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CHANGES IN PHOSPHOLIPID SUSCEPTIBILITY TOWARD PHOSPHOLIPASES INDUCED BY ATP DEPLETION IN AVIAN AND AMPHIBIAN ERYTHROCYTE MEMBRANES

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SUMMARY

About half of the sphingomyelin content of fresh and ATP-depleted chicken erythrocytes is hydrolysed by sphingomyelinase. Removal of sphingomyelin exposes the rest of the membrane phospholipids to hydrolysis by phospholipase C only in ATP-depleted but not in fresh cells. Addition of both sphingomyelinase and phospholipase C to ATP-depleted cells causes about 60-70 % hydrolysis of the total phospholipids accompanied by extensive (90 %) hemolysis.

The phospholipids of toad erythrocytes are partially available to phospholipase C activity in fresh cells (17–25 % hydrolysis) without prior sphingomyelinase treatment. However, in ATP-depleted toad cells phospholipase C hydrolyses 66 % of phospholipids and causes extensive lysis. Treatment of either fresh or ATP-depleted toad erythrocytes by sphingomyelinase together with phospholipase C induces hydrolysis of most of the phospholipids with complete lysis. Restoration of ATP to ATP-depleted cells endows them with resistance to the attack of phospholipase C.

The correlation between changes in ATP level and membrane organization as revealed by increased susceptibility toward phospholipases is discussed.

INTRODUCTION

The phospholipids present in the plasma membranes are considered to play an important role in the organization of the membrane and particularly in forming a seal or barrier against leakage of the cell content. The hydrolysis of phospholipids by phospholipases thus became a very suitable tool for the investigation of lipid organization and their role in the sealing property of the membrane. The availability of membrane phospholipids to phospholipases attack varies in different cell types [1, 2] and for the same cell varies with changes in the metabolic activity or physiological conditions [3], indicating that the organization of lipids is in a dynamic state. Thus it has been shown that lecithin and other phospholipids of human eythrocytes are protected against phospholipase C (*Bacillus cereus*) but became available to hydrolysis if the

cells are incubated with detergents [4] or in a hypotonic medium [5, 6]. However, as shown by Colley et al. [1], erythrocyte membrane phospholipids can be exposed in cells incubated in isotonic medium if sphingomyelin is removed by treatment with sphingomyelinase. Hydrolysis of human erythrocyte phospholipids by both sphingomyelinase and phospholipase C is accompanied by cell hemolysis [1].

It has been shown previously [3] that a heated preparation of phospholipase C (*Clostridium perfringens*) neither hydrolyse nor hemolyse fresh chicken erythrocytes. However, this preparation is able to hydrolyse most of the membrane phospholipids from ATP-depleted cells. In this case phospholipid hydrolysis is accompanied by cell hemolysis. In the light of these observations it might be inferred that the heated preparation of phospholipase C (*Cl. perfringens*) behaves toward ATP-depleted chicken erythrocytes as the combination of sphingomyelinase and phospholipase C (*B. cereus*) does toward human erythrocytes. Indeed, it is known that the phospholipase C from *Cl. perfringens* contains beside the well known lecithinase, also sphingomyelinase activity [7]. In the present work it was decided to use purified sphingomyelinase and phospholipase C in order to obtain further information on the changes in the availability of the phospholipids of fresh and ATP-depleted chicken erythrocytes to these enzymes. Furthermore, the question arises of whether the arrangement of phospholipids as exhibited by chicken erythrocytes is a general property of nucleated cells. In order to test this possibility toad erythrocytes were also studied.

MATERIALS AND METHODS

Cells

Chicken erythrocytes were collected from the neck of decapitated chickens into an Erlenmeyer containing heparin (100 units/ml blood). Human erythrocytes type O^+ were obtained by vein puncture from volunteer donors. Toad erythrocytes were obtained by heart puncture.

The cells were washed by centrifugation in Soln K (see below) as described [8] and either used immediately or after storage at 4 °C for periods not exceeding 24 h. Hemolysis was carried out and measured as described previously [8].

Medium

The medium used for washing and incubation of the cells contained 135 mM KCl; 5.4 mM NaCl and 0.8 mM $MgSO_4$ in 30 mM Tris · HCl buffer (pH 7.4) (Soln K).

ATP depletion and restoration

For ATP depletion, the cells were incubated in Soln K with addition of 1 mM KCN and 20 mM NaF at 37 °C for 12 h. Restoration of ATP was accomplished by incubating the washed ATP-depleted cells in Soln K containing: 5 mM glucose, 1 mM adenine, 5 mM inosine and 10 mM phosphate buffer (pH 7.4) at 37 °C for 8 h with gentle agitation as described [3].

Enzyme treatment and phospholipid extraction

Cell suspension containing 5 % cells (v/v) were incubated with different enzymes at 37 °C for 30 min. The reaction was stopped by dilution (3 times) with

cold Soln K followed by centrifugation at 10 000 × *g* · 10 min at 4 °C. The supernatant was used for measurements of the degree of hemolysis. The pellet was resuspended in 3-6 vol. of Soln K and the phospholipids extracted immediately according to Burger et al. [9]. The extracted phospholipids were finally dissolved in 1ml chloroform/methanol solution (2:1,v/v) and the phospholipid phosphorous determined as described by Barttlet [10].

Phospholipid analysis

Aliquots of equal volumes containing between 0.05–0.2 μmole phospholipids were applied to silica gel H (Merck) coated glass plates (20 cm × 20 cm) and phospholipids separated at 4 °C by use of two dimensional chromatography using chloroform methanol water (60:35:5, by vol.) as the first solvent. When the solvent approached 2 cm from the upper edge the plates were removed and dried at room temperature for 1 h. The dried plates were rerun at right angle to the first direction in a mixture of chloroform/methanol 28 % NH_3 (30:10:2, by vol.).

The individual phospholipids were visualized by use of iodine vapours. The spots were scraped and their phosphorous content was determined according to Barttlet [10]. The recovery of phospholipid phosphorous in the various spots was 100 ± 10 % for the total amount applied.

A commercial preparation of phospholipase C (*Cl. perfringens*) was obtained from Worthington Chemical Co. The enzyme was heated prior to use as described [11].

Phospholipase C (*B. cereus*) partially purified was obtained from Makor Chemicals, Jerusalem, Israel; a purified preparation of phospholipase C (*B. cereus*; EC 3.1.4.3) and partially purified sphingomyelinase was a generous gift from Professor L. L. M. Van Deenen from the Department of Biochemistry, University of Utrecht, the Netherlands. If not otherwise stated only the purified phospholipase C preparations were used.

All solvents and reagents used in this work were of analytical grade.

RESULTS

Hemolysis of fresh and ATP depleted human and chicken erythrocytes by phospholipase C preparations (Cl. perfringens and B. cereus)

It has been shown previously that a heated preparation of phospholipase C from *Cl. perfringens* causes hemolysis of ATP-depleted but not of fresh chicken erythrocytes [3]. Thus it was of interest to find out whether ATP depletion sensitized human erythrocytes to heated phospholipase C (*Cl. perfringens*) preparations as well as to phospholipase C of *B. cereus* which is known to have no hemolytic effect on fresh human cells. As can be seen from Table I, the *Cl. perfringens* preparation hemolysed both ATP-depleted and fresh human erythrocytes. However, the phospholipase C of *B. cereus* has practically no effect on either fresh or ATP-depleted human or chicken erythrocytes. As mentioned above, the heated phospholipase C of *Cl. perfringens* contains also a sphingomyelinase activity, therefore the possibility was considered that this activity is responsible for the lytic effect of the heated phospholipase C of *Cl. perfringens* on ATP-depleted cells. The results of Table II clearly show that addition of sphingomyelinase and phospholipase C (*B. cereus*) together or preincubation of ATP-depleted cells with sphingomyelinase followed by treatment

TABLE I

SUSCEPTIBILITY OF ATP-DEPLETED CHICKEN AND HUMAN ERYTHROCYTES TO HEMOLYSIS BY PHOSPHOLIPASE C (*Cl. perfringens* and *B. cereus*)

All experimental conditions as described in Materials and Methods. To the incubation medium which contained the heated phospholipase C of *Cl. perfringens* 5 mM of $CaCl_2$ was added. The concentration of the enzymes were 1.6 μg/ml. The partially purified *B. cereus* enzyme was used.

	% hemolysis			
	Human		Chicken	
Enzyme source	Fresh	ATP-depleted	Fresh	ATP-depleted
Cl. perfringens (heated)	42	53	9	49
B. cereus	9	10	6	11

TABLE II

EFFECT OF THE ORDER OF ADDITION OF PHOSPHOLIPASE C AND SPHINGOMYELINASE ON THE HEMOLYSIS OF FRESH AND ATP-DEPLETED CHICKEN ERYTHROCYTES

Samples of 3 ml of 2 % fresh and ATP-depleted chicken erythrocytes were first incubated at 37 °C with 0.08 units of sphingomyelinase; 0.27 units of phospholipase C or both for 30 min. The cells were then centrifuged and the degree of hemolysis was determined (on the supernatant) as previously described [8]. The pellet was washed 3 times in Soln K, resuspended in fresh Solution K and the enzymes were added under the same concentrations but in an inverse order. The degree of hemolysis was measured after an additional incubation of 30 min as above. N.D., not determined.

	First treatment		Second treatment	
Erythrocytes	Enzyme	% hemolyis	Enzyme	% hemolysis
Fresh	(1) Sphingomyelinase	6	Phospholipase C	10
	(2) Phospholipase C	0	Sphingomyelinase	2
	1+2	10		N.D.
ATP-depleted	(1) Sphingomyelinase	6	Phospholipase C	86
	(2) Phospholipase C	0	Sphingomyelinase	4
	1+2	92		N.D.

with *B. cereus* enzyme after removal of sphingomyelinase caused their complete hemolysis. Addition of the enzyme in reverse order had no effect. However, the incubation of fresh cells with both enzymes in either order or together does not cause any hemolysis.

Reversibility of ATP dependent susceptibility of chicken erythrocytes phospholipids towards hydrolysis by phospholipase C

In order to verify whether the hemolytic effects were quantitatively correlated with the hydrolysis of phospholipids, measurements of individual phospholipid

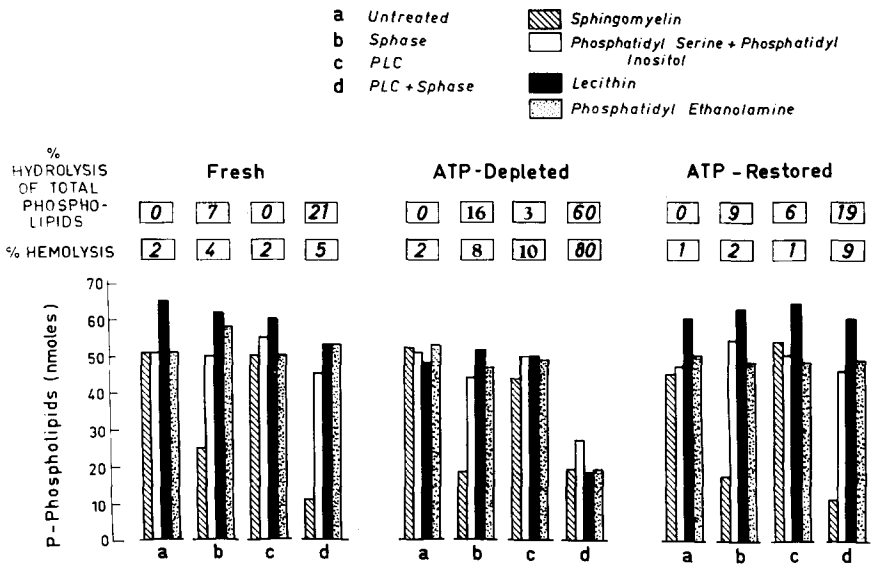

Fig. 1. Susceptibility of phospholipids of ATP-depleted chicken erythrocytes to enzymatic hydrolysis by phospholipase C of *B. cereus*. The enzyme concentrations were as follows: sphingomyelinase (sphase), 0.120 I.U./ml; phospholipase C, *B. cereus* (PLC), 0.4 I.U./ml. Equal aliquots (80 μl) of the phospholipids extracted from equal amounts of cells were used for chromatographic separations and the phospholipid phosphorous was determined as described in Materials and Methods.

content prior to and following enzymatic treatment were made. As shown in Fig. 1, sphingomyelin of fresh and ATP-depleted cells was susceptible to hydrolysis by partially purified sphingomyelinase. On the other hand, phospholipase C (*B. cereus*) alone failed to hydrolyse phospholipids of fresh and ATP-depleted cells. However, when phospholipase C was added together with sphingomyelinase, substantial hydrolysis of phospholipids occurred only in ATP-depleted cells, whereas phospholipids of fresh cells remained unsusceptible to phospholipase C activity.

It is also shown that when ATP was restored in ATP-depleted cells, they became resistent toward phospholipase C and thus behaved similarly to fresh cells (Fig. 1).

Hydrolysis of phospholipids of fresh, ATP-depleted and ATP-restored toad erythtrocytes

Preliminary results suggested that toad erythrocytes which are also nucleated cells, have a relatively low sphingomyelin content (unpublished results). Thus it was of interest to study the effect of sphingomyelinase and phospholipase C activity on these cells. As can be seen from Fig. 2, the sphingomyelin amounts to about 14 % of the total phospholipid phosphorous content and it is susceptible to sphingomyelinase digestion in both fresh and ATP-depleted toad cells (Fig. 2).

Surprisingly, treatment of ATP-depleted toad erythrocytes by phospholipase C (*B. cereus*) causes hydrolysis of 66 % of the phospholipids accompanied by a complete hemolysis in the absence of sphingomyelinase treatment. In addition,

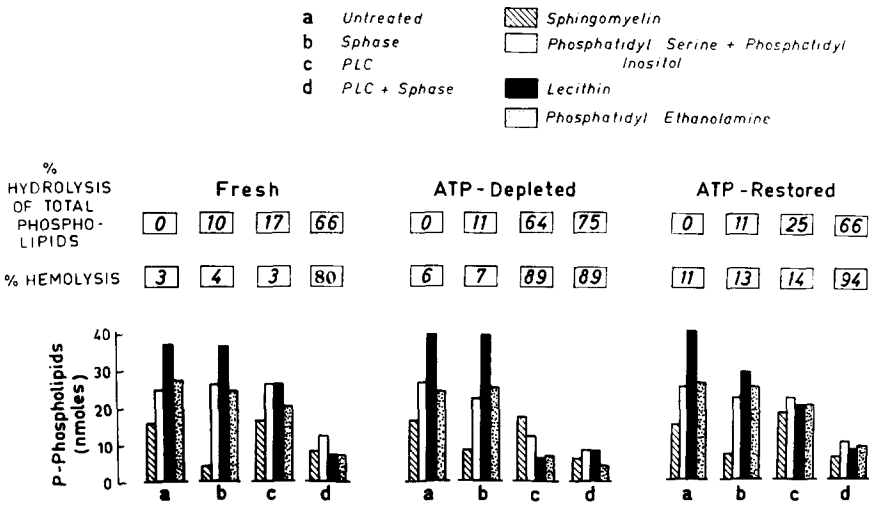

Fig. 2. Susceptibility of phospholipids of fresh and ATP-depleted toad erythrocytes to phospholipase C of *B. cereus* (PLC). Same experimental conditions and symbols as in Fig. 1. (40-μl aliquots were applied on the chromatography plates.)

phospholipase C (*B. cereus*) hydrolyses part of the phospholipids in fresh toad cells. As can be seen in Fig. 2, phospholipase C removes 17 % of the phospholipid phosphorous from toad fresh cells without any hemolysis in the absence of any sphingomyelin hydrolysis. Fresh toad eryhtrocytes were hemolysed only after combined action of both sphingomyelinase and phospholipase C (*B. cereus*). Hemolysis was induced in fresh toad erythrocytes only when sphingomyelinase acted prior to phospholipase C (Table III). The combined action of both enzymes resulted in about the same degree of phospholipid hydrolysis in both fresh and ATP-depleted cells and reached 66 and 75 %, respectively. Similar to the observation with chicken erythrocytes, restoration of ATP, in ATP-depleted toad erythrocytes also causes them to

TABLE III

EFFECT OF THE ORDER OF ADDITION OF PHOSPHOLIPASE C AND SPHINGOMYELINASE ON THE HEMOLYSIS OF FRESH AND ATP-DEPLETED TOAD ERYTHROCYTES

Same experimental conditions as in Table II. The enzyme concentration was: sphingomyelinase 0.03 I.U./ml; phospholipase C (*B. cereus*) 0.32 I.U./ml. N.D., not determined.

	First treatment		Second treatment	
Erythrocytes	Enzyme	% hemolysis	Enzyme	% hemolysis
Fresh	(1) Sphingomyelinase	4	(1) Phospholipase C	78
	(2) Phospholipase C	6	(2) Sphingomyelinase	10
	1+2	78		N.D.
ATP-depleted	(1) Sphingomyelinase	10	N.D.	
	(2) Phospholipase C	89	N.D.	
	1+2	89	N.D.	

behave like fresh cells upon incubation with phospholipase C (Fig. 2). However, it should be mentioned that toad erythrocytes seem to be more fragile than chicken erythrocytes and the various treatments, required for ATP depletion and ATP restoration caused a low deree of background hemolysis (about 10 %, see Fig. 2).

DISCUSSION

The results presented in this work confirm previous observations that sphingomyelin is exposed on the outer face of the red blood cell membrane. However, it appears that sphingomyelin is not the only factor which protects the rest of the phospholipids and determines their degree of hydrolysis by phospholipase C. The present data show that membranes of fresh chicken erythrocytes differ in the degree of phospholipids exposure from other red blood cells of species such as human, pig [1] and toad. Removal of sphingomyelin exposes the rest of the phospholipids to phospholipase C activity in the latter cases but not in chicken eythrocytes. In order to render these cells susceptible to phospholipase C it is necessary to deplete them of ATP in addition to removal of sphingomyelin.

Phospholipase C alone is able to cause marked hydrolysis with extensive hemolysis of ATP-depleted toad erythrocytes and hydrolyses as much as 17 and 25 % of the phospholipids of fresh and ATP-restored toad cells, respectively. In contrast to previous observations and to the results obtained with chicken erythrocytes, removal of sphingomyelin is not essential for hydrolysis of toad erythrocytes phospholipids by phospholipase C.

Preliminary results obtained in this laboratory indicate that phospholipase C acts upon rat erythrocytes in much the same way as on toad erythrocytes (unpublished results). Based on the above observation one can assume that the phospholipids of the erythrocyte membrane are protected against phospholipase C activity by two seemingly independent factors: (a) masking by sphingomyelin and (b) the state of membrane organization which might be reversibly altered by changing the ATP levels.

The degree of phospholipids hydrolysis is partially dependent on the relative amounts of sphingomyelin. Previous observations [1] and the present results, suggest that in cases such as human, pig and chicken (ATP-depleted erythrocytes) hydrolysis of sphingomyelin which amounts to 22–30 % of the total phospholipids exposes the rest of the phospholipids towards phospholipase C activity. On the other hand in cells where sphingomyelin is relatively low (10–15 %) such as toad or rat (unpublished results) phospholipase C is able to hydrolyse part of the other phospholipids without prior hydrolysis of sphingomyelin.

The final degree of phospholipid hydrolysis appears to be dependent in such cases as chicken or toad erythrocytes on the ATP level of the cells. Changes in the ATP levels of the cells might induce organizational changes in the membrane proteins-lipids or both by phosphorylation-dephosphorylation reactions of membrane components. Reported observations of phosphorylations of membrane lipids [12] and proteins [13, 14] in human erythrocytes and preliminary results obtained in this laboratory showing a positive correlation between phosphorylation of chicken erythrocytes membranes, ATP levels and reversible susceptibility of ATP-depleted cells towards phospholipase attack (unpublished results) support this interpretation.

The possibility that the changes observed in the degree of phospholipids exposure in ATP-depleted cells, are due to accumulation of small amounts of lysophospholipid compounds should also be considered. Such lyso-compounds possessing detergent-like activity might in sublytic concentrations expose membrane phospholipids to phospholipase C. Indeed it has been shown [4] that human erythrocyte phospholipids can be hydrolysed by phospholipase C after the addition of Triton X-100 in sublytic concentration.

Preliminary results (unpublished) in our laboratory have shown that addition of bovine serum albumin to ATP-depleted cells removes most of the lyso-compounds. This treatment, however, did not change the behaviour of these cells towards phospholipase C.

The results of this work further support the notion that a variable fraction of the phospholipids is organized in such a way as to be unavailable to phospholipases. This might be due to a closer association with some of the membrane components other than phospholipids. Indeed, it has been shown [15] that phospholipase C (*Cl. Welchii*) failed to hydrolyse complexes of asolectin and basic proteins such as cytochrome *c* and lysozyme. If similar associations exist between phospholipids and protein of chicken erythrocyte membranes it is expected to be reversible and correlated with ATP levels.

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