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Action of pure phospholipase A_2 and phospholipase C on human erythrocytes and ghosts

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SUMMARY

1. Pancreatic phospholipase A_2 (phosphatide acyl-hydrolase, EC 3.1.1.4) and phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* appeared not to be lytic for human erythrocytes, either before or after treatment of the cells with trypsin, pronase or neuraminidase. No significant breakdown of phospholipids could be observed.

2. Both phospholipases were found to evoke hemolysis in the presence of sublytic concentrations of sodium deoxycholate, whereas sublytic concentrations of Triton X-100 were effective only in combination with phospholipase C.

3. Treatment of human red cell ghosts with either phospholipase A_2 or phospholipase C resulted in a complete hydrolysis of lecithin, phosphatidylethanolamine and phosphatidylserine, whereas sphingomyelin was not attacked. Similar results were obtained with liposomes derived from human erythrocytes, indicating that the degree of hydrolysis depends only upon the chemical nature of the phospholipids involved.

4. In the native human erythrocyte membrane the fatty acid–ester linkage at C_2 and the phosphoryl–glycerol linkage at C_3 of the phosphoglyceride molecules apparently are not accessible to phospholipase A and C attack. Removal of the sialic acid residues from the membrane surface does not promote the action of these lipolytic enzymes. Changes in membrane architecture occurring during membrane isolation or as induced by nonlytic concentrations of detergents lead to exposure of membrane phosphoglycerides to phospholipases A and C.

Previous reports from this laboratory dealt with the action of crude phospholipases on intact erythrocytes. Whereas *Crotalus adamanteus* phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) has been shown not to be lytic[1], the action of *Clostridium welchii* phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) exhibited a complete hemolysis of intact erythrocytes from human and various animal species[2]. Snake venom phospholipase A has been shown to be lytic in combination with a

basic direct lytic factor[3–5], whereas action of this enzyme on ghosts caused complete hydrolysis of phospholipase A digestable phospholipids[4]. Recently, several groups have demonstrated that 70% of the total lipid phosphorus could be released from human erythrocyte ghosts by the action of partially purified bacterial phospholipase C preparations[6–11]. However, interpretation of this phenomenon is difficult not only because impure enzyme preparations have been used, but also because no information is available on the amount of degradation of the individual phospholipid classes.

Both pancreatic phospholipase A_2 (ref. 12) and *Bacillus cereus* phospholipase C (ref. 13) have been completely purified in this laboratory. The pure *B. cereus* enzyme has to be considered as a genuine phospholipase C, since it does not exhibit phosphatase activity towards phosphate esters other than diacylphosphoglycerides (P. Comfurius, R.F.A. Zwaal and B. Roelofsen, unpublished observations and P.P.M. Bonsen, unpublished observations). Moreover, the enzyme appears to be moderately stereospecific in its action since the activity on 1,2-diheptanoyl-*sn*-glycero-3-phosphorylcholine was found to be 16 times more than on 2,3-diheptanoyl-*sn*-glycero-1-phosphorylcholine (P.P.M. Bonsen, unpublished observations). The comparative action of both enzymes on intact human erythrocytes and red cell ghosts forms the main subject of this report.

For routine assays of hemolytic activity, 0.25 ml of washed, freshly collected erythrocytes (from acid–citrate–dextrose-treated blood; cells packed for 10 min at 3000 × *g*) were suspended in 5 ml isotonic saline (0.87% NaCl, pH 7.4, with $NaHCO_3$ or Tris; 0.25 mM $CaCl_2$). Aliquots of enzyme were subsequently added and the mixture was incubated for 1 h at 37° with gentle stirring, followed by centrifugation in a clinical centrifuge. 0.2 ml of supernatant was diluted with 10 ml water and the absorbance was measured at 418 nm using distilled water as blank. Absorbance of 100% hemolysis was determined by replacing NaCl with distilled water prior to the incubation procedure. 40 I.U. of both phospholipase A_2 and phospholipase C did not cause more than 2% hemolysis and no significant degradation (less than 5%) of phospholipids could be found after addition of EDTA and extraction of the cells according to Reed *et al.*[14]. Comparable results were observed when incubations were carried out in 0.6% NaCl.

In order to establish whether the presence of sialic acid residues at the surface of the erythrocyte prevents the attack of both phospholipases, the cells were preincubated with trypsin, pronase E or neuraminidase according to the methods of Seaman *et al.*[15,16]. These incubations did not cause any detectable hemolysis. The cells were subsequently washed 4 times with isotonic saline★ and aliquots were hemolysed to prepare ghosts according to Dodge *et al.*[17]. Sialic acid determinations were performed on the ghosts following the procedure of Warren[18]. The release of sialic acid from intact cells caused by trypsin, pronase and neuraminidase was found to be 65, 85 and 95%, respectively. When the pretreated cells were subsequently incubated with either phospholipase A_2 or phospholipase C, neither hemolysis nor hydrolysis of phospholipids could be established.

Heemskerk and Van Deenen[1] have shown that sublytic concentrations of deoxycholate together with the nonlytic *Crotalus admanteus* venom resulted in a complete lysis of rabbit erythrocytes. From this point of view it would be of interest whether

★Soybean trypsin inhibitor was added to the cells incubated with trypsin, prior to the washing procedure.

sublytic concentrations of deoxycholate or the non-ionic detergent Triton X-100 do evoke lysis of the cell in combination with pure phospholipases. As shown in Fig. 1, sodium deoxycholate appears to be sublytic at concentrations smaller than 0.65 mM and Triton X-100 at concentrations smaller than 0.016 mM. The combined action of either deoxycholate or Triton X-100 with phospholipase C does evoke hemolysis after 1 h at 37°, to a degree which is dependent on both detergent and phospholipase C concentration; however, the effect of Triton X-100 is considerably stronger than that of deoxycholate. In contrast, with phospholipase A_2 hemolysis was observed only above 0.5 mM deoxycholate to a degree which is independent of enzyme concentration, whereas sublytic Triton X-100 concentrations in combination with phospholipase A_2 did not show any lysis at all (Fig. 1). No breakdown of phospholipids could be detected in the unlysed cells, whereas these lipids were found to be rapidly hydrolysed in the ghosts produced by the lysed cells. In a control experiment it was found that 0.02 mM Triton X-100 did not inhibit the hydrolysis of purified egg lecithin by phospholipase A_2. Although an explanation for the observed phenomena is difficult to give, the possibility must be considered that low concentrations of these detergents do induce some alteration in the molecular organization of the red cell membrane, making it more susceptible to phospholipase C than to phospholipase A_2. This may be surprising, considering that phospholipase A_2 catalyses the formation of lyso-phospholipids which, in contrast to diglycerides, are lytic by themselves.

Since most of our present-day knowledge of the erythrocyte membrane is based upon experiments with red cell ghosts, the action of pure phospholipases on ghosts is equally interesting. Human erythrocyte ghosts were isolated according to Dodge *et al.*[17]. Suspensions of 45 mg ghosts in 20 ml 0.1 M Tris buffer (pH 7.4; 10 mM $CaCl_2$) were incubated under stirring with either phospholipase A_2 or phospholipase C. At different incubation times 4-ml aliquots were taken and enzyme activity was immediately blocked by addition of 20 mg EDTA. The ghost samples which had been incubated with

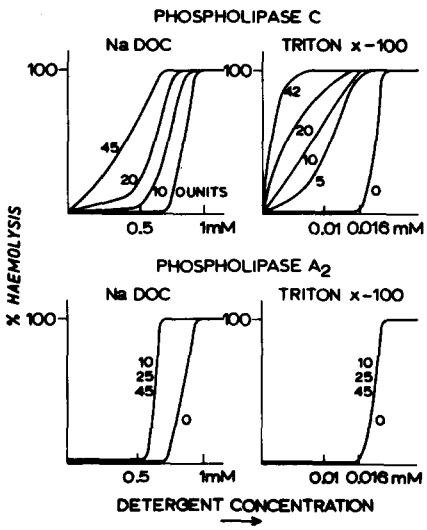

Fig. 1. Hemolysis by phospholipases in the presence of sodium deoxycholate and Triton X-100. Unit, I.U. of enzyme; NaDOC, sodium deoxycholate.

phospholipase C were washed twice with 40 ml of distilled water to remove the phosphorus containing split products. Lipids were extracted from the ghosts according to Reed *et al.*[14]. The pooled extracts were taken to dryness under reduced pressure and the residue was dissolved in 5 ml chloroform–methanol (1:1, v/v). Duplicate 1-ml samples were taken for total phosphorus determination after evaporation and destruction with 70% $HClO_4$ at 190° by a modification[19] of the procedure of Fiske–SubbaRow. The individual phospholipids were determined as phosphorus[19], after separation of duplicate 1-ml samples by means of two-dimensional thin-layer chromatography on silica gel H, according to the procedure of Broekhuyse[20]. The ghost samples which had been incubated with phospholipase A_2 were not washed with water but directly subjected to the lipid extraction procedure and further analysed as described above.

As shown in Fig. 2, both phospholipases caused a complete digestion of lecithin, phosphatidylethanolamine and phosphatidylserine, exhibiting only some differences with respect to the velocity with which these phospholipid classes were hydrolysed. However, in both cases, no detectable degradation of sphingomyelin could be demonstrated. These observations are responsible for the fact that only 70% of the initially present phospholipids are attacked, as is indicated by the two dashed lines in Fig. 2, levelling off at 30% retention. With sonicated* liposomes derived from human erythrocyte total lipids essentially the same results were obtained with respect to the individual phospholipid classes, after incubation with both phospholipases.

De Haas *et al.*[12] have already demonstrated that sphingomyelin fully resisted enzymic breakdown by pancreatic phospholipase A_2. Our results strongly suggest that under the described experimental conditions sphingomyelin cannot be considered as a substrate for *B. cereus* phospholipase C either[13], being in contrast to the action of crude phospholipase C from *Clostridium welchii*[2,11]. Furthermore, it has to be emphasized that those phospholipids which are substrates for the phospholipases used, can be completely degraded in human red cell ghosts and liposomes but cannot be attacked in the membrane of the intact erythrocytes. The present results using pure phospholipase A confirm our earlier observation that this enzyme does not attack the phospholipids present in the intact

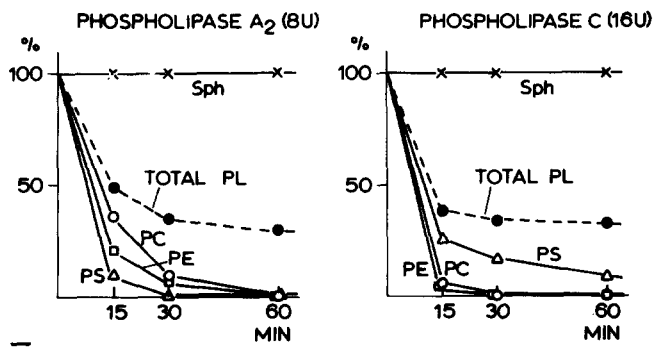

Fig. 2. Percent retention of phospholipids in human erythrocyte ghosts after incubation with phospholipases at 37°. Total PL, initially present total phospholipids; PC, lecithin; PE, phosphatidylethanolamine; PS, phosphatidylserine; Sph, sphingomyelin.

*Calcium was added after sonication up to a concentration of 10 mM.

erythrocyte membrane. It has been demonstrated now that this is not due to a hindrance of the approach of the enzyme by the sialic acid residues. In contrast to previous opinions pure phospholipase C behaved identically to phospholipase A and did not hydrolyse the phosphoglycerides present in the intact erythrocyte membrane and did not cause lysis. This observation requires a revision of membrane models depicting the erythrocyte membrane with the polar headgroups of the phospholipids being exposed freely to the outside. Only in the presence of sublytic concentrations of detergents or in isolated membranes is action of these pure phospholipases apparent. The contrasting results obtained with intact cells as opposed to ghosts indicate that the intact cell membrane and the ghost membrane are separate entities, most likely differing in the spatial arrangement of their components. The present observations seem to support that the "stroma" from the erythrocytes must be considered at best as a first derivative of the original membrane.

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