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LYTIC AND NON-LYTIC DEGRADATION OF PHOSPHOLIPIDS IN MAMMALIAN ERYTHROCYTES BY PURE PHOSPHOLIPASES

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SUMMARY

1. Sphingomyelinase (sphingomyelin cholinephosphohydrolase) from *Staphylococcus aureus* hydrolysed 75–80% of the sphingomyelin in human and pig erythrocytes and 50–60% of the sphingomyelin in ox and sheep erythrocytes, without producing haemolysis of the cells.

2. Although phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* alone was not lytic, the combination of sphingomyelinase and phospholipase C produced haemolysis of human and pig erythrocytes and extensive degradation of all the phospholipid classes in the ghosts thus produced. In contrast, the combination of these two enzymes failed to produce lysis of ox and sheep erythrocytes; in these cells the only phospholipid degraded was sphingomyelin.

3. Human erythrocytes treated with sphingomyelinase showed increased osmotic fragility relative to control cells.

4. In the intact erythrocytes studied, phosphoglyceride molecules are not accessible to phospholipases A_2 and C. Removal of choline phosphate residues from sphingomyelin exposes the phosphoglycerides to phospholipases A_2 and C in human and pig erythrocytes but not in ox and sheep red cells.

INTRODUCTION

Pure pancreatic phospholipase A_2 (phosphatide acylhydrolase, EC 3.1.1.4.) and pure phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* have both been shown to cause complete degradation of the main phospholipids in erythrocyte ghosts with the exception of sphingomyelin, resulting in the release of 70% of the total lipid phosphorus[1,2]. On the other hand, both enzymes appeared to be inactive towards the phospholipids of intact red cells when incubated under isotonic conditions, and no detectable haemolysis of the cells was observed[2]. Similar results were obtained when the cells had previously been treated with proteolytic enzymes. However, both phospholipases were able to produce lysis when the cells were swollen in hypotonic sucrose[3]; after partial haemolysis of

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the total cell population no significant degradation of any phospholipid class could be detected in the residual intact cells. This may indicate that degradation of only a very small amount of phospholipid by these two phospholipases is required to produce haemolysis.

Ibrahim and Thompson[4] also showed that, with intact cells, pancreatic phospholipase A_2 produced no phospholipid breakdown and no haemolysis. Crude sea snake venom, however, appeared to be able to produce extensive degradation of lecithin in intact human erythrocytes without causing the cells to lyse[4]. Op den Kamp *et al.*[5] showed that phospholipase C from *B. cereus* hydrolysed 80% of the phosphatidylethanolamine and 30% of the cardiolipin in the *Bacillus subtilis* protoplast. Although 20% of the total lipid phosphorus was removed by this attack no lysis of the protoplast was observed. On the other hand, pancreatic phospholipase A_2 attacked all the phospholipid classes in the bacterial protoplast and produced lysis. These results are taken to indicate that the effects of phospholipases on membranes vary both with the origin of the phospholipase and the type of membrane used.

The present paper deals with the degradation of sphingomyelin in the membranes of intact mammalian erythrocytes by sphingomyelinase (sphingomyelin cholinephosphohydrolase), and the effects that this has on the susceptibility of the cells to other lipolytic and proteolytic enzymes. Since human and pig erythrocyte membranes have similar phospholipid compositions, these two species are compared with ox and sheep red cell membranes, both of which have high sphingomyelin contents and contain very little lecithin[6,7].

MATERIALS AND METHODS

Pure phospholipase A_2 from porcine pancreas[8] was prepared in this laboratory by the group of professor G. H. de Haas. Pure phospholipase C was prepared by the method of Zwaal *et al.*[1]. Pure sphingomyelinase from *Staphylococcus aureus*[9,10] was a gift from Dr T. Wadström. Partially purified sphingomyelinase was prepared from *S. aureus* culture medium as follows: after 7 h growth the cells were removed by centrifugation and the supernatant medium was lyophilised. The freeze-dried material was dissolved in water (3 g in 100 ml) and the solution adjusted to 77% saturation with solid $(NH_4)_2SO_4$. The solution was allowed to stand at 4 °C for 1 h and was then centrifuged at $27000 \times g$ at 0 °C for 15 min. The precipitate was dissolved in 10 ml water and dialysed for 3 h against glycerol–water (1:1, v/v). The resulting solution (approx. 2.5 ml) was applied to a Sephadex G-100 column (1.8 cm × 135 cm), using 0.05 M Tris buffer (pH 7.6) containing 5 mM $CaCl_2$, 2 mM $MgCl_2$ and 50% (by vol.) glycerol as eluent. Fractions of 4 ml were collected and tested for sphingomyelinase activity in a two-phase diethylether buffer system, using a method similar to that previously described for phospholipase C (ref. 1) but with pure sphingomyelin as the substrate. The active fractions from 256–284 ml were pooled and concentrated by ultradialysis against 25% (by vol.) glycerol[11] and further concentrated by dialysis against 50% (by vol.) glycerol. The final preparation had an activity of 1 I.U. per 20 μl. No differences in effects on intact erythrocytes and ghosts were observed between the pure and the partially purified sphingomyelinases.

Fatty acid-poor bovine serum albumin was obtained from Calbiochem. Trypsin, thermolysin and pilz proteinase were all purchased from Merck.

Haemolysis procedure

Routine assays of enzyme-induced haemolysis were carried out under isotonic conditions for 1 h at 37 °C using freshly collected, washed erythrocytes as previously described[2]. The amounts of enzyme added were routinely: 10 I.U. of phospholipase A_2, 10 I.U. of phospholipase C and 5 I.U. of sphingomyelinase. The incubation mixtures contained 0.25 mM $CaCl_2$ and 0.2 mM $MgCl_2$.

Phospholipid analysis

After incubation with the enzymes the samples were centrifuged and the supernatants were removed. Enzymatic activity was inhibited by addition of a mixture of *o*-phenanthroline and EDTA (final concentration: 4 mM of each), prior to lipid extraction of the cells by the method of Reed *et al.*[12]. The extracts were taken to dryness under reduced pressure and the residue was dissolved in 100 μl of chloroform–methanol (1:1, v/v). The phospholipids were separated by two-dimensional thin-layer chromatography using the procedure of Broekhuyse[13], and determined as phosphorus after destruction with 70% $HClO_4$ at 190 °C by a modification[14] of the procedure of Fiske and SubbaRow. Since phospholipases readily attack phospholipids in ghosts, phospholipid analysis was performed only on samples showing less than 5% lysis.

RESULTS

When human erythrocytes were incubated with mixtures of phospholipase C and sphingomyelinase complete haemolysis was observed within 1 h, although neither of these enzymes produced lysis independently (Table I). Identical results were obtained with pig red cells. However, no lysis was produced by the combined action of the two enzymes on ox or sheep erythrocytes.

After treatment with phospholipase C alone there were no marked changes in the phospholipid pattern of human and pig erythrocytes (Fig. 1). Since sphingomyelin is not degraded by phospholipase C from *B. cereus*[2] it was used as an internal standard, and from this it was concluded that no significant breakdown of the other phospholipids had taken place. In contrast, sphingomyelinase, although producing no lysis, caused 75–80% degradation of sphingomyelin (Fig. 1). Prolonged incubation with sphingomyelinase failed to produce more extensive breakdown of sphingomyelin in the intact cells. It should be noted that the mixture of the two enzymes completely degrades all the major phospholipid classes in ghosts. Therefore, cells

TABLE I

PERCENTAGE HAEMOLYSIS PRODUCED BY PHOSPHOLIPASE C AND SPHINGOMYELINASE IN MAMMALIAN ERYTHROCYTES

	Human	*Pig*	*Ox*	*Sheep*
Blank (no enzyme added)	1	1	1	2
Phospholipase C	2	1	2	2
Sphingomyelinase	3	2	1	5
Phospholipase C + sphingomyelinase	100	100	1	4

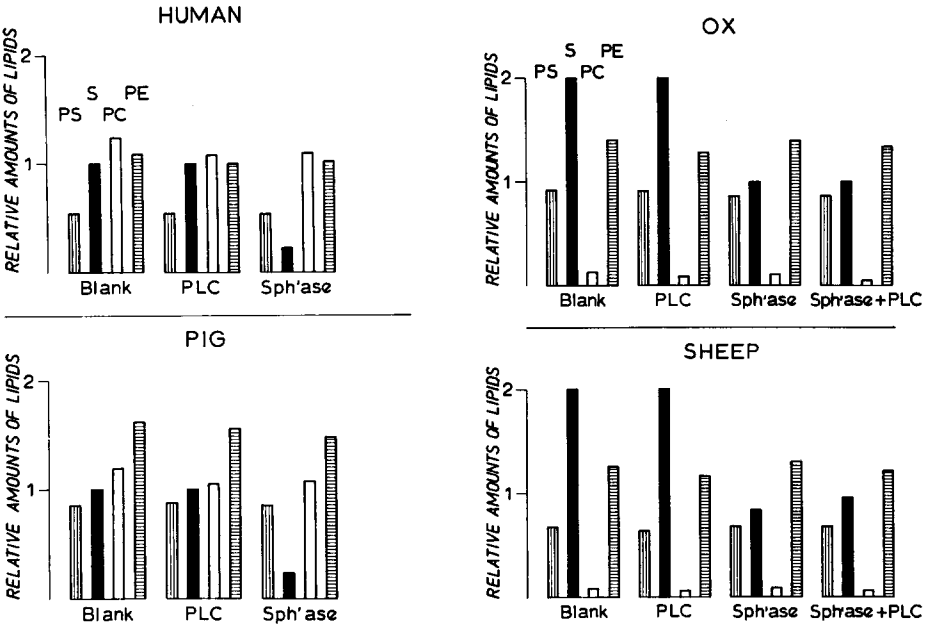

Fig. 1. Phospholipid patterns of human and pig erythrocyte membranes after treatment of intact cells with phospholipase C and sphingomyelinase. Blank, no enzyme added; PLC, phospholipase C; Sph'ase, sphingomyelinase; PS, phosphatidylserine; S, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine. Bars represent relative proportions of the phospholipids indicated at upper left.

Fig. 2. Phospholipid patterns of ox and sheep erythrocyte membranes after treatment of intact cells with phospholipase C and sphingomyelinase. Abbreviations are as in Fig. 1.

TABLE II

PERCENTAGE HAEMOLYSIS PRODUCED BY PHOSPHOLIPASES IN HUMAN ERYTHROCYTES EITHER IN THE PRESENCE OR IN THE ABSENCE OF 2% (w/v) BOVINE SERUM ALBUMIN

	% haemolysis
Blank (no enzyme added)	1
Blank + bovine serum albumin	2
Phospholipase A_2	1
Phospholipase A_2 + bovine serum albumin	2
Phospholipase C	2
Phospholipase C + bovine serum albumin	4
Phospholipase A_2 + phospholipase C	2
Phospholipase A_2 + phospholipase C + bovine serum albumin	4
Sphingomyelinase	4
Sphingomyelinase + bovine serum albumin	35
Sphingomyelinase + phospholipase A_2	29
Sphingomyelinase + phospholipase A_2 + bovine serum albumin	91
Sphingomyelinase + phospholipase C	100
Sphingomyelinase + phospholipase C + bovine serum albumin	100

incubated with the mixture of the two enzymes were not analysed for phospholipids, since after lysis both enzymes will readily degrade all the phospholipids in the ghosts thus produced.

The phospholipid patterns of cow and sheep erythrocytes after treatment with the two enzymes are shown in Fig. 2. Phospholipase C alone produced no significant decrease of the glycerophospholipids relative to sphingomyelin in either cow or sheep red cells, while sphingomyelinase degraded 50–60% of the sphingomyelin without causing the cells to lyse. Moreover, the phospholipid composition after the combined action of the two enzymes was not significantly different from that observed after the action of sphingomyelinase alone.

The influence of fatty acid-free bovine serum albumin on enzyme-induced haemolysis of human erythrocytes is shown in Table II. The non-lytic behaviour of phospholipase A_2 and C (both separately and in combination) was not influenced by the addition of bovine serum albumin. On the other hand, bovine serum albumin caused sphingomyelinase to produce some haemolysis. The combined action of phospholipase A_2 and sphingomyelinase produced partial lysis of the cells. This effect was markedly increased upon addition of bovine serum albumin. As expected, the combination of sphingomyelinase and phospholipase C produced complete haemolysis both in the presence and in the absence of bovine serum albumin. It should be noted that sphingomyelinase in combination with phospholipase A_2 was not lytic for ox erythrocytes.

When human and ox erythrocytes were first incubated with sphingomyelinase followed by treatment with trypsin, thermolysin or pilz proteinase (all obtained from Merck, Darmstadt) no lysis over blank was observed. However, the cells treated with sphingomyelinase alone had increased osmotic fragility relative to control cells (Fig. 3).

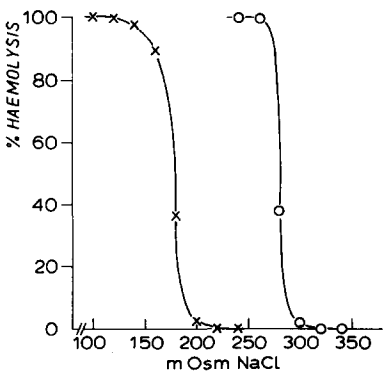

Fig. 3. Osmotic fragility of human erythrocytes after treatment with sphingomyelinase. ×—×, untreated; ○—○, sphingomyelinase treated.

DISCUSSION

Sphingomyelinase from *S. aureus*, unlike pancreatic phospholipase A_2 and phospholipase C from *B. cereus*, is able to attack the membrane of intact mammalian erythrocytes. In human and pig erythrocytes up to 80% of the sphingomyelin can be degraded without lysis; this is equivalent to approximately 20% of the total phospho-

lipids. Only 60% of the sphingomyelin can be degraded in ox and sheep erythrocytes, but in these cells this represents approximately 30% of the phospholipids. The failure of sphingomyelinase to lyse ox and sheep erythrocytes in these experiments is not in contradiction to the observations of Wadström and Möllby[9,10], since they only found lysis when the cells were cooled to 4 °C after incubation with sphingomyelinase at 37 °C.

It has been shown[3] that less than 5% degradation of the glycerophospholipids of swollen human erythrocytes by phospholipase A_2 and phospholipase C leads to lysis of the cells. Apparently this is not the case for sphingomyelin. Degradation of sphingomyelin in human and pig erythrocytes may expose the glycerophospholipids to the action of phospholipase C leading to lysis of the cells. However, this does not occur in ox and sheep erythrocytes.

Recently, Bretscher[15] proposed a model in which only choline-containing phospholipids (sphingomyelin and lecithin) are present on the outside of the cell membrane. This implies that in ox and sheep erythrocyte membranes, which have a very low lecithin content, the outer layer will contain almost all the sphingomyelin of the cell to the exclusion of the glycerophospholipids. Although this model may partially explain the present results, it does not account for the existence of a sizeable fraction of the sphingomyelin which is not available to sphingomyelinase in the intact cell, *e.g.* 40–50% of the sphingomyelin in ox and sheep erythrocytes.

Coleman *et al.*[16] were the first to report the production of "black dots" in erythrocyte ghosts by the action of phospholipase C from *Clostridium perfringens*. They suggested that these contained the diglycerides and ceramides produced by the action of the enzyme. Phospholipase C from *B. cereus* also produces black dots in erythrocyte ghosts (Figs 4a and b). In this case the dots cannot contain ceramides since this enzyme does not attack sphingomyelin. As the dots can be removed for the greater part by the action of pure pancreatic lipase (Fig. 4c) it is likely that they are composed predominantly of diglycerides. Pure sphingomyelinase, although degrading essentially all the sphingomyelin in erythrocyte ghosts does not lead to the appearance of black dots (Fig. 4d). These results lead to the suggestion that in erythrocytes, ceramides produced by sphingomyelinase action remain in position in the membrane, whereas enzymatically formed diglycerides migrate into discrete pools. This may explain why sphingomyelinase does not produce haemolysis, whereas in combination with phospholipase C lysis of the cells is observed.

Phospholipase A_2 in combination with sphingomyelinase produces lysis of human erythrocytes, although to a lesser degree than phospholipase C *plus* sphingomyelinase. The observation that no lysis of ox erythrocytes is produced by phospholipase A_2 *plus* sphingomyelinase is consistent with the suggestion that in these cells sphingomyelin breakdown does not lead to the exposure of glycerophospholipids to phospholipase action. In human red cells, however, this exposure is likely to occur with the resultant formation of lyso compounds which are powerful lytic agents. The increase in lysis observed when bovine serum albumin is added to the incubation of human red cells with the mixture of sphingomyelinase and phospholipase A_2, is probably due to the removal of fatty acids and lyso compounds from the membrane by the bovine serum albumin. This, similarly to the migration of diglycerides into pools after phospholipase C treatment, may lead to a further weakening of the membrane structure.

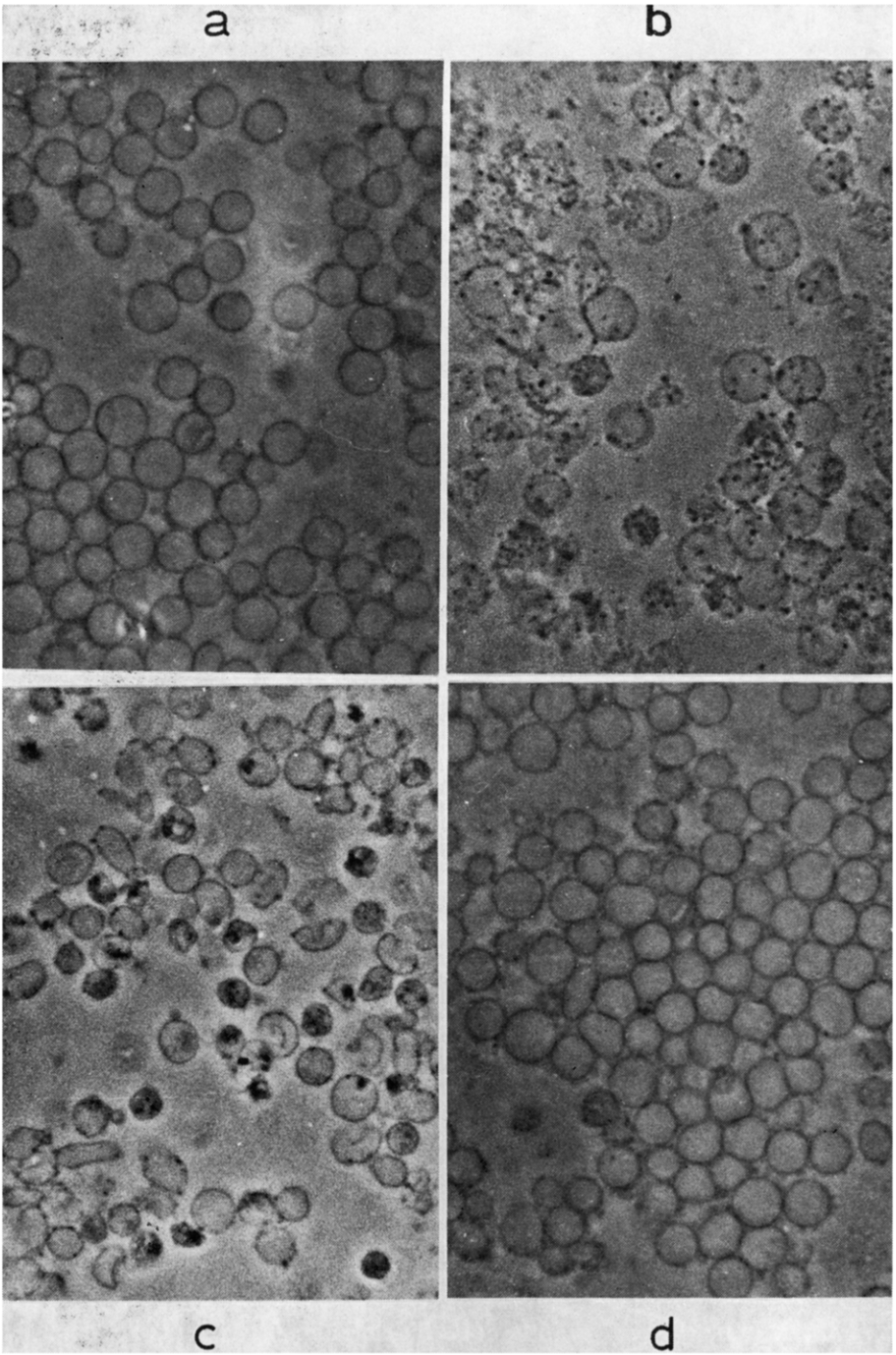

Fig. 4. Phase contrast micrographs of human erythrocyte ghost, prepared by the method of Dodge *et al.*[17]. The ghosts (20 mg) were suspended in 20 ml of 0.1 M Tris–HCl, pH 7.4 containing 5 mM $CaCl_2$ and incubated with enzymes for 1 h at 37 °C. (a) No enzyme added. (b) Phospholipase C (10 I.U.). (c) Ghosts from (b) treated with pure pancreatic lipase (200 μg). (d) Sphingomyelinase (5 I.U.).

Extensive degradation of sphingomyelin in erythrocyte ghosts does not lead to release of protein from the ghost membrane (Colley, C. M. and Zwaal, R. F. A., unpublished). Whether this also holds for the intact cell cannot be determined at present, since release of haemoglobin due to a few percent lysis (compare Table I) makes accurate detection of liberated membrane protein impossible. However, removal of choline phosphate from the sphingomyelin on the exterior of the cell will certainly contribute to the increased susceptibility of sphingomyelinase treated erythrocytes to osmotic shock. The ceramides thus produced in the cell membrane maintain the impermeability of the membrane for haemoglobin even though the physical strength of the membrane is definitely decreased.

It is remarkable that after degradation of sphingomyelin in the intact cell, release of sialopeptides by treatment with proteolytic enzymes does not cause the cell to lyse, whereas the breakdown of only a small fraction of the glycerophospholipids does produce haemolysis. It is concluded that phospholipids are not only important for the permeability properties of the cell but also play a significant part, probably in combination with proteins, in the maintenance of the physical strength of the cell membrane.

During the preparation of this paper, Gul and Smith[18] reported that *Naja naja* phospholipase A_2 hydrolysed phospholipids in washed human erythrocytes without causing the cells to lyse. This emphasises the comment in the introduction to this paper that phospholipases from different sources have different actions on the same membrane, since pancreatic phospholipase A_2 can only act on human erythrocytes after degradation of the sphingomyelin. The lysis observed under these conditions may therefore be due to the prior removal of choline phosphate from sphingomyelin. The observation[18] that the addition of bovine serum albumin to the incubation leads to lysis is consistent with the enhancement of the lytic effect of sphingomyelinase *plus* phospholipase A_2 by bovine serum albumin reported in this paper.

The failure of pancreatic phospholipase A_2 alone to attack phospholipids in intact erythrocytes, while the phospholipids are available to *Naja naja* phospholipase A_2, leads to the suggestion that the substrate requirements are not fulfilled for the former enzyme but that a proper enzyme–substrate complex can be formed with the latter.

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