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Phosphatidylinositol-specific phospholipase C of *Bacillus thuringiensis* as a probe for the distribution of phosphatidylinositol in hepatocyte membranes

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Phosphatidylinositol-specific phospholipase C (PI-PLC) produced by *Bacillus thuringiensis* has been used as a probe for the distribution of phosphatidylinositol in hepatocyte membranes. Approx. 50% of this phospholipid was hydrolysed in microsomal vesicles (endoplasmic reticulum) with no significant hydrolysis of the remaining membrane phospholipids. Latency of mannose-6-phosphatase was retained during treatment indicating that the vesicles remained impermeable. Stripping of the ribosomes did not increase hydrolysis of phosphatidylinositol; however, when the vesicles were opened using dilute sodium carbonate, hydrolysis increased to > 90%. Hydrolysis of phosphatidylinositol of Golgi membranes was 35% and of plasma membranes was 50%. After treatment with PI-PLC, radiolabelled secretory proteins were retained in Golgi membranes and trapped lactate dehydrogenase was retained in plasma-membrane preparations indicating that the vesicles remained closed. Hydrolysis of phosphatidylinositol increased to > 90% when the membranes were opened by treatment with dilute sodium carbonate. These observations indicate that PI-PLC of *Bacillus thuringiensis* is a suitable probe for the distribution of phosphatidylinositol in membranes, and that in liver membranes this phospholipid occurs on each side of the bilayer, a topography consistent with its diverse roles.

INTRODUCTION

Inositol phospholipids have been implicated in three important cell functions: (i) plasma-membrane phosphatidylinositol and its phosphorylated derivatives are the source of second messengers for hormones acting at the cell surface (Michell, 1977; Berridge & Irvine, 1984; Berridge, 1987), (ii) glycosyl-phosphatidylinositol in the plasma membrane acts as a covalently-linked anchor for a large number of cell surface proteins (Ferguson & Williams, 1988; Low & Saltiel 1988), (iii) phosphatidylinositol-glycans in which the oligosaccharide is directed towards the cytosol have recently been implicated as mediators of the action of insulin (Low & Saltiel, 1988). In order to understand the mechanisms involved in the biosynthesis of these disparate inositol phospholipids, it is necessary to establish the intramembrane topography of the phosphatidylinositol. Despite its importance, however, there is little direct information available concerning the topography of phosphatidylinositol in biological membranes. A potentially useful tool for investigations of membrane topography is the phosphatidylinositol-specific phospholipase C (PI-PLC), produced by bacteria, which has been used extensively in studies of phosphatidylinositol-anchored proteins. Previous studies with PI-PLC of *Staphylococcus aureus* showed that less than 5% of the phosphatidylinositol of mammalian erythrocytes was available for hydrolysis, but that this increased to 85–95% in permeable ghosts, suggesting that phosphatidylinositol is located on the cytoplasmic aspect of the membrane (Low & Finean, 1977). In the present study we have investigated the suitability of the enzyme from *Bacillus thuringiensis* as a probe for the distribution of phosphatidylinositol in hepatocyte membranes.

MATERIALS AND METHODS

Purification of PI-PLC

PI-PLC was purified from *Bacillus thuringiensis* as described previously (Low *et al.*, 1988).

Preparation of subcellular fractions

Rat liver total microsomes. These were prepared as described previously (Higgins & Fieldsend, 1987). In some experiments they were stripped of ribosomes by treatment with pyrophosphate (Paiment *et al.*, 1980) and in others they were opened to form flat sheets using sodium carbonate (Fujiki *et al.*, 1982; Higgins & Hutson, 1984). The integrity of the microsomal vesicles after incubation with PI-PLC was determined by measurement of the latency of mannose-6-phosphatase (Arion *et al.*, 1976; Higgins, 1981).

'Trans-enriched' Golgi vesicles. These were prepared from rat liver as described previously (Higgins, 1984). Viewed in the electron microscope, this preparation consists mainly of large membrane-bound vesicles containing lipoprotein particles having the same orientation

Abbreviation used: PI-PLC, phosphatidylinositol-specific phospholipase C.

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as in the intact cell (Ehrenreich *et al.*, 1973; Bergeron, 1979; Higgins, 1984). The integrity of the Golgi membranes after incubation with PI-PLC was checked by assay of the loss of labelled secretory protein contents as described previously (Higgins, 1984). In some experiments the Golgi membranes were opened to form flat sheets by treatment with sodium carbonate (Howell & Palade, 1982; Higgins, 1984).

Plasma membranes. These were prepared from rat liver essentially as described by Hubbard *et al.*, (1983). The preparation characterized by these investigators contains 47% sinusoidal plasma membrane, 23% bile cannalicular membrane and 7% lateral surface membrane, and is contaminated with 15–20% endoplasmic reticulum. Our preparation was enriched 30-fold with 5′-nucleotidase and contained 15–20% endoplasmic reticulum, as measured by glucose-6-phosphatase and NADPH–cytochrome *c* reductase activity. The integrity of the plasma-membrane vesicles after incubation with PI-PLC was checked by determination of loss of lactate dehydrogenase trapped in the membrane vesicles during homogenization (Higgins & Evans, 1978). In some experiments, the membrane vesicles were opened by treatment with sodium carbonate (Hubbard & Ma, 1983).

Treatment of subcellular fractions with phospholipase C

Subcellular fractions were resuspended in 30 mM-Tris buffer/40 mM-KCl, pH 7.4 (2–5 mg/ml). Phospholipase C was added to give a final concentration of 3–10 units/ml (1 unit catalyses the hydrolysis of 1 μmol of phosphatidylinositol per min) and the sample incubated for a range of times at 37 °C. The final volume was 1.0 ml for investigations of endoplasmic reticulum and 0.2 ml for investigations of Golgi or plasma membranes. The reaction was stopped by addition of 20 vol. of chloroform/methanol (2:1, v/v). The lipids were extracted as described previously (Higgins & Dawson, 1977) and separated by t.l.c. on silica gel (Merck 60F 254) using propionic acid/propanol/chloroform/water (2:3:2:1, by vol.) as solvent system with pure phospholipid spots as markers. The phospholipid-containing bands were revealed using iodine vapour, and phospholipid phosphorus was measured as described previously (Higgins & Fieldsend, 1987) using a modification of the method of Bartlett (1959).

RESULTS AND DISCUSSION

When rat-liver microsomes were incubated with PI-PLC, hydrolysis of phosphatidylinositol was rapid, with approx. 49% hydrolysis after 30 min and no substantial change during the next 60 min. No other phosopholipid was hydrolysed (Fig. 1). In three separate experiments the latency of mannose 6-phospatase of PI-PLC-treated microsomes averaged 92.2% (Table 1), while that of freshly-prepared microsomes was 95.2%. Microsomes therefore remain closed during treatment with PI-PLC. In three separate experiments, hydrolysis of phosphatidylinositol increased to 93.63±3.89% in microsomes opened by sodium carbonate treatment to produce flat sheets of membrane in which both sides are available to PI-PLC in the medium (Table 1). Sodium carbonate treatment of microsomes results in stripping of the ribosomes from the membrane surface in addition to formation of flat sheets. It is possible, therefore, that

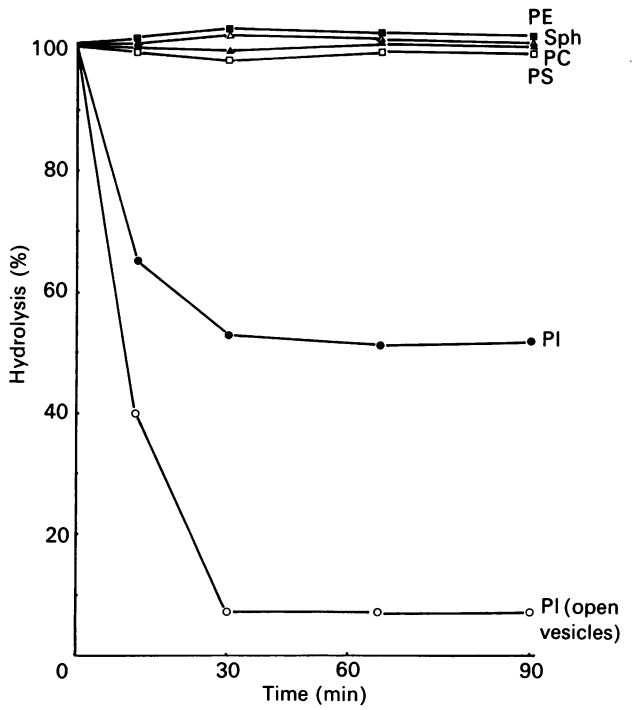

Fig. 1. Hydrolysis of phosphatidylinositol of microsomal membranes by phospholipase C of *Bacillus thuringiensis*

Microsomal vesicles and open sheets of membrane were prepared, incubated with PI-PLC and the lipids extracted and analysed as described in the Materials and methods section. Hydrolysis of individual phospholipids is plotted against time. The points are the averages of two determinations. There was no significant hydrolysis of phospholipids other than phosphatidylinositol (PI) in either open or closed vesicles, and only the data for hydrolysis of phosphatidylcholine (PC), sphingomyelin (Sph), phosphatidylethanolamine (PE), and phosphatidylserine (PS) for closed vesicles is plotted.

increased hydrolysis of phosphatidylinositol in opened vesicles is of the phosphatidylinositol normally protected by ribosomes. To check this, we prepared microsomal vesicles which were stripped of ribosomes but which remained sealed. The percentage hydrolysis of phosphatidylinositol in these vesicles was 41.92±2.54% in three separate experiments, similar to that in unstripped microsomes.

Our observations indicate that PI-PLC from *Bacillus thuringiensis* is an appropriate probe for investigating the distribution of phosphatidylinositol in membranes, and that phosphatidylinositol is present on both sides of the membrane bilayer in endoplasmic reticulum membranes. It is therefore topographically appropriately placed to serve as a precursor both for biosynthesis of phosphorylated forms of phosphatidylinositol at the cytosolic surface of the plasma membrane, and for biosynthesis of glycosylated forms of phosphatidylinositol which are located both intracellularly and at the cell surface.

Around 36% of the phosphatidylinositol of 'trans-enriched' Golgi membranes and 50% of the phosphatidylinositol of plasma-membrane vesicles were available for hydrolysis by PI-PLC (Table 1). This increased to more than 95% when the membranes were opened with sodium carbonate. During incubation there was no

Table 1. Availability of phosphatidylinositol for hydrolysis by phospholipase C from *Bacillus thuringiensis*

Subcellular fractions were prepared as described in the Materials and methods section and either used without treatment (intact vesicles) or opened with sodium carbonate (open vesicles). These were incubated with PI-PLC for 60 min at 37 °C, the lipids were extracted and the percentage composition of the phospholipids of each fraction and the percentage hydrolysis of the phosphatidylinositol determined as described in the Materials and methods section. The results are the average of four determinations ± S.D. In separate experiments the integrity of the membrane vesicles after treatment with PI-PLC compared with untreated vesicles was determined as described in the Materials and methods section. In microsomal membranes the integrity is expressed as the latency of mannose-6-phosphatase; in Golgi membranes as retention of labelled protein contents, and in plasma membranes as retention of lactate dehydrogenase. Sph, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

Subcellular fraction	Lipid	Composition (%)	Hydrolysis (%)		Membrane integrity (%)
			Intact vesicles	Open vesicles	
Endoplasmic reticulum (microsomes)	Sph	6.81 ± 0.77	46.42 ± 2.99	93.63 ± 3.89	92.5
	PC	50.20 ± 4.25			
	PS	7.28 ± 3.23			
	PI	12.64 ± 2.69			
	PE	23.10 ± 1.26			
Golgi membranes	Sph	10.79 ± 1.92	35.23 ± 0.81	94.33 ± 5.89	93.45
	PC	50.93 ± 3.15			
	PS	14.44 ± 0.77			
	PI	6.51 ± 0.43			
	PE	17.18 ± 1.25			
Plasma membranes	Sph	8.3 ± 0.68	49.99 ± 2.27	92.78 ± 4.20	100.00
	PC	55.1 ± 4.55			
	PS	13.7 ± 2.25			
	PI	5.0 ± 0.5			
	PE	22.1 ± 1.78			

significant loss of labelled secretory proteins from Golgi preparations, indicating that the vesicles did not become leaky (Table 1). This is consistent with our previous observations that Golgi membranes retain their integrity after treatment with phospholipase C from *Clostridium welchii* (Higgins, 1984). There is no unambiguous marker for the contents of plasma-membrane vesicles; however, we have previously used trapped lactate dehydrogenase as an indicator of the integrity of plasma-membrane vesicles during treatment with phospholipase C from *Clostridium welchii* (Higgins & Evans, 1978). On treatment of plasma-membrane vesicles with PI-PLC, there was no loss of trapped lactate dehydrogenase, indicating that vesicles did not become leaky.

Interpretation of our findings concerning the availability of membrane phosphatidylinositol for hydrolysis is complicated by the fact that it is impossible to obtain pure fractions of either Golgi or plasma membranes. The 'trans-enriched' Golgi fraction may contain endocytic vesicles derived from the plasma membrane (Kay *et al.*, 1984). Such vesicles would have the same topography as the Golgi vesicles, with the cytosolic side of the membrane to the outside. The percentage contribution of such endocytic vesicles to the Golgi preparation has not been reported, although from the morphological appearance of the fraction, which consists predominantly of larger lipoprotein-filled vesicles, this appears to be small (Erhrenreich *et al.* 1973; Higgins, 1984). A further factor to be taken into account in interpreting the results of experiments with Golgi membranes is the relatively large amount of phospholipid in the lipoprotein particles in the Golgi cisternae. However, in the present study this is not a problem as the phospholipids of the Golgi contents consist of phosphatidylcholine and sphingomyelin, with no detectable phosphatidylinositol (Higgins, 1984). We consider therefore that the hydrolysis of 36% of the Golgi phosphatidylinositol indicates that this phospholipid occurs in both leaflets of the membrane. In the case of the plasma membrane, interpretation is more complex because of the heterogeneity of isolated fractions. Plasma membranes tend to vesiculate and form outside-out vesicles on homogenization (Trams & Lauter, 1974; Losa, 1976; Schafer *et al.*, 1978; Higgins & Evans, 1978). Our results indicate that these vesicular elements do not become leaky during incubation, although it is not possible to exclude the presence of unsealed membranes derived from the lateral borders of hepatocytes. However, in order to account for 50% hydrolysis of phosphatidylinositol it would be necessary for the plasma membrane to comprise at least half unsealed membrane sheets. This is considerably more than estimated by Hubbard *et al.* (1983). Our results therefore suggest that phosphatidylinositol occurs on both sides of the plasma membrane of hepatocytes, a location consistent with its varied function in these cells.

Previous reports of the distribution of phosphatidylinositol in membranes other than those from erythrocytes have been indirect; the distribution of phosphatidylinositol has been inferred by subtraction. The only other direct investigation we are aware of is a preliminary investigation by Sundler *et al.* (1977) who found that about 60% of the phosphatidylinositol of Golgi membranes and microsomes was available for hydrolysis by a partially-purified PI-PLC from *B. cereus*. The qualitative differences between the findings of Sundler *et al.* (1977) and ourselves may be due to differences in the subcellular

fraction or in the PI-PLC. Sundler *et al.* (1977) did not perform control experiments to demonstrate that their membrane preparations remained closed after treatment with PI-PLC or that in open vesicles hydrolysis was close to completion. The higher level of hydrolysis they observed may have been a consequence of some leaky vesicles in their preparation.

The results reported here indicate that PI-PLC from *B. thuringiensis* is a useful probe for liver membranes and may be suitable for investigations of the topography of phosphatidylinositol in other membranes.

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REFERENCES

Arion, W. J., Ballas, L. M., Lange, A. J. & Wallin, B. K. (1976) J. Biol. Chem. **251**, 4901–4907
Bartlett, G. R. (1959) J. Biol. Chem. **234**, 466–468
Bergeron, J. J. M. (1979) Biochim. Biophys. Acta **555**, 493–503
Berridge, M. J. (1987) Annu. Rev. Biochem. **56**, 159–193
Berridge, M. J. & Irvine, R. F. (1984) Nature (London) **312**, 315–321
Ehrenreich, J. H., Bergeron, J. J. M., Siekevitz, P. & Palade, G. E. (1973) J. Cell Biol. **59**, 45–72
Ferguson, M. A. J. & Williams, A. F. (1988) Annu. Rev. Biochem. **57**, 285–320
Fujiki, Y., Hubbard, A. L., Fowler, S. & Lazarow, P. (1982) J. Cell Biol. **93**, 97–102
Higgins, J. A. (1981) Biochim. Biophys. Acta **640**, 1–15
Higgins, J. A. (1984) Biochem. J. **219**, 261–272
Higgins, J. A. & Dawson, R. M. C. (1977) Biochim. Biophys. Acta **470**, 342–356
Higgins, J. A. & Evans, W. H. (1978) Biochem. J. **174**, 563–567
Higgins, J. A. & Hutson, J. L. (1984) J. Lipid Res. **25**, 1295–1305
Higgins, J. A. & Fieldsend, J. L. (1987) J. Lipid Res. **28**, 268–278
Howell, K. E. & Palade, G. E. (1982) J. Cell Biol. **92**, 822–832
Hubbard, A. L. & Ma, A. (1983) J. Cell Biol. **96**, 230–239
Hubbard, A. L., Wall, D. A. & Ma, A. (1983) J. Cell Biol. **96**, 217–229
Kay, D. G., Khan, M. N., Posner, B. I. & Bergeron, J. J. M. (1984) Biochem. Biophys. Res. Commun. **123**, 1144–1148
Losa, G. (1976) Abstr. 10th Int. Congr. Biochem., Hamburg, p. 282
Low, M. G. & Finean, J. B. (1977) **162**, 235–240
Low, M. G. & Saltiel, A. R. (1988) Science **239**, 268–275
Low, M. G., Stienberg, J., Waneck, G. L., Flavell, R. A. & Kincade, P. W. (1988) J. Immunol. Methods **113**, 101–111
Michell, R. H. (1977) Biochim. Biophys. Acta **45**, 81–147
Paiment, J., Beaufay, H. & Godelaine, D. (1980) J. Cell Biol. **86**, 29–37
Schafer, A., Haase, W., Murer, W. & Kinne, R. (1978) Biochem. J. **172**, 57–62
Sundler, R., Sarcione, S. L., Alberlt, A. W. & Vegelos, P. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 3350–3354
Trams, E. G. & Lauter, C. J. (1974) Biochim. Biophys. Acta **345**, 180–197

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