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CHANGES IN LIPID METABOLISM AND CELL MORPHOLOGY FOLLOWING ATTACK BY PHOSPHOLIPASE C (*CLOSTRIDIUM PERFRINGENS*) ON RED CELLS OR LYMPHOCYTES

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SUMMARY

When intact human erythrocytes were treated with phospholipase C (*Clostridium perfringens*), up to 30 % of the membrane phospholipids were broken down without significant cell lysis. Only phosphatidylcholine and sphingomyelin were attacked. Ceramide (derived from sphingomyelin) accumulated, but 1,2-diacylglycerol (derived from phosphatidylcholine) was largely converted into phosphatidate. Up to 12 % of the cell phospholipid could be converted into phosphatidate in this way. Pig erythrocytes and lymphocytes showed a similar but smaller synthesis of phosphatidate after phospholipase C attack.

Phospholipase C also caused a marked morphological change in erythrocytes, giving rise to spherical cells containing internal membrane vesicles. This change appeared to be due to ceramide and diacylglycerol accumulation rather than to increased phosphatidate content of the cells.

INTRODUCTION

There exists a considerable body of information concerning the effects of phospholipase C treatment on isolated erythrocyte membranes (see ref. 1), but less information is available regarding the effects on intact cells of treatment with sublytic concentrations of this enzyme. In isolated erythrocyte membrane preparations the 1,2-diacylglycerol produced by phospholipase C action either accumulates as discrete lipid droplets associated with the membrane [1] or, in some species, is destroyed by membrane-associated lipase activity [2]. The results presented in this paper show that in intact, metabolically healthy human erythrocytes that are treated with sublytic concentrations of phospholipase C much of the 1,2-diacylglycerol produced does not accumulate. Instead, it is converted into phosphatidate by the action of diacylglycerol kinase in the membrane and phosphatidate accumulates in the cells. Similar changes were observed when pig lymphocytes were exposed to phospholipase C.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

Treatment of human, pig or sheep erythrocytes with phospholipase C also leads to a morphological change from a biconcave disc to a sphere containing internal vesicles. This process appears to depend on accumulation of ceramide (*N*-acylsphingenine) and diacylglycerol in the cell membrane, rather than on synthesis of phosphatidate.

MATERIALS AND METHODS

A freeze-dried culture filtrate of *Clostridium perfringens* (Burroughs-Wellcome) was used as an impure preparation of phospholipase C. In some experiments a pure phospholipase isolated from this material [3] or a pure preparation of *Bacillus cereus* phospholipase C (kindly donated by Dr R. F. A. Zwaal) was used.

Whole citrated human blood (Group O Rh positive) was obtained from Birmingham Regional Blood Transfusion Centre. Erythrocytes were sedimented and washed three times by centrifugation in 0.154 M NaCl; leucocytes were removed by aspiration after each centrifugation. The washed cells were suspended to a concentration of $2 \cdot 10^9$ cells/ml in a modified Krebs-Ringer medium which contained 11 mM glucose and in which CO_2/HCO^-_3 buffer was replaced by 18 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes-Ringer) [4]. Pig lymphocytes were isolated as described previously and were suspended at $2 \cdot 10^8$ cells/ml in the same medium [4]. Some experiments were carried out with pig and sheep red cells, prepared and treated in the same way as the human cells.

The studies of lipid labelling were carried out with cells which had been incubated at 37 °C in Hepes-Ringer medium for 1 h in the presence of $^{32}P_i$ (10 μCi/ml) in order to label the ATP pool of the cells. 1 ml samples of the labelled cell suspensions were then transferred into tubes which held 10–20 μl of a phospholipase solution containing up to 2 μg of an enzyme preparation which hydrolysed 10 μmol phosphatidylcholine/min per mg when incubated with a phosphatidylcholine emulsion. Incubation at 37 °C was continued for 10 min and incorporation was stopped by addition of 2.5 ml methanol and then 1.25 ml chloroform; phospholipase activity was rapidly terminated by this procedure. Lipids were isolated and analysed by chromatography on formaldehyde-treated papers [5], using the upper phase from a mixture of *n*-butanol/formic acid/water (4 : 1 : 5, v : v : v) as the solvent: this solvent system, unlike the acetic acid system used previously, moves phosphatidate well ahead of the area of the chromatogram which contains the major phospholipids (Allan, D., unpublished results). When the individual phospholipids in this spot (phosphatidylcholine, sphingomyelin, phosphatidylethanolamine and phosphatidylserine) were to be analysed they were separated by thin-layer chromatography [6].

Red cell lysis was monitored by measurements of absorbance at 410 nm in the supernatant solution after sedimentation of erythrocytes by centrifugation.

RESULTS

When human erythrocytes were treated with up to 2 μg/ml of the impure phospholipase C there was a progressive increase in the concentration and labelling with ^{32}P of a phospholipid which migrated just behind the solvent front on formaldehyde-treated papers, in the position of authentic phosphatidate (Fig. 1). The other

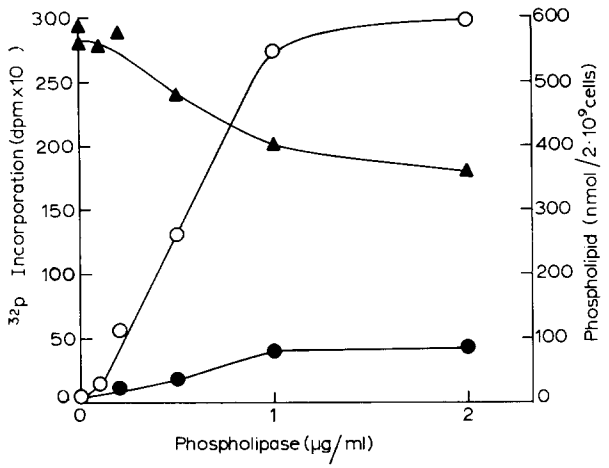

Fig. 1. Effect of phospholipase C treatment on the cell content and ^{32}P labelling of the phospholipids in human erythrocytes. Experimental details are in the text. Cell content of the major phospholipids (see Methods) (▲); Cell content of phosphatidate (●); Radioactivity in phosphatidate (○).

evidence for the identity of this lipid as phosphatidate was that it moved close to the solvent front during thin-layer chromatography [6] and on mild alkaline hydrolysis it gave rise, in 85–90 % yield, to a phosphate ester which was not distinguishable from authentic 1(3)-glycerol phosphate either by paper chromatography in methanol/formic acid/water (80 : 13 : 7) or in phenol saturated with water/acetic acid/ethanol (50 : 5 : 6) or by electrophoresis on paper for 2 h at 3000 V and pH 3.5 [7]. These procedures would clearly distinguish glycerol phosphate derived from phosphatidate both from the glycerophosphodiesters released by mild alkaline hydrolysis from phosphatidylinositol phosphate or phosphatidylinositol diphosphate (the only other phospholipids into which ^{32}P is incorporated by erythrocytes, see refs. 8 and 9) and from the other phosphodiesters derived from the major phospholipids of animal cells. The increase in phosphatidate content to about 12 % of total cell phospholipids and the high degree of labelling of phosphatidate with ^{32}P which occurred in phospholipase C-treated erythrocytes were not accompanied by appreciable cell lysis. The ^{32}P specific activity of phosphatidate in treated cells approached that of ATP and inorganic phosphorus. During the phospholipase treatment, up to 30 % of the cell phospholipids were hydrolysed. The lipids attacked were exclusively phosphatidylcholine and spingomyelin, about equal quantities of these being broken down. The majority of the liberated 1,2-diacylglycerol derived from phosphatidylcholine was therefore converted to phosphatidate but there was no evidence for the presence of ceramide phosphate, the hypothetical derivative of ceramide analogous to phosphatidate.

Similar results were obtained when a purified sample of the *Cl. perfringens* phospholipase C was employed. In contrast, treatment with the *B. cereus* phospholipase C gave rise to very little phosphatidate labelling. Its maximum effect was only about 0.2 % of the maximum effect of the *Cl. perfringens* enzyme, confirming that the *B. cereus* enzyme, as has been reported before [10, 11], has little or no ability to attack the phospholipids of the outer leaflet of the intact human erythrocyte mem-

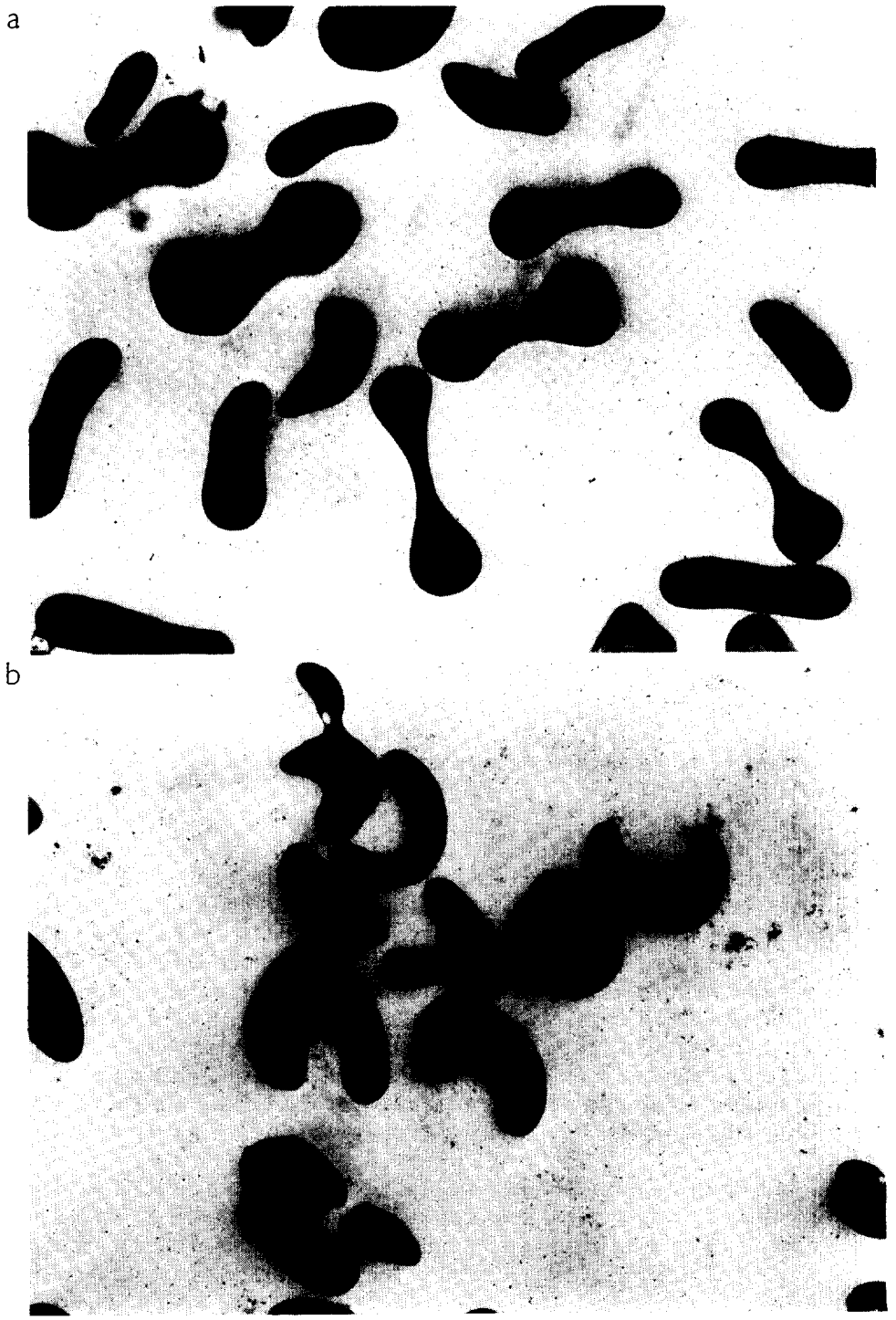

Fig. 2. See opposite page for legend.

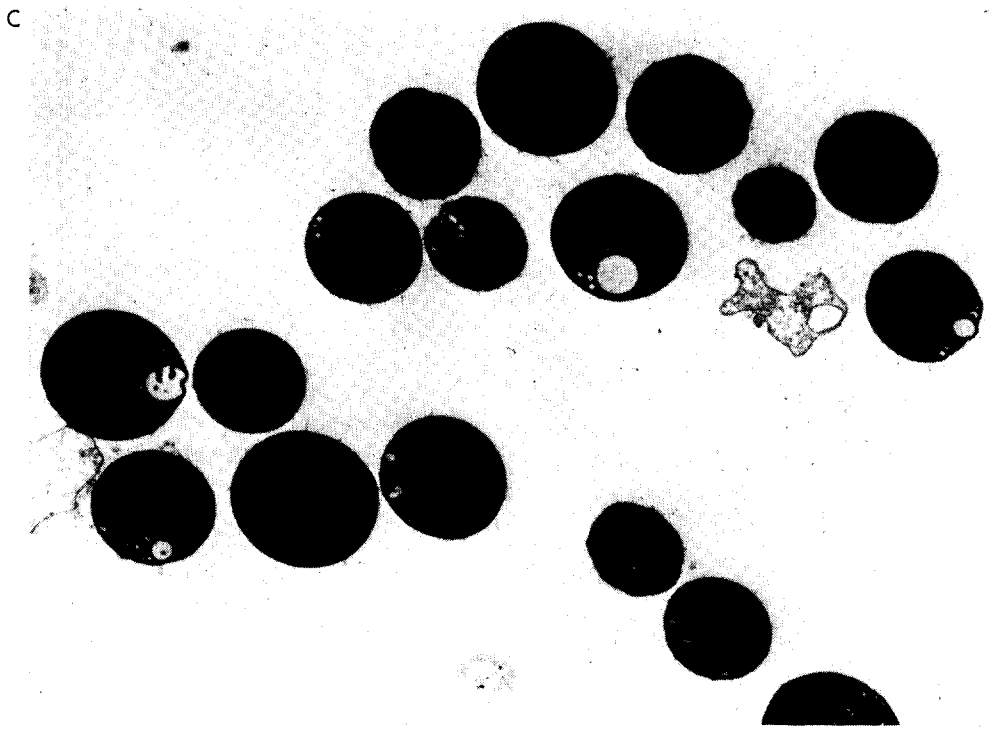

Fig. 2. Electron micrographs of control human erythrocytes (a) and erythrocytes treated for 20 min (b) and 60 min (c) with 1 μg/ml phospholipase C. Samples were fixed in suspension with 2% glutaraldehyde, washed with 0.2 M cacodylate buffer and stained with osmium tetroxide ($\times$ 4050).

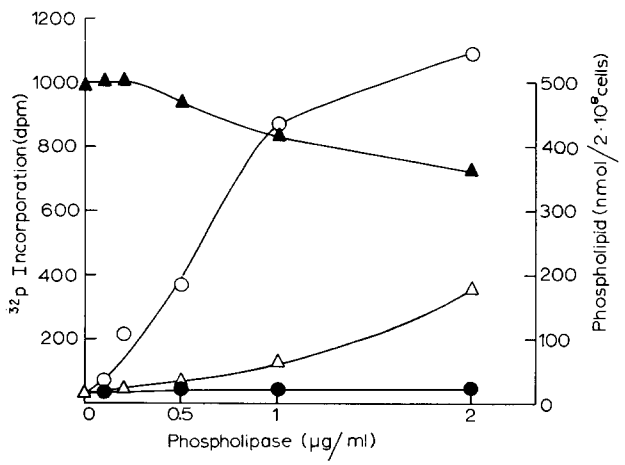

Fig. 3. Effect of phospholipase C treatment on the cell content and ^{32}P labelling of the phospholipids in pig lymphocytes. Experimental details are in the text. Cell content of the major phospholipids (see Methods) (▲); cell content of phosphatidate (●); radioactivity in major phospholipids (△); radioactivity in phosphatidate (○).

brane. Pig red cells treated with *Cl. perfringens* phospholipase C showed a labelling response similar to that of human cells but the magnitude of the effect was only about 10 % of that observed in the same number of human cells.

Treatment of human, pig or sheep erythrocytes with phospholipase C caused characteristic changes in their morphology (Fig. 2). The cells progressed from the initial biconcave disc (Fig. 2A), through a crescent-shaped form, to a cup-like shape (stomatocyte) (Fig. 2B) and eventually to a uniform sphere (Fig. 2C). Cell lysis became apparent in the final stages of this process. Thin sections of phospholipase-treated cells showed a rounded appearance with some cup-shaped pits in their surface: most cells had vesicles in their interiors. These effects occurred more rapidly in energy-depleted human cells and in cells which had been treated with phospholipase C at concentrations above that leading to maximum phosphatidate synthesis.

When pig lymphocytes were treated with phospholipase C in the same way as the erythrocytes they also showed a large increase in labelling of phosphatidate with ^{32}P, but only a small percentage increase in the contribution of phosphatidate to the total lipids of the cells (Fig. 3). Total ^{32}P incorporation into phosphatidate by pig lymphocytes varied considerably in different experiments: generally, it was between 5 and 50 % of the incorporation by an equivalent number of human red cells. In addition, an increase was observed in the labelling of the major phospholipids. This latter effect, which was localised mainly in the phosphatidylcholine after separation by thin-layer chromatography, may have been due to increased availability in the cells of 1,2-diacylglycerol, which is the immediate precursor of phosphatidylcholine.

DISCUSSION

Treatment of cells with a non-lytic concentration of phospholipase C would be expected to cause breakdown of phospholipids present in the outer lipid leaflet of the plasma membranes and therefore to generate 1,2-diacyglycerol in this leaflet. The information presented in this paper shows that in human erythrocytes or pig lymphocytes 1,2-diacylglycerol produced in this way can gain access to diacylglycerol kinase and ATP and thus be converted to phosphatidate. The simplest interpretation of this observation would be that the diacylglycerol crosses from the outer to the inner leaflet of the membrane, rather than that ATP can obtain access to the external leaflet. When diacylglycerol kinase, the membrane-bound enzyme responsible for the synthesis of phosphatidate from diacylglycerol, was first described by Hokin and Hokin [12] its maximum activity against externally added diacylglycerol was recorded as about 20 nmol phosphatidate formed/h per mg membrane protein. In the present experiments, in which the substrate has been introduced directly into the membrane by enzyme action rather than being added as an emulsion, the greatest observed activity was about an order of magnitude higher than this. Presumably this increased activity reflected better access of substrate to the enzyme.

The presence in erythrocytes and lymphocytes of a very active diacylglycerol kinase, which in most circumstances is not called upon to express its full activity, suggests that phosphorylation of diacylglycerol to phosphatidate may have some important role in the economy of cells. This point of view is strengthened by the fact that this is one of very few reactions involving the metabolism of a glycerolipid head-group which is retained in the mature erythrocyte. The rate at which ATP must be

utilised to synthesise phosphatidate under the artificially stressed conditions existing in a phospholipase-treated erythrocyte is equivalent to about half of the normal rate of glycolytically driven ATP synthesis in these cells (400 nmol ATP/h per 10^9 cells, see ref. 13). Obviously this situation normally does not exist, but for some reason the healthy cell has this capacity available to it should the need arise.

The Hokins [14] suggested that diglyceride kinase together with phosphatidate phosphohydrolase might form a cycle of reactions which comprised the $(Na^+ + K^+)$-ATPase of the red cell membrane. While the magnitude of the diglyceride kinase activity measured here might be consistent with this proposal, only much lower levels of phosphatidate phosphohydrolase have been observed.

A possible reason for the presence of this active diacylglycerol kinase might be to prevent the adverse effects on the cell which would follow upon accumulation of diacylglycerol. The information presented here and elsewhere [15] suggests that accumulation of diacylglycerol or ceramide in either leaflet of the lipid bilayer in the erythrocyte membrane leads to marked morphological changes, the membrane undergoing vesiculation with the diacylglycerol or ceramide-enriched surface to the interior. Erythrocytes normally contain only about 0.2 % of their glycerolipids as diacylglycerol and efficient maintenance of the concentration at this low level may be ensured by the presence of a high level of diacylglycerol kinase activity.

The rapid conversion of diacylglycerol into highly labelled phosphatidate makes a ^{32}P-labelled human red cell a highly sensitive test system for low levels of phospholipase C activity, providing that the enzyme in question can attack intact cells.

In pig lymphocytes treated with phospholipase C the degree of labelling of phosphatidate with ^{32}P was intermediate between that seen with human red cells and that observed for pig erythrocytes. It seems unlikely, therefore, that any substantial proportion of phosphatidate labelling in pig lymphocyte suspensions could be due to contaminating red cells, which accounted for only about 3 % of the total cells present. This deduction is supported by the observation that a pronounced (2- to 4-fold) increase in labelling of other phospholipids (mainly phosphatidylcholine)occurred in lymphocyte suspensions, probably due to equilibration of phosphatidate or diacylglycerol formed at the plasma membrane with the pool of these lipids in the endoplasmic reticulum. Enhanced labelling of the major phospholipids could not be due to red cells which cannot perform the necessary metabolic transformations.

The morphological changes seen with phospholipase C treatment of human erythrocytes were not correlated with increased phosphatidate concentration in the cells and were accelerated in starved cells, which had a decreased ability to phosphorylate diacylglycerol. Furthermore, phospholipase C treatment of sheep red cells led to the same morphological changes despite the fact that these cells showed no increase in ^{32}P labelling of phosphatidate. In this case it appears that no diacylglycerol is produced. This is consistent with the fact that in sheep red cells the only choline-containing phospholipid (the major component of the outer lipid leaflet) is sphingomyelin [16, 17], so that only ceramide is released by phospholipase C. These considerations suggest that accumulation of ceramide and/or diacylglycerol are the factors which initiate the morphological changes. This interpretation is supported by the independent observation that sphingomyelinase produces internal vesiculation in red cell ghosts [17].

Morphological changes similar to those seen with phospholipase C treatment have also been observed following the addition of a variety of compounds to red cells. The intermediate 'stomatocyte' or cup-shaped form has been most often described in studies using light microscopy but in some cases electron microscopy has shown the change proceeding to the stage of internal vesiculation [19, 20]. Agents which induce these effects include cationic amphipaths such as chlorpromazine and primaquine [19] or large hydrophobic molecules bearing hydroxyl group(s) (e.g. retinol [20] and cortisol [19]). Ceramide and diacylglycerol would fit into the latter class.

Internal vesiculation involves membrane fusion and it is interesting to note that retinol, diacylglycerol and other amphiphilic alcohols can also induce cell-cell fusion [21]. The mode of action of such membrane fusogens is unknown, but their initial effect may be to alter the packing of membrane lipids within either leaflet of the lipid bilayer and hence change the curvature of the membrane (see ref. 22).

This interpretation is supported by the evidence presented here and elsewhere [15] that insertion of diacylglycerol or ceramide into the outer leaflet of the red cell membrane (using phospholipase C) produces internal vesiculation, but that diacylglycerol introduced into the inside face of the membrane gives rise to evagination and outward budding of membrane vesicles.

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