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SOME CHARACTERISTICS OF A PHOSPHOLIPASE A_2 FROM SHEEP RED CELL MEMBRANES

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SUMMARY

1. Red cell membranes of sheep contain a phospholipase A_2 (phosphatide 2-acyl-hydrolase, EC 3.1.1.4), which degrades exogenous phosphatidylcholine.

2. The enzyme requires Ca^{2+} and is greatly stimulated by detergents such as Triton X-100 or deoxycholate. Its pH optimum is 8.0. Phosphatidylcholine hydrolysis is not affected by albumin or high ionic strength.

3. The enzyme has a broad temperature optimum between 30 and 45 °C and is relatively heat stable.

4. The phospholipase A activity is destroyed by 2-chloroethanol, but partially regained upon dialysis against an aqueous buffer.

5. The enzyme is completely inhibited in the presence of dodecylsulfate. After removal of the denaturant the enzymatic activity is restored.

INTRODUCTION

We have recently shown that membrane proteins from sheep red cells preferentially bind sphingomyelin which may be one of the reasons why this membrane type contains high proportions of this lipid and at the same time extremely low amounts of phosphatidylcholine [1]. During the course of these experiments we consistently observed the appearance of lyso-phosphatidylcholine after the binding dialysis of sheep red cell apoproteins with various lipid mixtures, suggesting the presence of a phospholipase A. This finding was unexpected in two ways.

First, our recombination technique, based on the use of 2-chloroethanol as solubilizing agent, led to an inactivation of all enzymatic membrane proteins, as far as they had been tested [2]. If the appearance of lyso-phosphatidylcholine was the result of a catalytic breakdown, this would mean that the phospholipase A in question escaped denaturation by 2-chloroethanol or, alternatively, this membrane protein was reactivated during the transfer to an aqueous medium in the presence of lipid.

Second, most of the current work on phospholipase A in cell membranes indicates that such an enzyme seems to be completely absent from red cell membranes

generally [3–7]. However, Paysant et al. [8] have described various experiments which indicated that red cell membranes from rats have phospholipase A activity towards phosphatidylglycerol from bacteria. Later they could show [9] that human erythrocytes contain a similar enzyme which also splits phosphatidylethanolamine and that a considerable increase in activity was observed after treatment of red cell haemolysate with trypsin. This proteolytic treatment also resulted in a breakdown of phosphatidylcholine.

In the following study we describe experiments elucidating the problem of the identity and properties of a phospholipase A in sheep red cell membranes.

MATERIALS AND METHODS

Substrate

Phosphatidylcholine (pure) from egg yolk (Merck) was further purified by preparative thin-layer chromatography, dissolved in chloroform (10 mg/ml) and stored at −20 °C.

[^{14}C]phosphatidylcholine (uniformly labeled, 1.8 Ci/mmole, from *Chlorella pyrenoidosa*), a product of New England Nuclear Company, was used as substrate for phospholipase A determination.

For determination of the enzyme specifity [^{14}C]phosphatidylcholine (labeled predominantly in the 2-position) was prepared as follows. Human erythrocyte phosphatidylcholine, isolated by thin-layer chromatography, was converted to lyso-phosphatidylcholine, by the action of phospholipase A_2 (*Crotalus adamanteus*, from Koch-Light Laboratories Ltd.) as described by Saito and Hanahan [10]. Following a slight modification of the method described by Smith and Silver [11] this preparation was then reacylated with [1-^{14}C]linoleic acid in the presence of acyl transferase, coenzyme A, ATP and $MgCl_2$ in phosphate buffer at pH 7.4 using fresh liver homogenate as the enzyme source. The lipids were extracted [12] and purification of phosphatidylcholine was achieved by preparative thin-layer chromatography. Subsequent hydrolysis with phospholipase A_2 (Crotalus adamanteus) showed that at least 94 % of the label was present in the 2-position. This substrate had a specific activity of $1.6 \cdot 10^2$ mCi/mole.

Sheep erythrocyte membranes

Erythrocytes were obtained from adult sheep, with no attempt at differentiation of Low potassium (LK) or High potassium (HK) types. The fresh blood was collected in sodium citrate anticoagulant mixtures. The plasma and buffy coat (containing the leucocytes) were separated from the red cells by centrifugation and cautiously removed. The sediment was washed 3 times at 0–4 °C with 2 vols of phosphate buffer (310 mosM, pH 8.0). The washed red cells were not examined for reticulocytes and residual white blood cells.

Erythrocyte membranes were then prepared by the procedure of Dodge et al. [13], modified as in ref. 2 and suspended in 0.05 M glycyl–glycine (pH 8.0) at a concentration of 3–5 mg protein per ml.

Sheep erythrocyte membrane recombinate

Red cell membranes of sheep were dissolved in 2-chloroethanol–water (9:1, v/v) at an apparent pH of 2 [2]. Recombination of the solubilized membranes was

obtained by dialysis against 10 mM Tris–HCl, 10 mM $CaCl_2$ and 1 mM $MgCl_2$ at pH 7.6. The recombinate was isolated by centrifugation (27000 rev./min, 30 min, 4 °C) and taken up in 0.05 M glycyl–glycine (pH 8.0) at a concentration of 3–5 mg protein per ml.

Sheep erythrocyte membrane proteins

Red cell membranes of sheep were solubilized in 2-chloroethanol and the membrane proteins separated from the lipids by gel chromatography on Sephadex LH-20 in 2-chloroethanol–water (9:1, v/v)[2]. The lipid-free protein was pooled and reaggregated by dialysis against 10 mM Tris–HCl, 10 mM $CaCl_2$ and 1 mM $MgCl_2$ at pH 7.6. The protein suspension was centrifuged (27000 rev./min, 30 min, 4 °C) and the sediment resuspended in 0.05 M glycyl–glycine (pH 8.0) at a concentration of 3–5 mg protein per ml.

All preparations were frozen at −20 °C until use and could be stored for months without loss of activity.

Determination of phospholipase A activity

The standard incubation mixture contained 0.05 M glycyl–glycine (pH 8.0), 8 mM $CaCl_2$, Triton X-100 (3 mg/ml), 1 mg phosphatidylcholine and [^{14}C]phosphatidylcholine ($27.5 \cdot 10^{-3}$ nmoles) in a volume of 0.2 ml. The reaction was started by adding 0.3 ml of the enzyme preparation containing 1 mg of protein. After incubation at 37 °C for 60 min, the reaction was stopped by the addition of 2 ml of chloroform–methanol (2:1, v/v). The homogenate was shaken vigorously for 3 min and centrifuged (10 min, 8000 rev./min) for complete separation into two phases. The aqueous phase and protein layer were removed by aspiration. For determination of total radioactivity, samples of the chloroform phase were taken. The chloroform was evaporated and the lipids dissolved in 5 ml of methanol before adding 10 ml of a scintillation fluid (toluene containing 7 g 2-(4′-tert-butylphenyl)-5-(4″-biphenylyl-1,3,4-)-oxadiazole (Ciba-Geigy, Basel)).

Samples of the extracted lipids were separated by thin-layer chromatography on Silica Gel HR (Merck) with a solvent system of chloroform–methanol–water (14:6:1, v/v/v)(Merck). The spots were localized by exposure to iodine vapour and the zones corresponding to the different phospholipids were scraped out into the counting vials, adding 5 ml of methanol and 10 ml of the scintillation solution. The radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer (Model 2450).

More than 80 % of the radioactivity applied to the thin-layer plate was recovered in the three spots corresponding to free fatty acids, phosphatidylcholine and lyso-phosphatidylcholine. The radioactivity remaining at the origin was less than 1 % in both incubation with and without enzyme. Phospholipase A activity was expressed as percentage of phosphatidylcholine hydrolyzed.

Determination of protein

Protein concentration of the enzyme preparation was determined by the method of Lowry et al. [14].

RESULTS

Incubation of sheep erythrocyte membranes with phosphatidylcholine as substrate resulted in release of free fatty acids and the formation of lyso-phosphatidylcholine (Fig. 4). Lecithinase activity could not be detected in human erythrocyte membranes.

Detergents

Only slight phospholipase activity was obtained in standard reaction mixtures without detergent. Addition of deoxycholate or Triton X-100 to the phosphatidylcholine dispersion greatly enhanced the activity of the enzyme system. Fig. 1A shows the activating effect of increasing amounts of deoxycholate and Triton X-100 on hydrolysis of phosphatidylcholine. Optimal activity was obtained with 1.5 mg per 0.5 ml of standard incubation mixture for both detergents. As demonstrated, Triton X-100 was more effective as an activator compared to deoxycholate. Therefore, Triton X-100 has been used for all further experiments. When dodecylsulfate was added to

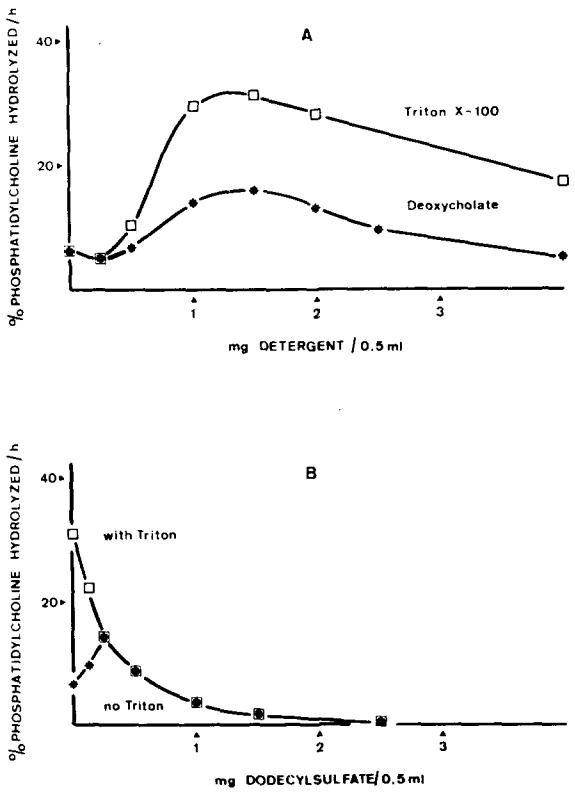

Fig. 1. A. Effect of detergents on phospholipase A activity. Standard assays, carried out as described in Materials and Methods, contained deoxycholate (0–4 mg) (∗−∗) or Triton X-100 (0–4 mg) (□−□). B. Interaction of dodecylsulfate and Triton X-100 in phospholipase A reaction. The amount of phosphatidylcholine hydrolyzed is plotted as a function of dodecylsulfate concentration in standard assay in the presence (□−□) and in the absence of 3 mg/ml Triton X-100 (∗−∗).

the reaction mixture, we observed a slight activation up to 0.25 mg per 0.5 ml of reaction mixture (Fig. 1B); above 0.25 mg, inhibition occurred. The experiment was repeated in the presence of the optimal concentration of Triton X-100 (1.5 mg/0.5 ml): in this case only the inhibitory effect could be noted.

pH Optimum and ionic conditions

The optimal pH of the assay system was shown to be in the range of 8.0, though significant enzyme activity was still found at pH 6.0 (65 % of maximal) and at pH 9.5 (80 % of maximal). NaCl added to the assay system up to a concentration of 2 M was without any significant influence on the enzyme reaction. However, a slight inhibitory effect could be observed with increasing ionic strength.

As shown in Fig. 2, we obtained about 20 % of phosphatidylcholine hydrolysis when Ca was omitted in the standard incubation mixture. Increasing amounts of EDTA, added to the assay system in order to remove endogenous Ca, caused a progressive diminution of the phospholipase A activity. The enzyme was completely inhibited at an EDTA concentration of 0.4 mM. Subsequent supply of Ca fully restored the initial enzyme activity, indicating that EDTA inhibited the phospholipase A through its ability to complex Ca. Fig. 2 also demonstrates the influence of increasing amounts of Ca and Mg added to the incubation mixture up to a concentration of 20 mM. As shown, Ca stimulated the phospholipase A with a maximum activation at concentrations around 8 mM. There was, however, no significant activation when Mg was used.

Ultrasonication

The phospholipase A was assayed after exposure to 20 KHz ultrasound for varying times at 20 °C (MSE ultrasonic disintegrator Model 7100). Irradiation up to 60 s gradually increased the enzyme activity up to 1.3-fold. With longer sonication times the phospholipase activity was diminished.

When the membrane suspension was subjected to repeated slow freezing and thawing, the enzyme activity was not significantly changed.

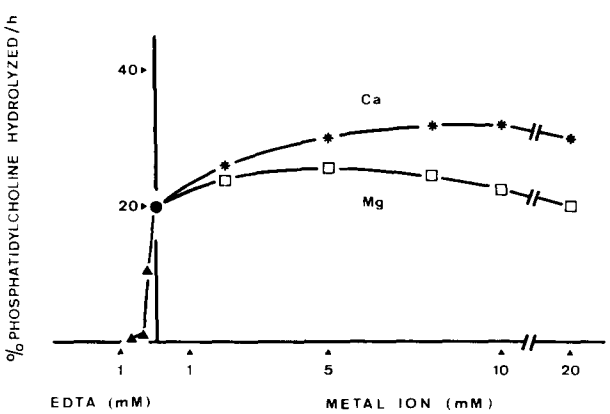

Fig. 2. Dependence of phospholipase A activity on Ca^{2+} and Mg^{2+}. Standard assays were carried out as described in Materials and Methods. Calcium chloride (∗—∗), magnesium chloride (□—□) or EDTA (▲—▲) were added as indicated.

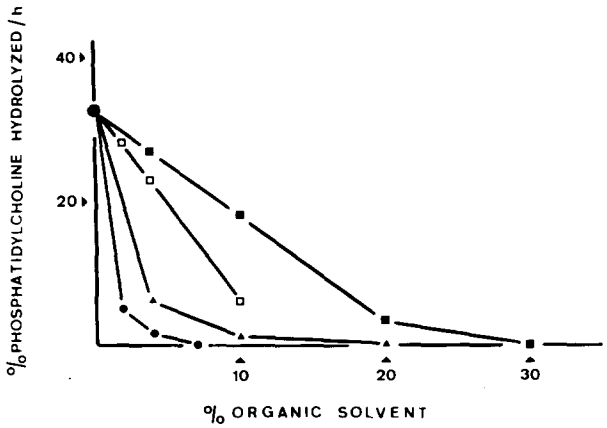

Fig. 3. Effect of organic solvents on phospholipase A activity. Standard assays, carried out as described in Materials and Methods, contained varying amounts of organic solvents. ■—■, ethanol; □—□, diethyl ether; ▲—▲, 2-chloroethanol; ●—●, butanol.

Organic solvents

A number of organic solvents were examined for their influence on phospholipase activity. As illustrated in Fig. 3 each of these compounds inhibited the hydrolysis of phosphatidylcholine. The enzyme retained half of its activity in 10 % ethanol solution and still showed significant phosphatidylcholine hydrolysis in a saturated ether medium. In ethanol complete loss of enzymatic activity was not observed under a concentration of 30 %. However, the degree of inhibition by butanol and 2-chloroethanol was much more pronounced. Thus, the enzyme was inhibited by only 5 % butanol and 15 % 2-chloroethanol.

Effect of other variables

Attempts were made to increase the enzyme activity by adding albumin, which is known to bind fatty acids. However, bovine serum albumin (fatty acid-poor, from Calbiochem; devoid of detectable phospholipase activity) present in the incubation mixture up to 20 mg/ml, did not influence the phospholipase activity.

In order to compare the properties of the phospholipase A of sheep red cell membranes with those of other well-known phospholipases, it was of interest to investigate the effect of Silica gel on the enzyme activity [15]. Silica gel HR, added to the reaction mixture up to a concentration of 0.4 mg/ml, did not stimulate the breakdown of phosphatidylcholine, but was slightly inhibitory.

Effect of temperature, heat stability

The phospholipase A showed a rather broad temperature optimum between 30 °C and 45 °C. With temperatures below 0 °C and over 70 °C no enzyme activity was observed.

The enzyme system showed high stability against heat treatment. It retained most of its activity when incubated for 10 min at 70 °C, but rapidly lost its activity between 70 °C and 80 °C. Complete loss of enzymatic activity was obtained when the enzyme preparation was heated for 10 min at 100 °C.

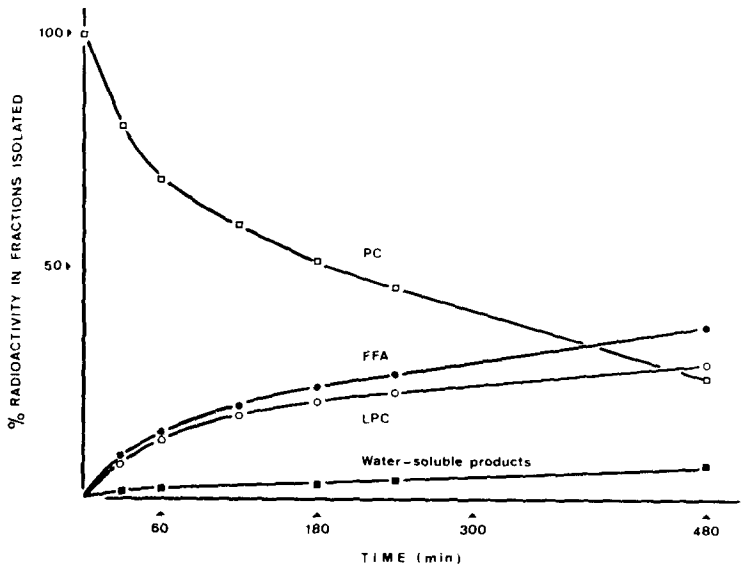

Fig. 4. Time-dependence of the degradation of phosphatidylcholine and formation of lyso-phosphatidylcholine, free fatty acids and water-soluble products. Standard assays were carried out as described in Materials and Methods. Phosphatidylcholine (PC), □—□; free fatty acids (FFA), ●—●; lyso-phosphatidylcholine (LPC), ○—○; water-soluble products, ■—■.

Time of incubation

The time-course both for the degradation of phosphatidylcholine and the formation of lyso-phosphatidylcholine, free fatty acids and water-soluble radioactive compounds is shown in Fig. 4. At all time intervals the recovery of radioactivity of the sum of the four fractions was within 70–75 % of the radioactivity added to the incubation mixture. Both the disappearance of phosphatidylcholine and the formation of lyso-phosphatidylcholine and free fatty acids were found to be non-linear with time. Up to one hour the formation of lyso-phosphatidylcholine paralleled the appearance of free fatty acids. After longer incubation times the amount of free fatty acids formed was greater than the amount of lyso-phosphatidylcholine produced. The corresponding increase in water-soluble products indicated a further breakdown of lyso-phosphatidylcholine by a lysophospholipase.

Determination of kinetic constants

The effect of substrate concentration on the initial reaction velocity of the phospholipase A is shown in Fig. 5. The experimental values are plotted according to the method of Lineweaver and Burk [16]. The reaction apparently follows Michaelis–Menten kinetics, with a K_m value of $7 \cdot 10^{-4}$ M and a V of 9 nmoles per min per mg protein.

Specificity of position and substrate

When (2-[1-^{14}C]linoleoyl)phosphatidylcholine was used as a substrate for the phospholipase A determination, no radioactive lyso-phosphatidylcholine was found as a degraded product, and all radioactivity was recovered as free fatty acids (Fig. 6).

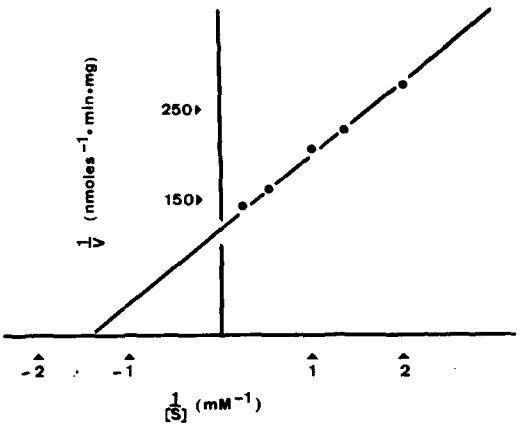

Fig. 5. Lineweaver–Burk plot of phosphatidylcholine hydrolysis by phospholipase A of sheep erythrocyte membranes. Varying amounts of phosphatidylcholine were incubated 10 min at 37 °C with 1 mg of sheep stroma under standard assay conditions as described in Materials and Methods.

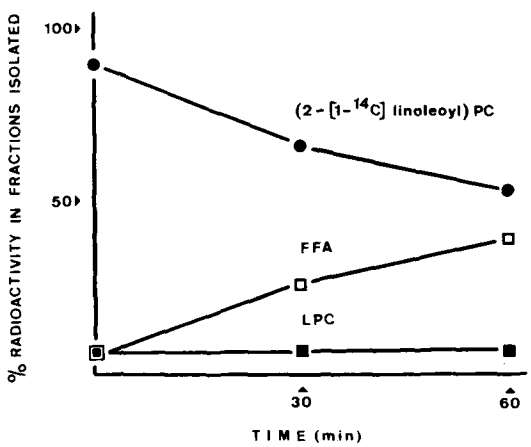

Fig. 6. Hydrolysis of (2-[1-^{14}C]linoleoyl) phosphatidylcholine and formation of radioactive lysophosphatidylcholine and free fatty acids by phospholipase A. Standard assays, carried out as described in Materials and Methods, contained $27.5 \cdot 10^{-3}$ nmoles of (2-[1-^{14}C]linoleoyl) phosphatidylcholine. (2-[1-^{14}C]linoleoyl) phosphatidylcholine, ● — ●; free fatty acids (FFA), □ — □; lyso-phosphatidylcholine (LPC), ■ — ■.

The phospholipase A of sheep erythrocyte membranes seemed therefore specific for the fatty acid at the 2-position. However, the use of a doubly-labeled substrate, e.g. ([1-^{3}H]palmitoyl-[2-^{14}C]linoleoyl) phosphatidylcholine, would be desirable to ascertain such a specificity.

Preliminary experiments have suggested that the enzyme preferentially catalysed the hydrolysis of phosphatidylcholine and showed very low activity towards phosphatidylethanolamine. This point is further discussed in the accompanying paper [17].

Phospholipase A activity in sheep erythrocyte membrane proteins and recombinate

From Fig. 3 one can conclude that the phospholipase A assay could not be carried out when the concentration of 2-chloroethanol in the incubation mixture exceeded 10 %. Thus, after solubilization of sheep erythrocyte membranes in 2-chloroethanol–water (9:1, v/v) at pH 2 no phospholipase activity was discernible (Table I).

Solubilized membranes and isolated membrane proteins were reaggregated by dialysis against an aqueous buffer solution. When 2-chloroethanol was removed, phospholipase activity could be partially recovered in both lipid-combined protein and reaggregated lipid-free membrane proteins.

Table I demonstrates that the specific activity of the apoprotein and recombinate in the standard assay (with Triton X-100 and 8 mM Ca) was very low as compared to the original ghosts. However, it was markedly enhanced when Triton X-100 was replaced by deoxycholate and Ca was increased up to 30 mM. Accordingly, the reconstituted enzymatic system required deoxycholate and a higher level of Ca^{2+} for maximal stimulation.

As previously observed (Fig. 1B), dodecylsulfate strongly inhibited the phospholipase activity. After storage of the stroma suspension in the presence of 2 % dodecylsulfate for 4 h, the detergent was removed by dialysis against 10 mM Tris-HCl buffer (pH 8.0) for 2 days and subsequent gel chromatography on Sephadex LH-20, using 2-chloroethanol–water (9:1, v/v) as solubilizing and eluting solvent. The apoprotein was then transferred to an aqueous buffer (10 mM Tris–HCl, 10 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.6) by dialysis, centrifuged and resuspended in 0.05M glycyl–glycine buffer (pH 8.0). Phospholipase determination in the reaggregated membrane proteins after dodecylsulfate treatment showed that the enzymatic activity was partly restored (Table II). Upon treatment of the stroma suspension in 2 % dodecylsulfate –8 M urea, enzyme reactivation was diminished. Pretreatment of stroma with 2 % dodecylsulfate in combination with sulfhydryl reagents, such as dithiothreitol, led to inactivation.

TABLE I

PHOSPHOLIPASE A ACTIVITY IN SHEEP ERYTHROCYTE MEMBRANE PROTEINS AND RECOMBINATE

Phospholipase A activity was determined in standard assays, as described in Materials and Methods, incubating 2 h at 37 °C, with either 1.5 mg Triton X-100–8 mM calcium or 1.5 mg deoxycholate–30 mM calcium.

Enzyme Source	Phospholipase A activity (% phosphatidylcholine hydrolyzed/mg (2 h))	
	Triton X-100+ 8 mM Calcium	Deoxycholate+ 30 mM Calcium
Membrane suspension	44	33
Membrane solution (in 90 % 2-chloroethanol pH 2)	–	–
Recombined membranes	4	16
Apoprotein suspension	1	11

TABLE II

INFLUENCE OF DODECYLSULFATE TREATMENT ON PHOSPHOLIPASE A ACTIVITY

Phospholipase A activity was determined in standard assays, as described in Materials and Methods, incubating 2 h at 37 °C, with either 1.5 mg Triton X-100–8 mM calcium or 1.5 mg deoxycholate–30 mM calcium.

Treatment of membrane suspension*	Phospholipase A activity (% phosphatidylcholine hydrolyzed/mg (2 h))	
	Triton X-100 8 mM Calcium	Deoxycholate 30 mM Calcium
Control 1 (ghosts without treatment)	44	33
Control 2 (ghosts suspended in 2 % dodecylsulfate)	—	—
2 % dodecylsulfate (removed by dialysis and gel chromatography)	4	23
2 % dodecylsulfate–8 M urea (removed by dialysis and gel chromatography)	2	7
2 % dodecylsulfate–dithiothreitol (removed by dialysis and gel chromatography)	—	—

* Details are given in the text.

DISCUSSION

Previous studies have attempted in a number of ways to find a phospholipase enzyme in erythrocytes. With the notable exception of a phospholipase A specific for phosphatidylglycerol in rat erythrocytes [8], no appreciable phospholipase A-like activity could be found either in erythrocyte haemolysate [3, 5, 6] or ghosts [4, 7] of various species. It should be mentioned that it is not always clear which species has actually been tested for phospholipase activity, mainly due to different aims of the studies involved. Recent work by Paysant et al. [9], however, demonstrated the existence of a very low phospholipase activity towards phosphatidylglycerol and phosphatidylethanolamine in human red cell haemolysate. After pretreatment with trypsin the enzyme activity was greatly enhanced and in this case also a considerable amount of phosphatidylcholine was degraded. So far we have been unable to detect phospholipase activity in human red cell ghosts, either before or after [17] trypsin treatment.

The present study demonstrates the evidence of an enzymatic system in sheep red cell membranes which catalyses the hydrolysis of exogenous phosphatidylcholine into free fatty acids and 1-acyl-lyso-phosphatidylcholine. With incubation times longer than one hour lyso-phosphatidylcholine is further degraded into free fatty acid and water-soluble products. Most likely, two enzymes are involved, a phospholipase A_2 and a lysophospholipase; this is in agreement with previous findings of Mulder and co-workers showing the existence of a lysophospholipase in erythrocyte haemolysate of human [5, 6], rabbit and ox [6].

Some properties of the phospholipase A from sheep erythrocyte membranes do agree with the general characteristics of the well-known phospholipases from other sources (*Crotalus adamanteus* venom [10, 18–21], *Escherichia coli* [22], post-heparin plasma [23], pig pancreas [24, 25], rat spleen [26], rat brain [27] and rat liver [28–31]). However, we do not observe a complete identity to one of these enzymes.

Although phospholipase A activity is observed in isolated membrane proteins of sheep erythrocytes, the possibility that lipids are necessary for correct functioning cannot be excluded. Phosphatidylcholine, present as substrate in the incubation mixture, may cause a lipid-reactivation of the enzyme.

Organic solvents have been shown to produce a depression in enzymatic activity, an effect which is more pronounced with solvents of increased solubilizing power for membranes, such as 2-chloroethanol and butanol. However, it was found that the inhibitory action of 2-chloroethanol is reversible. This solvent, known to be effective for the total solubilization of erythrocyte membranes [32], was reported to cause loss of all enzymatic activities [33]. However, since after removal of 2-chloroethanol by dialysis the phospholipase A partially regains activity, it appears to behave differently from other membrane-bound enzymes. As 2-chloroethanol promotes the formation of α-helix conformation [34], it may convert the enzyme to a structure which apparently renatures when transferred to water. A similar partial reactivation is observed after elimination of dodecylsulfate and urea. It remains as yet unknown whether this effect is due to a reversible denaturation of the enzyme protein or if it is concerned with reversible perturbations not related to conformational changes.

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