PHOSPHOLIPASE C (*BACILLUS CEREUS*) ACTS ONLY AT THE INNER SURFACE OF THE ERYTHROCYTE MEMBRANE

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1. Introduction

Lister et al. [1] observed that the extent of hydrolysis of erythrocyte membrane phospholipid by phospholipase C from *Bacillus cereus* was related to the extent of haemolysis of the erythrocytes. They also noted that the extent of phospholipid hydrolysis was decreased if the hypotonically-lysed ghosts were restored to isotonic conditions or were incubated at 37°C in the lysing medium. They suggested that phospholipase C might act at the inner face of the membrane or alternatively that during haemolysis the membrane structure may be modified so as to make the phospholipid molecules more accessible to the enzyme. From similar experiments with both phospholipase A_2 Woodward and Zwaal [2] concluded that during the process of ghost formation from intact erythrocytes there was a change in membrane structure which made polar head groups of phospholipids more readily available to phospholipase C.

Several observations made in this laboratory during studies of erythrocyte membrane structure have emphasised that *Bacillus cereus* phospholipase C will hydrolyse phospholipids in erythrocyte membranes only if the enzyme is introduced when the ghost is permeable so that it may penetrate into or through the membrane.

2. Experimental and results

Phase contrast microscope observations (fig. 1) of the action of phospholipase C on erythrocyte ghosts prepared in a range of haemolysing buffers of different osmolarities showed that not all of the ghosts displayed the dense droplets and reduced diameters characteristic of ghosts modified by phospholipase C [3]. The proportions which were modified depended on the osmolarities of the haemolysing buffers and correlated closely with the extent of hydrolysis of phospholipid in the ghost preparation (fig. 2). This is conveniently expressed as a percentage of the maximum phospholipid hydrolysis (about 70%) attained when all ghosts in the preparation were modified (i.e. ghosts prepared in 20 imosM buffer). The unmodified ghosts were appreciably darker than the modified ones suggesting that they had retained haemoglobin. When the sample was suspended in a 5% solution of bovine serum albumin (fig. 1b) unmodified ghosts appeared less dark than the surrounding medium thus indicating that they were also impermeable to albumin. Centrifugal fractionation of this suspension facilitated the isolation of a pure sample of modified ghosts (fig. 1c). On the basis of phospholipid to cholesterol ratios the phospholipids of these ghosts were found to have been hydrolysed to the maximum extent. The overall extent of phospholipid hydrolysis in each preparation in fig. 2 can be accounted for in terms of a maximum hydrolysis of phospholipid in the permeable ghosts and none in the impermeable ones.

In another experiment erythrocytes were haemolysed at 25°C in 80 imosM buffer and phospholipase C was added to samples of the ghosts at different times after haemolysis. Phase contrast micrographs (fig. 3) showed that the proportion of ghosts which was not susceptible to enzyme attack increased as the time interval between haemolysis and addition of phospholipase C was increased. Such ghosts were very

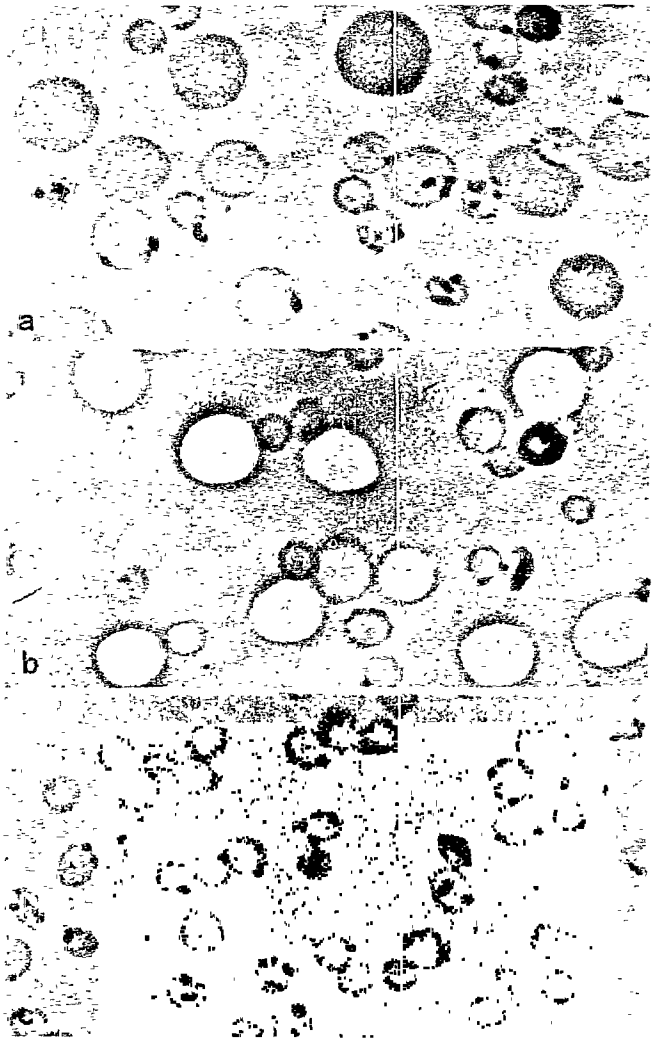

Fig. 1. Phase contrast micrographs of phospholipase C treated pig erythrocyte ghosts. After incubation with phospholipase C (see fig. 2) a sample of the ghosts was suspended in a) haemolysing buffer (60 imosM) and b) haemolysing buffer containing 5% BSA. The ghosts which have excluded BSA appear pale due to their lower internal refractive index. c) Fraction of permeable ghosts obtained by sedimentation (103,000 *g* × 30 min) of preparation as in b) through layer of haemolysing buffer containing 10% BSA Magnification × 1100.

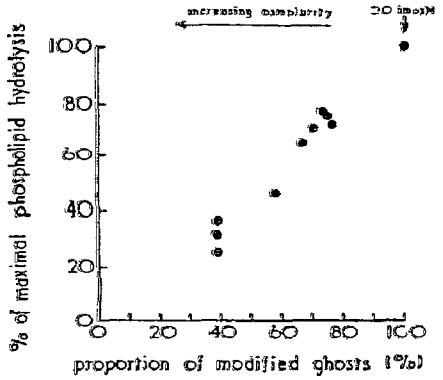

Fig. 2. Correlation of the extent of phospholipid hydrolysis with the proportion of ghosts which is modified by phospholipase C. Pig erythrocytes were haemolysed in a range of bicarbonate buffers (pH 7.4) of different osmolarities (20 imosM to 100 imosM). The washed ghosts were incubated for 1 hr at 30°C with phospholipase C from *Bacillus cereus* (Makor Chemicals Ltd., Jerusalem, Israel) at a concentration of 67 μg/ml. The proportion of modified ghosts in each preparation was estimated from phase contrast micrographs. Each point represents an analysis of three micrographs involving a total of about 250 ghosts. Mean standard deviation for the experiment was below 3%. Maximal hydrolysis represents hydrolysis of 72% of membrane phospholipids.

dark and the membranes were evidently impermeable to haemoglobin. There was a close correspondence between the proportion of haemoglobin-permeable ghosts and the overall level of phospholipid hydrolysis expressed as a percentage of maximum hydrolysis (fig. 4). It is suggested that under the conditions of this experiment haemolysed cells were able to regain impermeability (spontaneously reseal) and would then resist attack by externally-located phospholipase C. There was no indication that the molecular configuration of the membrane had been irreversibly modified by the process of haemolysis so as to make it susceptible to phospholipase C action.

3. Discussion

This conclusion was confirmed in further experiments in which erythrocytes were haemolysed and resealed in the presence of dextran (1.5%) and phospholipase C but under conditions which limited the enzyme action (i.e. at 0°C). Haemolysis of erythrocytes in hypotonic buffer containing dextran has been shown to increase the retention of haemoglobin in ghosts [5] presumably because the cells were able to reseal rapidly after losing only a small proportion of their haemoglobin. At the same time dextran was able to enter the cells [6]. Consequently in the present experiment it seemed likely that phospholipase C would also have entered the cells during the brief haemolysis

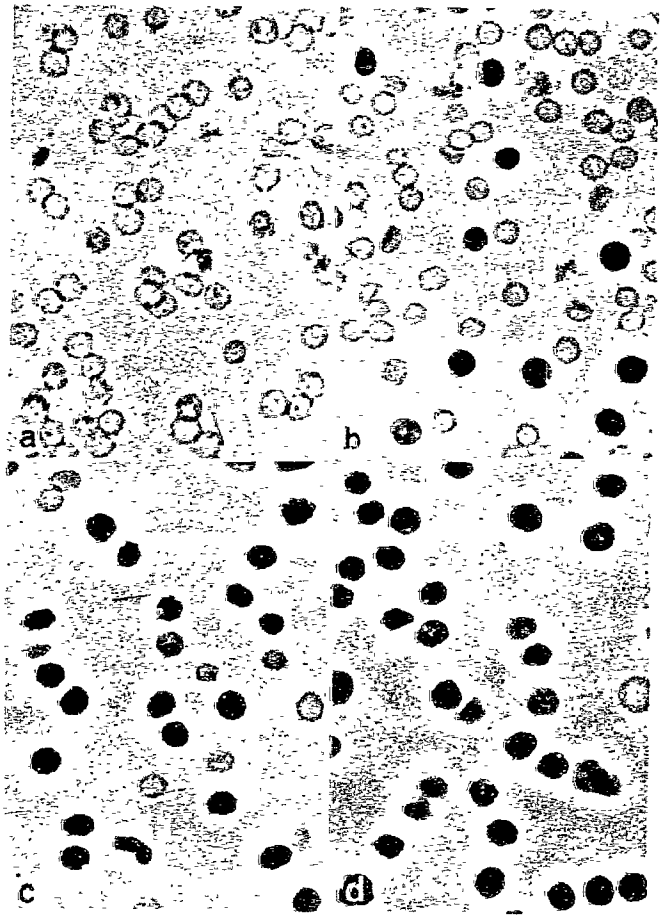

Fig. 3. Phase contrast micrographs of phospholipase C treated human erythrocyte ghosts. Phospholipase C was added (see fig. 4) a) before haemolysis, b) 1 min after haemolysis, c) 30 min after haemolysis and d) incubated in the absence of phospholipase C. Samples for microscopy were diluted with 80 imosM $NaHCO_3$. The ghosts which have retained haemoglobin appear darker due to their high internal refractive index. Magnification × 440.

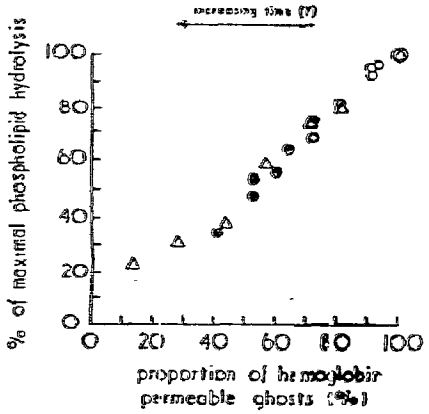

Fig. 4. Correlation of the extent of phospholipid hydrolysis with the proportion of haemoglobin-permeable ghosts. (●) Pig erythrocyte ghosts and (△) human erythrocyte ghosts incubated with phospholipase C (Makor Chemicals Ltd.,). (○) Pig erythrocyte ghosts incubated with a highly purified enzyme kindly supplied by Dr. R.F.A. Zwaal [4]. Phospholipase C (20 μg/ml) was either present at the time of haemolysis or added at different times (T) after haemolysis in 80 imosM bicarbonate buffer (pH 7.4). The haemolysates were incubated with the enzyme at 25°C for 1 hr. The proportion of pale (haemoglobin-permeable) ghosts in each preparation was determined (see fig. 2) from phase contrast micrographs. The amount of phospholipid hydrolysis obtained when phospholipase C was present during haemolysis was used as the maximal value.

and would be present inside the resealed ghosts. When the preparation was incubated at 30°C the ghosts haemolysed again and dense droplets appeared on the membranes. Control preparations to which phospholipase C either was not added at all or was added after the initial haemolysis and resealing showed neither rehaemolysis nor droplets.

These experiments provide strong evidence that an essential requirement for the hydrolysis of phospholipids in erythrocyte membranes by phospholipase C is that the enzyme must at some time be able to penetrate the membrane either to assume a critical position within the membrane or to gain access to the cytoplasmic surface. A modification of membrane structure so as to make phospholipid head groups more readily accessible to external enzyme is not necessarily implicated.

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