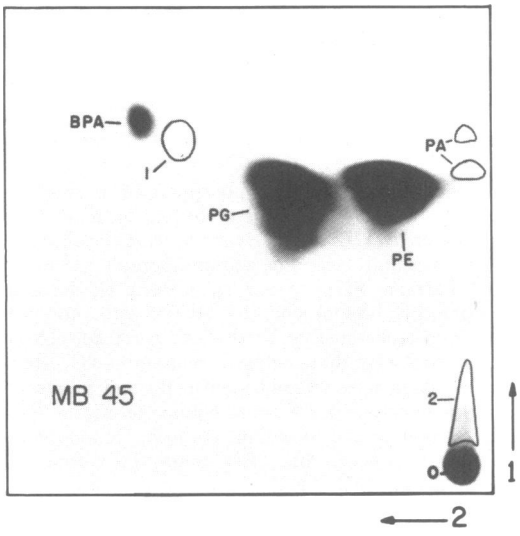

FIG. 1. *Autoradiogram of a two-dimensional chromatogram of the ^{32}P-labeled phospholipids of MB 45. Minor phospholipids which may not show up in the photograph are circled. Solvents for directions 1 and 2 are given in Materials and Methods. PE, Phosphatidylethanolamine; PA, phosphatidic acid; O, origin. Phospholipids 1 and 2 are unidentified.*

were the most nonpolar phospholipids detected in MB 45. A comparison of the R_f values of PX and known phospholipid standards determined in several solvent systems is given in Table 1. In all solvent systems PX had R_f values similar to those of synthetic BPA.

Tests performed directly on chromatograms (Table 2) to determine the nature of the chemical groups present in PX indicated that it was an acidic phospholipid without vicinal hydroxyl, free-amino or amide groups. A positive reaction for plasmalogens indicating the presence of alkenyl hydrocarbon chains was observed only with PX.

The nature of the backbone of PX was determined by mild alkaline methanolysis (Table 3). Chromatography of the deacylated derivates of PX, PG, and cardiolipin in two solvent systems showed that PX was similar to PG in having glycerophosphorylglycerol as the backbone structure. These results indicated the possibility that PX was an acylated derivative of PG.

Two acylated derivates of PG have been reported, acylphosphatidylglycerol and BPA, which can be distinguished by analyzing the derivatives produced by acetolysis (17). Theoretically, both PG and BPA would produce only one acetolysis derivative, 1,2-diacylglycerol

TABLE 1. *R_f values of phospholipids chromatographed on silica gel-loaded paper*

Phospholipid	R_f values of solvent systems:[a]		
	A	B	C
Phosphatidic acid	0.69	0.05	0.04
Phosphatidylethanolamine	0.63	0.43	0.16
Cardiolipin	0.80	0.63	0.33
Phosphatidylglycerol	0.60	0.63	0.36
Bisphosphatidic acid (tetrapalmitoyl)[b]	0.78	0.79	0.62
Phospholipid X	0.77	0.79	0.62

[a] See Materials and Methods for composition of solvent systems.

[b] Obtained from Serdary Research Lab., London, Ontario.

TABLE 2. *Results of tests performed on chromatograms for phospholipid X[a]*

Test reagent	Result	Interpretation
Rhodamine 6G stain	Blue spot	Acidic phosphatide
Phosphate spray	Blue spot	Phosphate present
Periodate-Schiff reagent	NR[b]	No vicinal hydroxyls
Reitsema spray	Orange spot	Plasmalogen form present
Ninhydrin reagent	NR	No free-amino group
Clorox-benzidine spray	NR	No amide group

[a] Tests were performed on the chromatograms after development in solvent B.

[b] NR, No reaction as compared to a positive control.

TABLE 3. *Chromatographic comparison of deacylation derivatives of phospholipids*

Phospholipid	Deacylated derivative	Rgp[a]	
		Solvent E	Solvent F
	Glycerophosphate	1.00	1.00
Phosphatidylglycerol	Glycerophosphorylglycerol	1.20	2.01
Cardiolipin	Glycerophosphorylglycerophosphorylglycerol	0.87	1.38
Phospholipid X		1.24	1.99

[a] Rgp, R_f using glycerophosphate as an internal standard with a mobility of 1.00 units.

TABLE 4. *R_f values of acetolysis products of phospholipid X and lipid standards*

Lipid	Acetolysis products	R_f solvent G[a]	R_f solvent H[a]
Tripalmitin	None		
Phosphatidylglycerol	1,2-Diacylglycerol monoacetate	0.46	0.40
	1,3-Diacylglycerol monoacetate[b]	0.57(Tr)[c]	0.49(Tr)
1-Monopalmitin	Monoacylglycerol diacetate	0.28	0.38
Bisphosphatidic acid (tetrapalmitoyl)	1,2-Diacylglycerol monoacetate	0.46	0.40
	1,3-Diacylglycerol monoacetate[b]	0.57(Tr)	0.49(Tr)
Phospholipid X	1,2-Diacylglycerol monoacetate	0.46	0.40
	1,3-Diacylglycerol monoacetate[b]	0.57(Tr)	ND[d]

[a] See Materials and Methods for composition of solvent systems.

[b] 1,3-Diacylglycerol monoacetate is an artifact of acetolysis due to acyl migration in the diglyceride (12) and present only in minor amounts.

[c] (Tr), Trace.

[d] ND, Not detected.

monoacetate, whereas acylphosphatidylglycerol would produce equal proportions of 1,2-diacylglycerol monoacetate and monoacylglycerol diacetate. Results of acetolysis (Table 4) indicated that PX produced only 1,2-diacylglycerol monoacetate. Traces of 1,3-diacylglycerol monoacetate, produced from PX, PG, and BPA, have been shown to be artifacts of the procedure due to acyl migration (17).

The similarity of PX and PG was also evident in a comparison of their infrared spectra (Fig. 2), which were nearly identical. The greater absorption in PX at 6 μm was the most obvious difference in the spectra. Absorption at this wavelength can be due to the presence of vinyl ether groups (11) which are found in plasmalogens.

The possibility of plasmalogens being present in PX, indicated by the Reitsema reagent and infrared spectrum, was confirmed by the selective cleavage of *O*-alk-1-enyl groups with methanol-hydrochloride and chromatography of the resulting derivatives (Table 5). Four ^{32}P-labeled derivatives were observed on autoradiograms and together constituted 18.2% of the total PX. The derivatives are tentatively identified as tri-, di-, and monoacylglycerophosphorylglycerol and the nonacylated derivative, glycerophosphorylglycerol.

The ratios of glycerol-phosphate-acyl ester in PX were estimated from an average of three determinations to be 2.00:1.08:3.62. The theoretical ratio for BPA is 2:1:4. PG analyzed simultaneously with PX gave the expected ratios of 2.00:1.03:2.01. The lower than theoretical value obtained for ester groups in PX would be expected if a proportion of the hydrocarbon chains were alkenyl groups.

An estimation of the molecular weight of PX based on the moles of phosphorus in a weighed sample showed it to be similar to the theoretical molecular weight of BPA (Table 6).

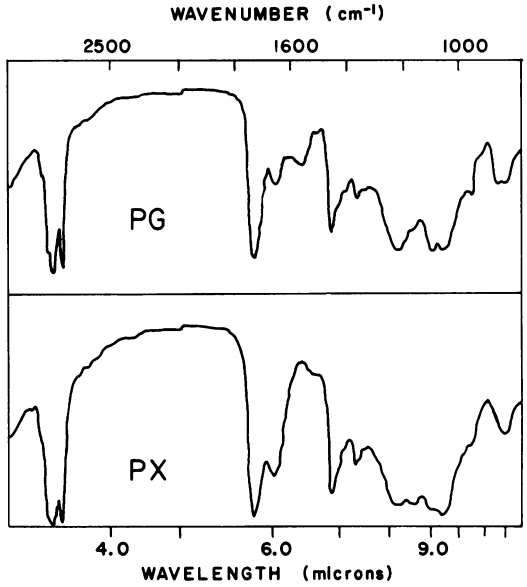

FIG. 2. *Infrared spectra of PG and PX.*

DISCUSSION

A relatively nonpolar phospholipid, found in the gram-negative marine isolate MB 45 and several other marine strains analyzed (9), was separated, purified and identified both chromatographically and by chemical analysis to be BPA. The presence of BPA had been suspected in *Escherichia coli* (3) but was later shown to be acylphosphatidylglycerol (7) which had been identified previously in *Salmonella*

TABLE 5. *Detection of O-alkenyl groups in ^{32}P-labeled phospholipid X after treatment with methanolic hydrochloride*

Determination	R_f solvent I[a]	% Total lipid phosphorous	Tentative identification of derivatives
Phospholipid X	0.63	81.8	
Derivative 1	0.54	11.3	Triacylglycerophosphorylglycerol
Derivative 2	0.41	3.2	Diacylglycerophosphorylglycerol
Derivative 3	0.29	2.6	Monoacylglycerophosphorylglycerol
Derivative 4	0	1.1	Glycerophosphorylglycerol

[a] See Materials and Methods.

TABLE 6. *Comparison of theoretical and experimentally determined molecular weights of phosphatidylglycerol and related phospholipids*

Phospholipid	Theoretical mol wt[a]	Experimental mol wt[b]
Phosphatidylglycerol	723	775
Acylphosphatidylglycerol	961	
Bisphosphatidic acid	1,200	
Phospholipid X		1,280

[a] Molecular weight was calculated with palmitic acid as the acyl groups.

[b] Calculations were based on the weight of the sample related to its phosphate content and assuming each molecule contained only one phosphate group.

typhimurium (14). However, the presence of BPA, which can be thought of as completely acylated PG, has not been shown previously to occur in bacteria.

PX, after extensive chemical analysis, fits all the criteria for BPA. PX has the same glycerophosphorylglycerol structure as BPA and its ratio of glycerol-phosphate-ester groups is in agreement with the ratio of BPA if the presence of alkenyl groups is taken into account. Based on the tentative identification of the derivatives of methanolic-hydrochloride treatment, 7.5% of the hydrocarbon chains of PX would be nonester groups. Assuming four hydrocarbon chains per molecule of PX, the theoretical ratio of glycerol-phosphate-acyl ester would be 2.00:1.00:3.70. In fact, a ratio of 2.00:1.08:3.62 was found. The formation of three acylated or lyso derivatives by cleavage of the alkenyl groups of PX would also be the maximum number expected for BPA since acylphosphatidylglycerol would produce a maximum of two derivatives and PG only one acylated derivative. Acetolysis of PX indicated that it could not be acylphosphatidylglycerol as monoacylglycerol diacetate was not detected. Chromatographic comparisons with standards, infrared spectrum, and a molecular weight estimation are all in agreement with the identification of PX as BPA.

It is concluded that PX is BPA mixed with approximately 18% of its plasmalogen analogues. The data also suggest that the plasmalogen analogues may contain one to four alkenyl groups substituted for acyl groups. However, more detailed analyses of the plasmalogen content of the BPA of MB 45 would be necessary to definitely establish the structure of these analogues.

The function, biosynthesis, and distribution of BPA are areas which require further investigation since they may lead to a greater understanding of membrane function and adaptation to different environments.

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