Formation of bis(phosphatidyl)inositol and phosphatidic acid by phospholipase D action on phosphatidylinositol

Neville G. CLARKE, Robin F. IRVINE and Rex M. C. DAWSON

Department of Biochemistry, ARC Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

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Phosphatidylinositol is readily attacked by phospholipase D. Although some phosphatidic acid is produced by hydrolysis, the main products are bis(phosphatidyl)inositols most likely formed by a transphosphatidylation reaction between two substrate molecules.

It has been generally assumed that phospholipase D, which is abundant in many tissues of higher plants, will not attack phosphatidylinositol in spite of its wide specificity of action towards phospholipids (Galliard, 1973; Heller, 1978). Recently we have shown that in extracts of celery stalk, a tissue rich in phospholipase D (Quarles & Dawson, 1969), there is a phosphodiesterase (phospholipase C) that converts phosphatidylinositol into diacylglycerol and inositol 1:2-cyclic phosphate or inositol monophosphate (Irvine *et al.*, 1980). However, we show here that with extracts from cauliflower florets, a tissue rich in phospholipase D and low in phosphodiesterase activity, the former enzyme attacks phosphatidylinositol producing both phosphatidic acid (hydrolysis) and bis(phosphatidyl)inositol (phosphatidyl transfer).

Experimental

Phosphatidylinositol was prepared from pig liver phospholipids. Fractionation was carried out on an alumina column followed by purification by preparative t.l.c. (Hazlewood & Dawson, 1975). The final product was rich in arachidonic acid, was chromatographically homogeneous and produced only glycerophosphoinositol on deacylation by mild alkaline treatment.

A portion of the substrate (18 mg) in chloroform/methanol (1:1, v/v) solution was taken to dryness *in vacuo* and incubated with the following: 14 ml of 0.3 M-Tris/maleate buffer, pH 7.0; 4.9 ml of 75 mM-CaCl₂; 4.9 ml of freshly prepared 2 mM-sodium dodecyl sulphate; 23 ml of cauliflower floret enzyme preparation; 23 ml of diethyl ether. The cauliflower floret enzyme was prepared by blending the florets with an equal volume of water, squeezing through muslin and collecting the supernatant after centrifuging at 100 000 g for 60 min.

The mixture was incubated for 1–4 h at 27°C and treated with 131 ml of chloroform and then, after shaking, with 66 ml of methanol. On mixing and centrifuging, the lower phase was recovered, taken down to low volume and put on t.l.c. plates (two to three plates, silica gel Merck 254) as a strip for preparative chromatography. After development of the plates in chloroform/methanol/conc. NH₃/water (65:35:2:3, by vol.), the lipoidal metabolic products formed from phosphatidylinositol were located by spraying with 2,4-dichlorofluorescein containing 1 mg of butylated hydroxytoluene/100 ml. The strips were scraped, the silicic acid inserted into short columns with sintered glass discs at the ends and the lipids were eluted with chloroform/methanol (1:1, v/v). The eluates were taken down to dryness and dissolved in a small amount of chloroform for analysis. Analysis of lipid groups was as follows: phosphate (Bartlett, 1959); inositol (Dawson & Freinkel, 1961); glycerol (Hazlewood *et al.*, 1980); fatty acyl ester (Renkonen, 1961). Deacylation of the phospholipids was carried out by using transacylation to monomethylamine followed by paper ionophoresis in volatile buffers at pH 3.6 or 9 (Clarke & Dawson, 1981). Acetolysis of phospholipids was carried out by heating about 20 µg of lipid phosphorus with 0.2 ml of acetic acid/acetic anhydride (3:2, v/v) for 3 h at 145°C. The mixture was evaporated *in vacuo* and the residue was heated for 5 min at 100°C in 2 M-HCl (1 ml) to hydrolyse any cyclic structures formed, and the acid was removed by evaporation at low temperature.

Results

T.l.c. of the lipids present in the incubation mixture showed extensive breakdown (>60%) of the phosphatidylinositol within 1 h. Although some water-soluble phosphorus had been released, prob-

Table 1. Analysis of two major components formed by the action of phospholipase D on phosphatidylinositol

	Molar ratios based on phosphate group			
	Phosphate	Glycerol	Inositol	Fatty acid ester
U1	2.0	2.20	1.15	4.12
U2	2.0	2.14	1.09	3.76

ably through phosphodiesterase action (Irvine *et al.*, 1980), the main lipid products were two unknown components running close together but faster than the substrate (U1, R_F 0.51, 25% of lipid phosphorus formed; U2, R_F 0.46, 64% of lipid phosphorus formed; phosphatidylinositol, R_F 0.29). The other phospholipids formed were phosphatidic acid (R_F 0.13, 8% of lipid phosphorus formed) and a minor (3% of lipid phosphorus formed) fast-running component (R_F 0.68), which was not further investigated. More prolonged incubation increased the yield of phosphatidic acid and water-soluble phosphorus formed but the unknown components U1 and U2 were still predominant even after 24 h. Leaving out sodium dodecyl sulphate from the incubation medium, although decreasing the extent of the catabolism of phosphatidylinositol, did not alter the general picture of the metabolites produced. Similar products were obtained with a commercial purified specimen of cabbage phospholipase D (Sigma; 75 units/mg of protein).

Both U1 and U2 were isolated by preparative t.l.c., and when rerun in the same chromatographic system ran independently to each other. Analysis of these components (Table 1) indicated that both approximated to the stoichiometric ratio for phosphorus/glycerol/inositol/fatty acyl ester of 2:2:1:4 (Table 1), suggesting that they were bis(phosphatidyl)-inositol formed by the transfer of a phosphatidyl moiety to the inositol ring of a substrate molecule. Deacylation of U1 and U2 gave water-soluble phosphorus products that could not be separated by paper ionophoresis at pH 3.6 and pH 9.0 and with mobilities distinct from other inositol- and/or glycerol-containing phosphate esters (Table 2). Acid hydrolysis (2M-HCl, 10 min, 100°C) of the deacylation products from U1 and U2 produced predominantly glycerophosphate with a small amount of inositol monophosphate, whereas with the deacylation product of U2 a trace of bis(phospho)inositol was also visible on the ionophoretogram. Similar phosphorus-containing products were obtained by a direct acid hydrolysis of the intact phospholipids (U1, U2).

To obtain unequivocal evidence for a dual esterification of phosphate groups to the inositol ring [as would occur if the substances were bis(phosphatidyl)inositols], attempts were made to hydrolyse

Table 2. Ionophoretic mobilities of deacylation products of U1 and U2 in comparison with some other phosphate esters

	M_{P_i}	
	pH 9 buffer	pH 3.6 buffer
Deacylation products of U1 and U2	0.65	0.89
Glycerophosphoinositol	0.49	0.65
Inositol 1-phosphate	0.73	0.72
Glycerol 1-phosphate	0.85	0.85
Glycerol 1:2-cyclic phosphate	0.80	1.04

first the intact phospholipids (U1, U2) with phospholipase C (*Bacillus cereus*) and secondly the deacylation products with a purified preparation of glycerophosphoinositol phosphate hydrolase (Dawson & Hemington, 1977). No bis(phospho)inositol could be detected even though the enzymes produced inositol monophosphate (or its cyclic form) from phosphatidylinositol and glycerophosphoinositol respectively.

However, the acetolysis procedure was much more successful and bis(phospho)inositol was formed in a yield of 89% from U2 and 75% from U1. The same procedure produced bis(phospho)inositol from phosphatidylinositol phosphate (diphosphoinositide) and inositol monophosphate from phosphatidylinositol. On ionophoresis at pH 3.6 a single bis(phospho)inositol spot was visible (M_{P_i} 1.05), whereas at pH 9.0 a main spot was visible at M_{P_i} 0.91 with subsidiary spots at 0.84 and 0.96. Similar products were observed after the acetolysis of diphosphoinositide; these are presumably equilibrium isomers of bis(phospho)inositol, formed possibly by acid phosphoryl migration, which can be separated by ionophoresis at high pH values.

Discussion

The present results clearly indicate that phosphatidylinositol can be attacked by phospholipase D. However, although the enzyme is acting as a hydrolase with some liberation of phosphatidic acid, the main reaction catalysed appears to be pre-

dominantly a transferase activity, with a phosphatidyl unit from one molecule of substrate being transferred to a second molecule (transphosphatidylation). The basic structure of the products is indicated by the group analytical data, and the finding that bis(phospho)inositol is formed in substantial yield on acetolysis and glycerophosphate on alkaline deacylation followed by acid hydrolysis. The most likely explanation of the two products formed by phospholipase D action with closely similar properties is that the phosphatidyl unit may have been transferred to different positions of the five vacant (2–6) hydroxy groups available on the inositol ring of the acceptor phosphatidylinositol. This might cause slightly different mobilities of the acylated products on t.l.c., yet any electrokinetic differences may be insufficient to cause dissociation on ionophoresis of the deacylated water-soluble products.

The ability of phospholipase D to transfer a phosphatidyl moiety from phospholipids to other acceptors apart from water is well known (Yang *et al.*, 1967; Dawson, 1967) and by using water-soluble aliphatic primary alcohols, a whole variety of phospholipids can be formed from phosphatidylcholine, e.g. phosphatidylethanol and phosphatidylethylene glycol, as well as phosphatidic acid, the product of hydrolase action. The present reaction is, however, more equivalent in principle to the ability of the enzyme to form cardiolipin [bis(phosphatidyl)glycerol] by the action of phospholipase D on phosphatidylglycerol (Stanacev *et al.*, 1973) and, in fact, the compounds from phosphatidylinositol are inositol-containing analogues of cardiolipin. We have repeated the precise incubation conditions described by Stanacev *et al.* (1973) and confirmed that, with phosphatidylglycerol as substrate, phospholipase D produced traces (<1% yield) of a substance that behaved chromatographically like cardiolipin. However, in contrast with a phosphatidylinositol substrate, phosphatidic acid was

always the major product formed from phosphatidylglycerol. This parallels the greater superiority of the inositol moiety compared with glycerol as an acceptor during intramolecular cyclizing catalysed by phospholipase C (Michell & Allan, 1975). In addition we also detected substantial amounts of phosphatidylethanol, which presumably is due to transphosphatidylation from the phosphatidylglycerol to ethanol present in the ether added to activate the reaction in the Stanacev *et al.* (1973) incubation system.

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