The Action of Phosphatidylinositol-Specific Phospholipases C on Membranes

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A phospholipase C prepared from lymphocytes readily hydrolysed pure phosphatidylinositol but was relatively ineffective against phosphatidylinositol in erythrocyte 'ghosts' and rat liver microsomal fraction and also against sonicated lipid extracts from these membranes. In contrast, a phospholipase C prepared from *Staphylococcus aureus* readily hydrolysed phosphatidylinositol in sonicated lipid extracts but had only low activity against purified phosphatidylinositol. Unlike the enzyme from lymphocytes, the *S. aureus* phospholipase C did not require Ca^{2+} for its activity and was inhibited by cations. The previously reported specificity of this enzyme was confirmed by our observation of hydrolysis of approx. 75% of the phosphatidylinositol in ox, sheep and cat erythrocyte 'ghosts' together with no detectable effect on the major erythrocyte membrane phospholipids. The phosphatidylinositol of rat liver microsomal fraction was hydrolysed only to a maximum of 15%. Some preliminary experiments showed that approx. 60% of the phosphatidylinositol of ox or sheep erythrocytes could be hydrolysed without causing substantial haemolysis.

Turnover of the headgroup of phosphatidylinositol is specifically stimulated in a wide variety of tissues by a range of stimuli. The significance of this phenomenon is not fully understood, but it has been suggested that it may reflect the involvement of phosphatidylinositol in cell-surface receptor mechanisms (Michell, 1975). Thus, although phosphatidylinositol is quantitatively only a minor membrane phospholipid, it may play an important role in the reception and modulation of extracellular stimuli, and this has provoked an interest in its structural significance in membranes.

One approach to the assessment of the structural significance of phosphatidylinositol in membranes is to study the effects of modification *in situ* by phospholipases. Phospholipases C have been used extensively to modify phospholipids in biological membranes and have provided information relating to the distribution of some classes of phospholipids in the structure of the erythrocyte. The phospholipases C from *Clostridium perfringens* and *Bacillus cereus* have wide substrate specificities, but the sphingomyelinase C produced by *Staphylococcus aureus* only hydrolyses sphingomyelin and lysophosphatidylcholine. *S. aureus* was also reported to produce another phospholipase C which hydrolysed only phosphatidylinositol and lysophosphatidylinositol (Doery *et al.*, 1965). We have prepared both this phospholipase C and soluble phosphatidylinositol-specific phospholipase C from lymphocytes (Allan & Michell, 1974a) and have made a preliminary study of their effects on erythrocyte membranes and on liver microsomal fractions.

Experimental

Materials

A soya-bean lipid fraction designated 'phosphatidylcholine', type II S (commercial grade), was obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Analysis of this crude material showed that approx. 15% of the phospholipid was phosphatidylinositol. Several neutral lipid components were also distinguishable by t.l.c. This soya-bean lipid fraction was homogenized and dialysed as described by Doery *et al.* (1965) and kept at -20°C . Small batches were sonicated (see below) immediately before use in the phospholipase C assay.

Phosphatidylinositol and sphingomyelin were purified from pig liver (Lapetina & Michell, 1973) and bovine brain (Ansell & Spanner, 1961) respectively. Lipid extracts from tissues and membrane preparations were also used as substrates, and these were prepared as described by Bramley *et al.* (1971). The lower phases were evaporated to dryness and dissolved in chloroform. Chloroform solutions of the lipids (except the soya-bean lipid fraction) were evaporated to dryness under vacuum and substrate dispersions were prepared by sonication of the dried lipid in water with an MSE probe sonicator (60W) for approx. 10 min in an ice-water bath.

Protease peptone was obtained from Difco Laboratories (Detroit, Mich., U.S.A.).

Sheep and ox blood were collected at the local abattoir, with 10 mM-EDTA, pH 7.4, as anti-coagulant, and were used within 6 h of removal from the animal. Cat blood was obtained from animals which

had been injected with *p*-aminohippuric acid in studies of renal filtration conducted in the Department of Physiology, University of Birmingham. Heparin was used as anti-coagulant and the blood was used within 24 h of collection.

Erythrocytes were washed in 0.155 M-NaCl/1 mM-Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid]/NaOH, pH 7.0, and then (a) used for making erythrocyte 'ghosts' (see under 'Methods') or (b) further washed in 0.29 M-sucrose/10 mM-NaCl/1 mM-Hepes, pH 7.0, and used in experiments where intact erythrocytes were required.

Staphylococcus aureus (Newman) was obtained from Dr. J. H. Pearce of the Department of Microbiology, University of Birmingham.

Methods

Preparation of *S. aureus* culture supernatant. The growth medium used was similar to that described by Dolman & Wilson (1940); 22 g of Difco proteose peptone was dissolved in 500 ml of water and dialysed against 500 ml of water for 2 days. The resulting difusate, together with ammonium lactate (5 g), KH_2PO_4 (1 g), K_2HPO_4 (1 g), NaCl (5 g), $\text{Mg}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ (0.4 g) and $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (0.2 g), was made up to a final volume of 1 litre and the pH adjusted to 7.4. The medium was distributed between two 2-litre conical flasks and autoclaved. Each flask was inoculated with 3 ml of an overnight broth culture of *Staphylococcus aureus* (Newman) and incubated for 24 h at 37°C. Aeration of the cultures was performed by rotation at 250 rev./min. After removal of the bacteria by centrifugation, the supernatant was freeze-dried in an Edwards centrifugal freeze-dryer. The dried material was redissolved in approx. 50 ml of water, dialysed for 2 days at 4°C against water, freeze-dried and then stored at -20°C until used. This material exhibited a small amount of haemolytic activity that could be decreased by dialysing the freeze-dried supernatant against 50 mM-sodium acetate, pH 4.0, for 24 h. The non-diffusible material was centrifuged and the supernatant dialysed and freeze-dried as above. Most of the haemolytic activity, but only 5% of the phospholipase C, was associated with the precipitate. The supernatant, however, contained 75% of the phospholipase C. The total activity recovered from 1 litre of culture supernatant was 4000–8000 units. The specific activity was 2–4 units/mg of protein. [Protein was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.]

Assay for phospholipase C activity. The assay mixture contained 0.5 ml of sonicated soya-bean 'phosphatidylcholine' (see under 'Methods') (20 mg/ml), 0.2 ml of 100 mM-Hepes, pH 7.0, and 3.0 ml of a solution of the freeze-dried culture supernatant. Under these conditions the phosphatidylinositol concentra-

tion was approx. 0.8 mM. Incubation was for 2 h at 37°C and the reaction was stopped by adding 6 ml of chloroform/methanol/conc. HCl (500:500:3, by vol.). Samples of the upper aqueous phase were assayed for organic phosphorus by the method of Galliard *et al.* (1965). Because of the non-linearity (with time) of this assay and the low specific activity of the enzyme, 1 unit of phospholipase C activity was arbitrarily defined as the amount of activity releasing 0.1 μmol of organic phosphorus into the upper phase in 2 h at 37°C. The assay was used for comparative purposes only.

Phospholipase C activity against pure phospholipids was assessed by substituting sonicated suspensions of pig liver phosphatidylinositol (2 mM) or bovine brain sphingomyelin (5 mM) for soya-bean 'phosphatidylcholine' in the above assay.

Assay for haemolytic activity. δ -Haemolytic activity was assayed by the method of Caird & Wiseman (1970). The δ -haemolysin, unlike the α - and β -haemolysins, has relatively high activity against human erythrocytes. One haemolytic unit produces 50% haemolysis of a 1% (v/v) suspension of human erythrocytes (2 ml) after incubation for 1 h at 37°C followed by 16 h at 4°C.

Preparation of permeable erythrocyte 'ghosts'. Erythrocytes were washed with 0.155 M-NaCl/1 mM-Hepes, pH 7.0, and 20 ml of the washed packed erythrocytes was haemolysed with 290 ml of 1 mM-Hepes, pH 7.0, at 4°C. The haemolysate was centrifuged at 15000g for 20 min and the pellet washed with 3×280 ml of 10 mM-NaCl/1 mM-Hepes, pH 7.0, to remove haemoglobin. All the above operations were carried out at 4°C. 'Ghosts' were used either fresh within 5 h of haemolysis or were stored at -20°C.

Permeability of the 'ghosts' to macromolecules was assessed by suspending 'ghosts' in 5% (w/v) bovine serum albumin or dextran (mol.wt. 87000 approx.) and examining them under the phase-contrast microscope as described previously (Low *et al.*, 1973).

Preparation of rat liver microsomal fractions. Microsomal fractions were prepared by a method similar to that described by Wirtz & Zilversmit (1969). A rat liver homogenate was prepared in 0.25 M-sucrose/1 mM-EDTA, pH 7.4 (4 ml/g of tissue). The homogenate was centrifuged at 600g for 15 min and the supernatant centrifuged at 15000g for 20 min; these pellets were discarded. The supernatant was then centrifuged at 105000g for 60 min and the pellet was suspended to the volume of the original homogenate in 10 mM-NaCl/1 mM-Hepes, pH 7.0, and centrifuged at 105000g for 60 min. The washed microsomal fraction was finally suspended in 10 mM-NaCl/1 mM-Hepes to a phospholipid concentration of approx. 2 mM. Microsomal fractions were used on the day of preparation or were stored at -20°C until required.

Treatment of membranes with phospholipase C. A portion (0.5 ml) of washed erythrocytes, packed erythrocyte 'ghosts' or microsomal suspensions (all containing 1–1.5 μ mol of phospholipid), 0.1 ml of 100 mM-Hepes, pH 7.0, and 0.4 ml of freeze-dried culture supernatant (in the same buffer as the membranes) were incubated for 2 h at 37°C (control incubations contained no culture supernatant). The lipids were extracted either by the method of Bramley *et al.* (1971) or, for erythrocytes, by a modified extraction procedure in which 2.5 ml of methanol was added first, followed by 1.25 ml of chloroform. The extraction mixture was centrifuged and the residual pellet re-extracted with 1.5 ml of chloroform/methanol (1:2, v/v) and 0.4 ml of water. The pooled extracts were then washed by adding chloroform and 2 M-KCl (Bramley *et al.*, 1971). The lipids were analysed by chromatography on formaldehyde-treated papers as described previously (Galliard *et al.*, 1965). Incubations were carried out in triplicate and the whole of each lipid extract was applied to the chromatogram. The results are expressed as the ratio of the amount of phosphorus contained in the phosphatidylinositol spot to the amount in the fast-running spot containing the major phospholipids. If the assumption was made that no hydrolysis of these phospholipids took place, then the amount of phosphatidylinositol hydrolysis could be readily calculated from the change in this ratio during incubation with the phospholipase C.

Haemolysis of erythrocytes during these incubations was monitored by measuring the E_{425} of the supernatants.

Treatment of lipid extracts with *S. aureus* culture supernatants. Portions (0.5 ml) of a sonicated suspension of the lipid extract (containing 1–1.5 μ mol of phospholipid; see under 'Materials'), 0.2 ml of 100 mM-Hepes, pH 7.0, and 0.3 ml of freeze-dried culture supernatant dissolved in water were incubated for 2 h at 37°C. The incubation mixtures were extracted and analysed as described above for membranes.

Preparation of lymphocyte supernatant. The soluble fraction was prepared essentially as described by Allan & Michell (1974a), except that the freeze-thawed lymphocytes were diluted with an equal volume of 0.155 M-NaCl/1 mM-Hepes, pH 7.0, before centrifugation. The hydrolysis of phosphatidylinositol by this enzyme was determined in the presence of 10 mM-Hepes/2 mM-EDTA/2 mM-CaCl₂, pH 7.0, from the loss of phosphorus in the lower phase of the lipid extract (for pure phosphatidylinositol) or by the chromatographic procedure described above (for lipid mixtures).

Chromatography of lipids. Phosphatidylinositol was separated from the major phospholipids by chromatography on formaldehyde-treated papers (Galliard *et al.*, 1965). The major phospholipids were analysed on silicic acid-impregnated paper (Whatman SG81) in the solvent di-isobutyl ketone/acetic acid/benzene/

water (80:35:10:6, by vol.). Neutral lipids were identified by t.l.c. as described previously (Freeman & West, 1966).

Results

*Action of *S. aureus* culture supernatant on extracted lipids*

The culture supernatant caused the release of water-soluble phosphorus compounds when incubated with soya-bean lipid fraction or with rat liver lipid extracts. However, the culture supernatant showed relatively little activity towards pure phosphatidylinositol. Incubation with 10 mg/ml (20 units/ml; see under 'Methods') for 2 h at 37°C resulted in the hydrolysis of only 50 nmol of the pure phospholipid (1 mM).

The hydrolysis of phosphatidylinositol in several lipid extracts was confirmed by separation of the phosphatidylinositol from the major phospholipids by paper chromatography. Lipid extracts prepared from rat liver, cat erythrocyte 'ghosts' or rat liver microsomal fractions all showed 45–55% hydrolysis of phosphatidylinositol when incubated with 20 units/ml for 2 h at 37°C.

The substrate specificity of the enzyme was investigated by analysing the major phospholipids by chromatography on silicic acid-impregnated papers. Incubation of ox, sheep or cat 'ghost' lipid extracts with 50 units/ml for 2 h at 37°C broke down less than 5% of the phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine. In this chromatographic system phosphatidylinositol was not well resolved from sphingomyelin and an apparent decrease in sphingomyelin content could be accounted for by phosphatidylinositol hydrolysis.

S. aureus is known to produce a Mg²⁺-dependent phospholipase C which will hydrolyse both pure sphingomyelin and sphingomyelin in erythrocyte membranes (Doery *et al.*, 1965; Colley *et al.*, 1973). The amount of sphingomyelinase in the culture supernatants used in these studies was, however, very low. Incubation of pure sphingomyelin (2.5 mM) with 20 units/ml for 2 h at 37°C caused the hydrolysis of less than 5 nmol. Inclusion of MgCl₂ (10 mM) in the incubation mixture did not significantly increase the amount of hydrolysis.

The mode of action of the enzyme was confirmed as that of a phospholipase C by t.l.c. of rat liver lipids after treatment with culture supernatant. A large increase in 1,2-diacylglycerol was observed. There was no significant production of unesterified fatty acid, suggesting that the phospholipase A in *S. aureus* cultures described by Doery *et al.* (1965) was absent. The appearance of a faint band with a mobility similar to that of monoacylglycerol may have been due to the action of the phospholipase C on lysophosphatidyl-

Table 1. Effect of cations on phospholipase C activity of *S. aureus* culture supernatant on a soya-bean lipid fraction

Standard assay conditions were used (see under 'Methods'). Each incubation contained 2.5 units of phospholipase C and the following additions. Values are the means of duplicate incubations from two separate experiments.

Additions	Relative phospholipase C activity (%)	
	100	100
No additions	100	100
150mM-NaCl	23	30
150mM-KCl	12	—
150mM-Choline chloride	76	63
1mM-CaCl ₂	98	96
10mM-CaCl ₂	11	—
2mM-EDTA	142	164
2mM-EDTA+2mM-CaCl ₂	145	175
2mM-EDTA+3mM-CaCl ₂	140	—

inositol (Doery *et al.*, 1965) or to a diacylglycerol lipase.

Inclusion of KCl and NaCl in the phospholipase C assay medium (see under 'Methods') considerably decreased the activity of the enzyme (Table 1). Choline chloride was less inhibitory at the same concentration (Table 1). An inhibitory effect of KCl on the pH 7.0 activity of the phosphatidylinositol-specific phospholipase C from lymphocytes has been reported previously (Allan & Michell, 1974*a,b*). CaCl₂ produced substantial inhibition at lower concentrations (Table 1); this was probably due to the precipitation of the substrate that takes place at this concentration of Ca²⁺. Conversely, addition of EDTA to the assay medium increased the phospholipase C activity by 40–70% (Table 1). It is unlikely that this effect is due to the chelation of Ca²⁺ bound by the substrate, as inclusion of equimolar Ca²⁺ and EDTA or a 1 mM excess of Ca²⁺ (2mM-EDTA) in the assay also increased phospholipase C activity (Table 1). EDTA (2mM) produced a similar increase in the activity of the enzyme towards purified phosphatidylinositol. These results suggest that the phospholipase C from *S. aureus* does not depend on Ca²⁺ for its activity. The phospholipase C from lymphocytes, however, requires a free Ca²⁺ concentration of approx. 1 μ M for maximal activity at pH 7.0 (Allan & Michell, 1974*b*).

Action of *S. aureus* culture supernatant on erythrocyte 'ghosts' and on liver microsomal fractions

The effect of phospholipase C on the phosphatidylinositol content of membranes is shown in Figs. 1–4. In the three species of erythrocyte 'ghosts' examined, substantial (approx. 75%) phosphatidylinositol

Fig. 1. Effect of *S. aureus* culture supernatant on the phosphatidylinositol content of ox erythrocyte membranes ●, Ox erythrocyte 'ghosts' (2); △, ox erythrocytes 'ghosts' in 150mM-NaCl (1); ○, ox erythrocytes (1). PI/PL is the amount of phosphatidylinositol expressed as a percentage of the sum of the other phospholipids. PI/PL was determined in triplicate incubations with the numbers of membrane preparations given in parentheses. Values are the means $\pm$ S.E.M. of all determinations.

Fig. 2. Effect of *S. aureus* culture supernatant on the phosphatidylinositol content of sheep erythrocyte membranes ●, Sheep erythrocyte 'ghosts' (2); △, sheep erythrocytes (2). See also legend to Fig. 1.

hydrolysis took place (Figs. 1–3). The major membrane phospholipids, however, were not hydrolysed by the phospholipase C. Incubation of ox, sheep or cat 'ghosts' with 50 units/ml for 2 h at 37°C broke

Fig. 3. Effect of *S. aureus* culture supernatant on the phosphatidylinositol content of cat erythrocyte membranes

●, Cat erythrocyte 'ghosts' (4); ▲, cat erythrocyte 'ghosts' in 150mM-NaCl (1); ○, cat erythrocyte 'ghosts' in 150mM-choline chloride (1). See also legend to Fig. 1.

Fig. 4. Effect of *S. aureus* culture supernatant on the phosphatidylinositol content of rat liver microsomal fraction ■, Microsomal fraction (2); □, microsomal lipid extracts (1). See also legend to Fig. 1.

down less than 5% of the membrane phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin.

Rat liver microsomal fractions showed only a small decrease (10–15%) in phosphatidylinositol content when incubated with the phospholipase C (Fig. 4). Phosphatidylinositol was more extensively hydrolysed in the lipid extracts prepared from the

microsomal fraction (Fig. 4). Freezing and thawing (two cycles) did not significantly change the amount of phosphatidylinositol that was hydrolysed.

The possibility that impermeability of the erythrocyte 'ghost' membranes restricted access of the phospholipase C to the phosphatidylinositol could be ruled out. The erythrocyte 'ghosts' used were judged to be permeable to macromolecules by their inability to retain haemoglobin during washing or to exclude macromolecules added to the suspending media (see under 'Methods').

The effect of increasing the salt concentration in the incubation mixtures is also shown. NaCl at 0.15M inhibited the hydrolysis of phosphatidylinositol of ox and cat 'ghosts' by the phospholipase C (Figs. 1 and 3). A smaller effect was observed if choline chloride (0.15M) was used instead of NaCl (Fig. 3).

Action of *S. aureus* culture supernatant on intact erythrocytes

The culture supernatants prepared by the method described in this paper had very low δ -haemolytic activity (<0.1 haemolytic unit/mg of protein; see under 'Methods'), but most preparations were still sufficiently haemolytic to preclude their use in experiments with intact erythrocytes. However, one preparation was obtained with sufficiently low haemolytic activity and was used in a limited number of experiments with sheep and ox erythrocytes (Figs. 1 and 2). In both cases approx. 60% breakdown of phosphatidylinositol took place, whereas haemolysis, although significantly increased compared with controls, was slight (2–5%). These incubations were carried out in 0.29M-sucrose/10mM-NaCl/10mM-Hepes at pH 7.0, an iso-osmotic medium which avoids the inhibition of the phospholipase C observed at higher ionic strengths (see above).

Action of lymphocyte supernatant on extracted lipids and permeable membranes

The lymphocyte enzyme had relatively little activity towards membrane phosphatidylinositol. Incubation of cat erythrocyte 'ghosts' (two experiments) or of rat liver microsomal fraction (two experiments) with 0.1 ml (in a 1 ml incubation mixture) of the supernatant for 1 h at 37°C gave less than 10% phosphatidylinositol hydrolysis. Under equivalent incubation conditions pure phosphatidylinositol would have shown approx. 50% hydrolysis in 10 min. Preliminary experiments showed that phosphatidylinositol in lipid extracts was relatively resistant to the action of this enzyme (unpublished work).

Discussion

The phospholipase C (*S. aureus*) used in this study was essentially similar in yield and specificity to that described by Doery *et al.* (1965). The retention of

most of the phospholipase C activity in the supernatant after precipitation of δ -haemolysin at pH4 served to distinguish this phospholipase C activity from that claimed to be present in a purified preparation of δ -haemolysin (Caird & Wiseman, 1970). Further, the crude culture filtrate itself appeared to contain relatively little δ -haemolysin.

The specificity of the phospholipase C for phosphatidylinositol was clearly demonstrated by the absence of significant hydrolysis of the major membrane phospholipids under conditions where substantial phosphatidylinositol hydrolysis took place.

The observed hydrolysis of at least 75% of the phosphatidylinositol in erythrocyte 'ghost' membranes indicates that most of this lipid is accessible in the membrane to the specific phospholipase. The remainder may be chemically resistant or physically inaccessible, but, as the present phospholipase C preparations are not sufficiently active to ensure maximum hydrolysis, especially in cat erythrocyte membranes where the initial phosphatidylinositol concentration is relatively high, further discussion of this point must be postponed until the enzyme preparation has been further purified.

The use of an impure enzyme preparation also introduces the possibility that accessibility of phosphatidylinositol to the phospholipase may be modified by contaminants such as proteolytic enzymes. It should be noted, however, that this same preparation hydrolyses only a small proportion of the phosphatidylinositol in liver microsomal membranes. Much more of this phospholipid is hydrolysed when the enzyme preparation is applied to the sonicated microsomal lipid extract, which suggests that accessibility of phosphatidylinositol in these membranes is limited by interaction with non-lipid components such as protein or by the membrane permeability barrier. Nevertheless, the inaccessibility of most of the microsomal phosphatidylinositol to the phospholipase contrasts with previous reports that microsomal phosphatidylinositol is readily accessible to exchange proteins (Wirtz *et al.*, 1972; Harvey *et al.*, 1973, 1974), although it is not clear what proportion of the membrane phosphatidylinositol is involved in the exchange process.

The inaccessibility of phosphatidylinositol both in liver microsomal fraction and in erythrocyte membranes to the phospholipase C from lymphocytes is consistent with the observation that this enzyme preparation is also relatively ineffective against phosphatidylinositol mixed with other lipids. In this case one can suggest that lipid-lipid interactions can limit accessibility of the phosphatidylinositol to the phospholipase and that this enzyme must have very specific substrate requirements, which will limit its usefulness in studies of biological membranes.

Preliminary observations on the effects on intact erythrocytes of one virtually non-haemolytic super-

natant from a *S. aureus* culture demonstrated that approx. 60% of the membrane phosphatidylinositol could be hydrolysed at the outer surface without causing substantial haemolysis. This suggests that a high proportion of the phosphatidylinositol is located in the outer leaflet of the erythrocyte membrane, but confirmation and extension of this observation requires a more consistently non-haemolytic enzyme preparation.

The present experiments have demonstrated that membrane phosphatidylinositol can be specifically modified *in situ* by a phospholipase C from *S. aureus*. We consider that this enzyme, after further purification, will be useful in determining the location of phosphatidylinositol in biological membranes and the possible role of this phospholipid in the activity and regulation of membrane-bound proteins such as adenylate cyclase (Levey, 1971) and muscarinic cholinergic and α -adrenergic receptors (Michell, 1975).

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