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PHOSPHOLIPASE A_2 FROM SHEEP ERYTHROCYTE MEMBRANES

Ca^{2+} DEPENDENCE AND LOCALIZATION

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Summary

The calcium dependence and the time course of phosphatidylethanolamine and phosphatidylcholine degradation by sheep erythrocyte membrane suspensions in presence of Triton X-100 were investigated. One enzyme with phospholipase A_2 specificity was found to be responsible for both phosphatidylethanolamine and phosphatidylcholine degradation.

The localization of this enzyme in the membrane of the sheep erythrocyte was investigated by proteolytic treatment of sealed erythrocyte ghosts from the outside and of ghosts which had both sides of the membrane exposed to chymotrypsin. The inability of sealed ghosts to take up chymotrypsin was followed by flux measurements of [^{14}C]dextran carboxyl previously trapped in the ghosts. No efflux of the marker was found during the proteolytic treatment. By comparing the residual phospholipase activities in the membranes from both ghost preparations, we concluded that the phospholipase is oriented to the exterior of the sheep erythrocyte.

Introduction

A phospholipase A_2 (EC 3.1.1.4) in sheep erythrocyte membranes has been identified and partly characterized in this laboratory [1]. Kramer et al. [1] found the enzymatic activity to be strongly stimulated by the detergents Triton X-100 and deoxycholate, and also by divalent cations, especially calcium. Kramer measured the enzymatic activity with exogenous phosphatidylcholine as substrate, and the phospholipase in this system showed a preference for

Abbreviations: EGTA, ethyleneglycol-bis-(2-aminoethyl ether)-*N*,*N*'-tetraacetic acid; SDS, sodium dodecyl sulfate.

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phosphatidylcholine, however, the endogenous phospholipids were not taken into consideration. The low phosphatidylcholine content of ruminant erythrocyte membranes was shown to be at least partly caused and upheld by this enzyme [2]. By testing erythrocyte membranes from other species, Zwaal et al. [2] found phospholipase activity directed towards phosphatidylethanolamine in ruminants and also in rat, rabbit and pig erythrocytes. No activity towards phosphatidylcholine or any other phospholipid could be detected in the last three species. The amount of endogenous phosphatidylethanolamine degraded in all species was much lower than that of phosphatidylcholine split in sheep erythrocyte membranes during the same incubation time.

From this observation the question arose, whether the phosphatidylethanolamine splitting in rat, rabbit and pig erythrocytes was indeed caused by a phospholipase specific for phosphatidylethanolamine. Another question concerns the phosphatidylethanolamine degradation in ruminants' erythrocytes, namely whether two enzymes are responsible for the respective cleavage of phosphatidylcholine and phosphatidylethanolamine, and if so, whether both enzymes are located at the same side of the erythrocyte membrane.

Zwaal treated whole erythrocytes and washed erythrocyte membranes with pronase and compared the phospholipase activities of the two preparations. He found the same degree of inactivation of the enzyme using phosphatidylcholine as substrate in both cases and concluded that the enzyme is oriented towards the external side of the membrane. If sheep erythrocyte membranes contain two enzymes with phospholipase activity, with a preference for endogenous substrates, one would expect the enzyme responsible for phosphatidylethanolamine degradation on the cytoplasmic side of the membrane. The asymmetric distribution of phospholipids in the sheep erythrocyte membrane is such that phosphatidylethanolamine is mainly oriented towards the cytoplasm, as Colley et al. showed [3].

In the first part of the present study we further characterize the membrane-bound phospholipase activity, in order to compare the properties of phosphatidylethanolamine degradation with those observed by Kramer et al. [1] for the phosphatidylcholine degradation. We analyzed the time course of the degradation of both substrates and studied the calcium dependence of the two phospholipase reactions. In the second part the localization of the enzymatic activity against phosphatidylethanolamine and phosphatidylcholine in the erythrocyte membrane was resolved. The results presented here may lead to a better understanding of the possible function of these membraneous phospholipase activities.

Materials and Methods

Materials

[^{14}C]Linoleic acid, [^{14}C]dextran carboxyl (M_r = 20 000) and [^{14}C]]phosphatidylcholine were purchased from New England Nuclear; Chelex 100 from Biorad Laboratories; lysophosphatidylethanolamine, *Naja naja* venom from Koch-Light Laboratories; α-chymotrypsin (bovine pancreas) B grade from Calbiochem; Kunitz-Soy-Bean-Trypsin-Inhibitor from Merck; 2-(4′-*tert*-butylphenyl)-5-(4′-biphenyl-1,3,4)-oxadiazole from Ciba-Geigy; protease type VI

from Sigma Chemicals. All the other chemicals were reagent grade purchased from either Merck or Fluka. For thin-layer chromatography Silicagel HR 60 from Merck was used.

Methods

Washed membranes were prepared by a modified [4] method of Dodge et al. [5] from sheep blood obtained from the local slaughterhouse. The protein content of the membrane preparations used was between 4 and 8 mg/ml as determined by the method of Lowry et al. [6]. For two-dimensional thin-layer chromatography the method of Broekhuyse [7] was used, and for phosphorus assay in the silicagel the method of Boettcher et al. [8]. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed following the method of Fairbanks et al. [9]. The non-hemoglobin protein content of ghost suspensions was estimated spectroscopically according to the method described by Bodemann and Passow [10].

Preparation of 2-[1-^{14}C]linoleyl-phosphatidylethanolamine

The method of Smith et al. [11] was modified. To 1 mg of lysophosphatidylethanolamine (1.93 μmol) and 25 μCi (0.4 μmol) [^{14}C]linoleic acid, 20 μmol ATP, 0.5 μmol CoA and 10 μmol $MgCl_2$ in 1 ml 40 mM potassium phosphate buffer (pH 7.4) were added and incubated with 0.3 ml of a preparation of rat liver microsomes and cytosol. These were prepared as follows [12].

1 g of fresh rat liver was homogenized in a Potter -Elvehjem homogenizer in 5 ml of a 20 mM Tris-HCl buffer (pH 7.2) containing 8.5% v/v sucrose. The homogenate was centrifuged for 10 min at 12 000 × *g*. The top of the supernatant containing fractions rich in fat was discarded. The remaining supernatant was then dialysed against 40 mM potassium phosphate buffer (pH 7.4) for 4 h.

0.3 ml of dialysed supernatant were added to the above mixture. After 2 h incubation at 37° C with mild shaking, the lipids were extracted according to Renkonen et al. [13]. The lipids were separated on thin-layer plates in chloroform/methanol/acetic acid/water (45 : 20 : 6 : 1). The centre of the plate was protected by a glass plate and the edge stained with iodine vapor. The band corresponding to phosphatidylethanolamine was scratched out and eluted with chloroform/methanol (2 : 1) over a small column. The solvent was evaporated under N_2 and the radioactive phosphatidylethanolamine taken up in an appropriate volume of chloroform/methanol (2 : 1). The overall yield relative to the lineoleic acid initially added was 12%. 98% of the recovered radioactivity was in the phosphatidylethanolamine fraction. Degradation by *Naja naja* venom showed 96% of [^{14}C]linoleic acid to be esterified to position 2. Specific activity was 7.7 Ci/mol phosphatidylethanolamine as determined by phosphorus assay according to Lowry et al. [14].

Phospholipase assay

50 000—70 000 cpm [^{14}C]phosphatidylcholine or 2-[^{14}C]linoleyl-phosphatidylethanolamine and 0.2 mg of lipids extracted from human erythrocyte membranes by the method of Folch et al. [15] were incubated with membrane preparations containing 1 mg of membrane protein in 50 mM sodium-glycyl-

glycine buffer (pH 8) containing 0.02% NaN_3, 0.2% Triton X-100 and 1 mM $CaCl_2$ in an end-volume of 500 µl. After the indicated time (see Results) of incubation in a shaking bath at 37° C the reaction was stopped by adding 2.2 ml of chloroform/methanol (5 : 8) and the lipids extracted according to Renkonen et al. [13]. An aliquot of the organic phase was assayed for total radioactivity and another aliquot separated on thin-layer plates in chloroform/methanol/acetic acid/water (16 : 6 : 0.5 : 1) for the assay with phosphatidylcholine and in 45 : 20 : 6 : 1 for the assay with phosphatidylethanolamine, respectively. After staining with iodine vapor the individual lipids were identified, then destained and scraped into counting vials. 5 ml methanol were added and 10 ml toluene containing 7 g of 2-(4′-*tert*-butylphenyl)-5-(4′-biphenyl-1,3,4)-oxadiazole/l. The radioactivity was measured in a Packard Tri-carb liquid scintillation counter (Model 2450). The radioactivity corresponding to lysophosphatidylcholine, phosphatidylcholine, fatty acids and to phosphatidylethanolamine and fatty acids, respectively, were compared and the activity of the phospholipase expressed as per cent degradation of the original radioactive phospholipid in a given time. Control for non-specific degradation was provided by membranes heat inactivated for 30 min at 100° C. Degradation after inactivation at 100° C was less than 2%.

Preparation of 'calcium-free' membranes and calcium/EGTA buffers

To determine the dependence of the phospholipase activity on very low concentrations of Ca^{2+} the procedures below were followed: 50 mM Tris and 50 mM maleic acid (pH 6.8) with NaOH containing 0.02% NaN_3 were passed through a Chelex-100 column. The Ca^{2+} was exchanged for Na^+, so the pH of the buffer was not changed. The Ca^{2+} concentration in the ion-exchanged buffer was $0.83 \cdot 10^{-6}$ M, as determined by atomic absorption. All glass vessels, pipettes etc. were rinsed with concentrated HCl, soaked in 2 N HCl overnight and rinsed thoroughly with bidistilled water. Only bidistilled water was used for solutions. Membranes were suspended in 1 mM EGTA in the above buffer; after short shaking the membranes were sedimented for 20 min at 90 000 × *g* in a Beckmann ultracentrifuge. The sediment was taken up in 1 mM EGTA in Tris/malate buffer by sonicating at 20 kHz for 30 s in an MSE ultrasonidesintegrator Model 7100. This treatment has a slightly activating effect on the phospholipase activity [1]. The membrane suspension contained 0.7 mg protein/450 µl and $8 \cdot 10^{-6}$ M Ca^{2+}. Ca^{2+}/EGTA buffers were prepared from 100 mM EGTA and 100 mM $CaCl_2$ solutions both pH 6.8. (The pH optimum for phosphatidylethanolamine (data not shown) and phosphatidylcholine degradation [1] lies between pH 6 and 8.5.) The buffers were mixed according to Schatzmann [16] to yield free Ca^{2+} concentrations between 10^{-7} and $4 \cdot 10^{-5}$ M.

Preparation of sealed ghosts

Essentially the method of Schwoch and Passow ([17], see also [10]) was used.

To localize the phospholipase in the membrane we tried to find two preparations of erythrocyte ghosts which were as similar as possible, differing only in the accessibility of the two sides of the membrane to proteolytic enzymes. We

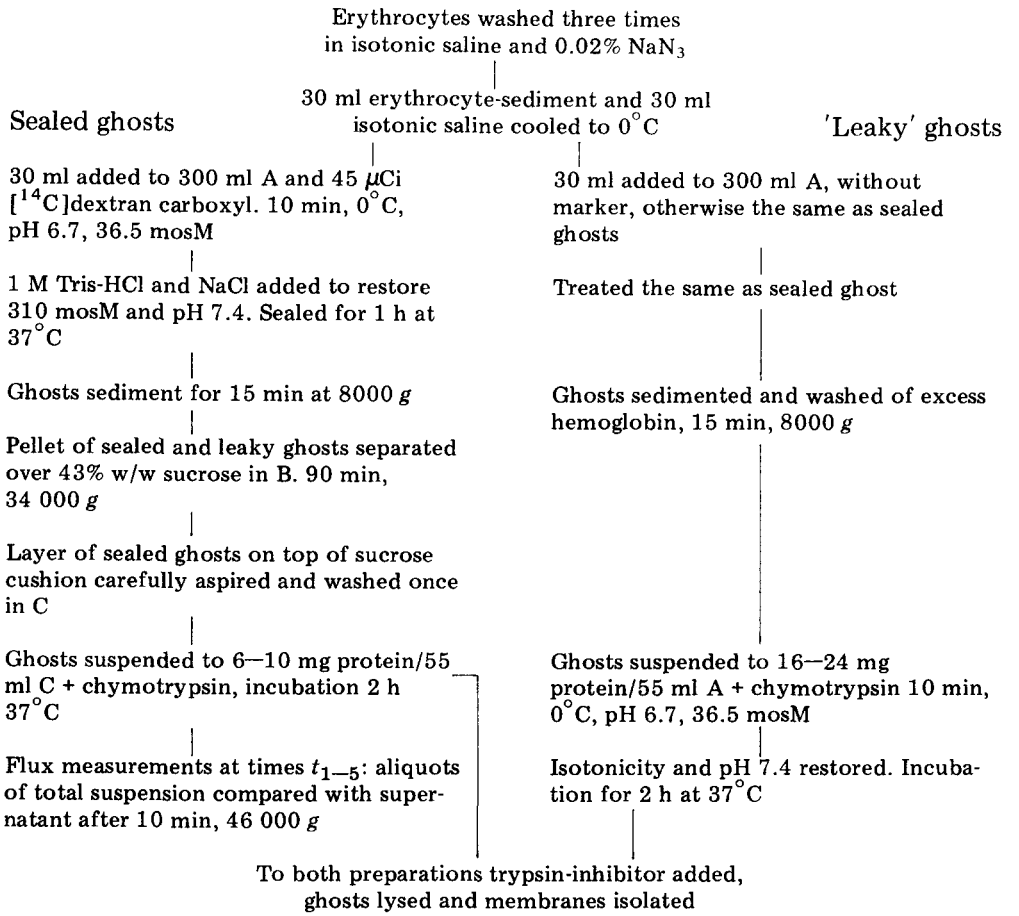

Fig. 1. Schematic representation of the preparation of sealed (left hand colum) and 'leaky' ghosts (right hand column). (A) Hemolysis medium containing: 1.2 mM acetic acid, 4.0 mM $MgSO_4$ at pH 4.0; (B) 25 mM Tris-HCl pH 7.5, 50 mM NaCl; (C) 10 mM sodium phosphate, pH 7.35, 145 mM NaCl. For further details see text.

thus hoped to avoid differential inactivation of the phospholipase due to different membrane preparations and not solely due to a specific orientation of the phospholipase activity in the membrane.

We followed the flow-sheet in Fig. 1. In the first step care was taken to discard the buffy coat and a pellet of erythrocytes which stuck to the bottom of the tube after each centrifugation. With the term 'leaky' ghosts we design erythrocyte ghosts with membranes accessible to chymotrypsin from both sides. Due to the method of preparation (compare Fig. 1), these ghosts might initially be sealed enclosing chymotrypsin.

Acetylcholinesterase assay

To determine the sidedness of the sealed ghosts obtained by the above method, acetylcholinesterase activity was determined in absence and in presence of Triton X-100 and compared with whole erythrocytes [18]: 30 mg

acetylthiocholine, 5 mg 5,5'-dithio-bis(2-nitrobenzoic acid) in 100 ml 100 mM sodium phosphate (pH 7.4) with or without Triton X-100 (1%) was the substrate solution. 1 ml of this was incubated with 5 µl ghost or erythrocyte suspension.

Results

Phosphatidylethanolamine degradation in rat, rabbit and pig erythrocyte membranes

No significant phospholipase activity could be measured in erythrocyte membranes from these three species, neither with [^{14}C]phosphatidylethanolamine or [^{14}C]phosphatidylcholine nor with endogenous phospholipids as substrate. The incubations for the phospholipase assay were as described in Materials and Methods.

Experiments with sheep erythrocyte membranes

Time course of phosphatidylethanolamine and phosphatidylcholine degradation. The time dependence of phosphatidylcholine and phosphatidylethanolamine splitting over a range of 1—16 h was measured (Fig. 2). The curves are very similar for both substrates, they show a linearity between 1 and 3 h, from

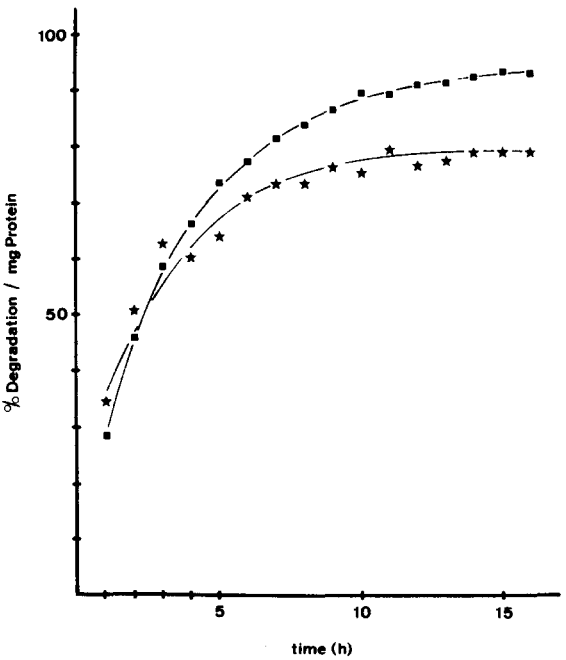

Fig. 2. Time dependence of phosphatidylcholine and phosphatidylethanolamine degradation. Washed membranes (1 mg membrane protein) were incubated with 50 000—70 000 cpm [^{14}C]phosphatidylcholine or [^{14}C]phosphatidylethanolamine, respectively, 0.2% Triton X-100, 0.2 mg human erythrocyte membrane lipids. 1 mM $CaCl_2$ in 50 mM sodium-glycyl-glycine in an end-volume of 0.5 ml. Per cent of initial radioactivity degraded is plotted against time. ■———■, phosphatidylethanolamine degradation; ★———★, phosphatidylcholine degradation.

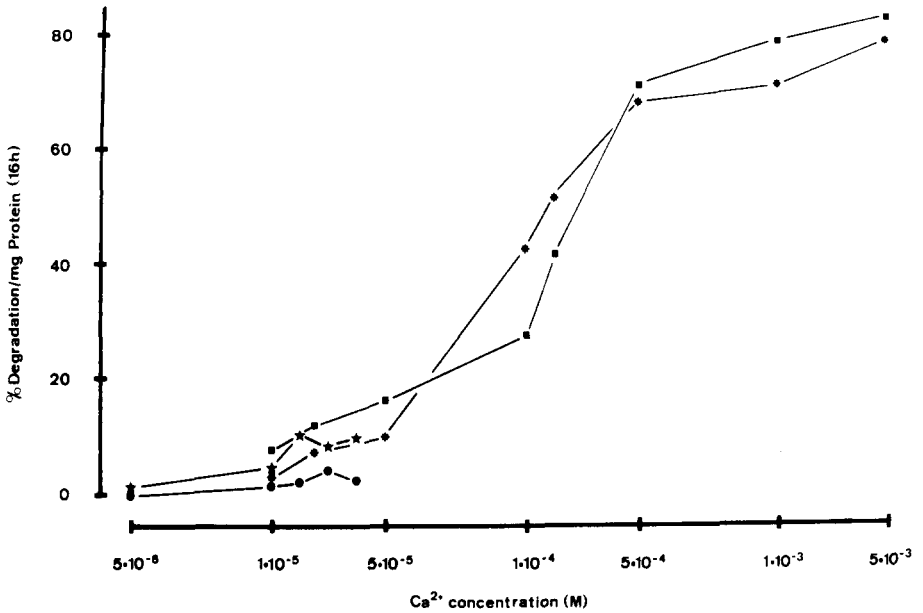

Fig. 3. Dependence of the phospholipase activity on calcium concentration. Membranes were suspended in 1 mM EGTA, centrifuged and the sediment either taken up in 1 mM EGTA and the desired calcium concentration added with Ca^{2+}/EGTA buffers (see Materials and Methods): ●———●, phosphatidylethanolamine degradation; ★———★, phosphatidylcholine degradation; or taken up in Na-Tris/malate buffer and the calcium added from a $CaCl_2$ stock solution: ■———■, phosphatidylethanolamine degradation; *———*, phosphatidylcholine degradation. Phospholipase was determined as described in the legend to Fig. 2 and incubation was for 16 h.

where onward the curves flatten. Although the time course is no longer linear, we chose 16 h as an incubation time to measure the highest possible product concentrations, especially in the experiments with low calcium concentrations and partly inactivated enzyme after proteolytic treatment.

Dependence of phospholipase activity on calcium concentration. Preliminary experiments soon showed that at calcium concentrations above 10^{-5} M the enzyme is active, thus conventional methods of calcium dosage could not be used.

As is shown in Fig. 3, the critical calcium concentration both for phosphatidylethanolamine and phosphatidylcholine degradation lies between $5 \cdot 10^{-5}$ and $5 \cdot 10^{-4}$ M. The activities of systems with Ca^{2+}/EGTA buffers and calcium diluted from stock solutions are comparable. However, it was consistently observed that the enzyme activities towards phosphatidylethanolamine in Ca^{2+}/EGTA buffers are lower than in diluted $CaCl_2$ solutions, the contrary is the case for phosphatidylcholine degradation. The activities were calculated from the radioactivity of the one labelled substrate present in the beginning of the incubation. The concentration of total substrate, i.e., phosphatidylethanolamine and phosphatidylcholine, was identical for the incubations with [^{14}C]phosphatidylcholine and [^{14}C]phosphatidylethanolamine as substrate. An inhibition or activation of the degradation of one substrate in presence of the other thus cannot be shown in our system (see Discussion).

TABLE I

ADSORPTION OF [^{14}C] DEXTRAN CARBOXYL TO ERYTHROCYTE MEMBRANES

Erythrocytes were hemolysed in presence of [^{14}C]dextran carboxyl (see Fig. 1 for details) and kept at 0°C during the following procedures. At the indicated times, aliquots of 0.1 ml of total suspension and supernatant after centrifugation for 10 min at 46 000 × *g* and the pellet corresponding to 0.1 ml of ghost suspension were assayed for radioactivity. Data are expressed as cpm.

Fraction	Time (min)			
	0	15	30	60
Total	1969	2097	2150	2268
Supernatant	2152	2160	1723	1689
Pellet	91	79	66	88

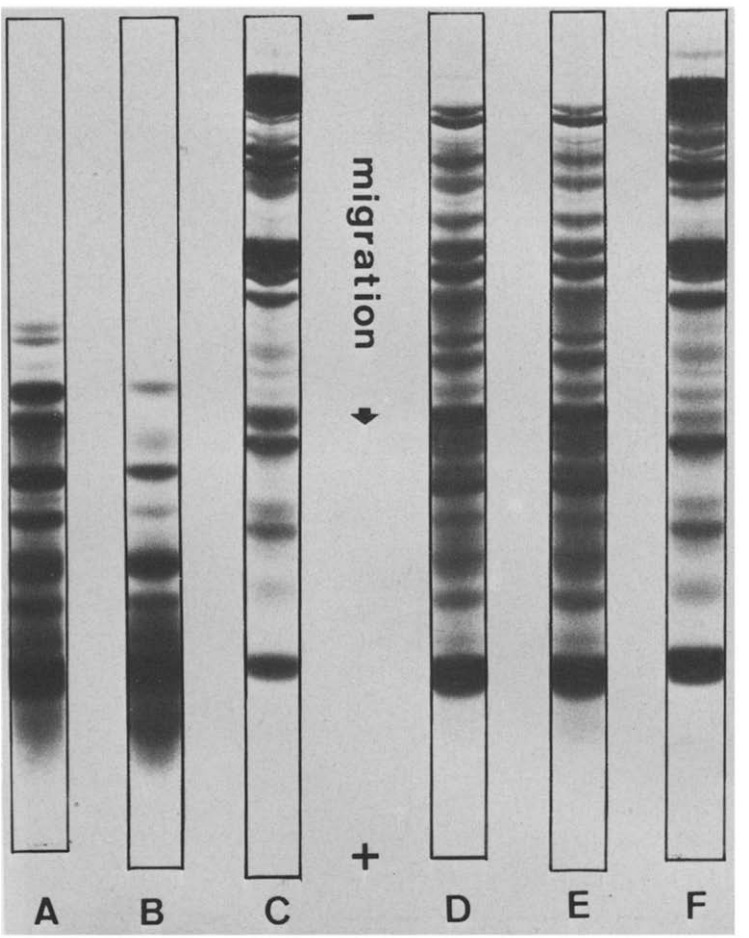

Fig. 4. SDS-polyacrylamide gel electropherograms of chymotrypsin-treated ghost suspensions. Ghosts were treated as described in Fig. 1. Membranes were run on 5.8% gels. A, B, C: 'leaky' ghosts; D, E, F: sealed ghosts. A and D: ghosts treated with 5.3 mg of chymotrypsin; B and E: ghosts treated with 21.2 mg of chymotrypsin; C and F: controls.

Proteolysis of ghosts with chymotrypsin. [^{14}C]Dextran carboxyl with a molecular weight of 20 000 was used as a marker for the tightness of the sealed ghosts, because of its similar size as chymotrypsin (M_r = 20 600). We therefore assumed that if [^{14}C]dextran carboxyl does not leak from the ghosts chymotrypsin cannot enter these. Flux measurements of [^{14}C]dextran carboxyl during the 2 h incubation of ghosts with chymotrypsin indeed showed that the

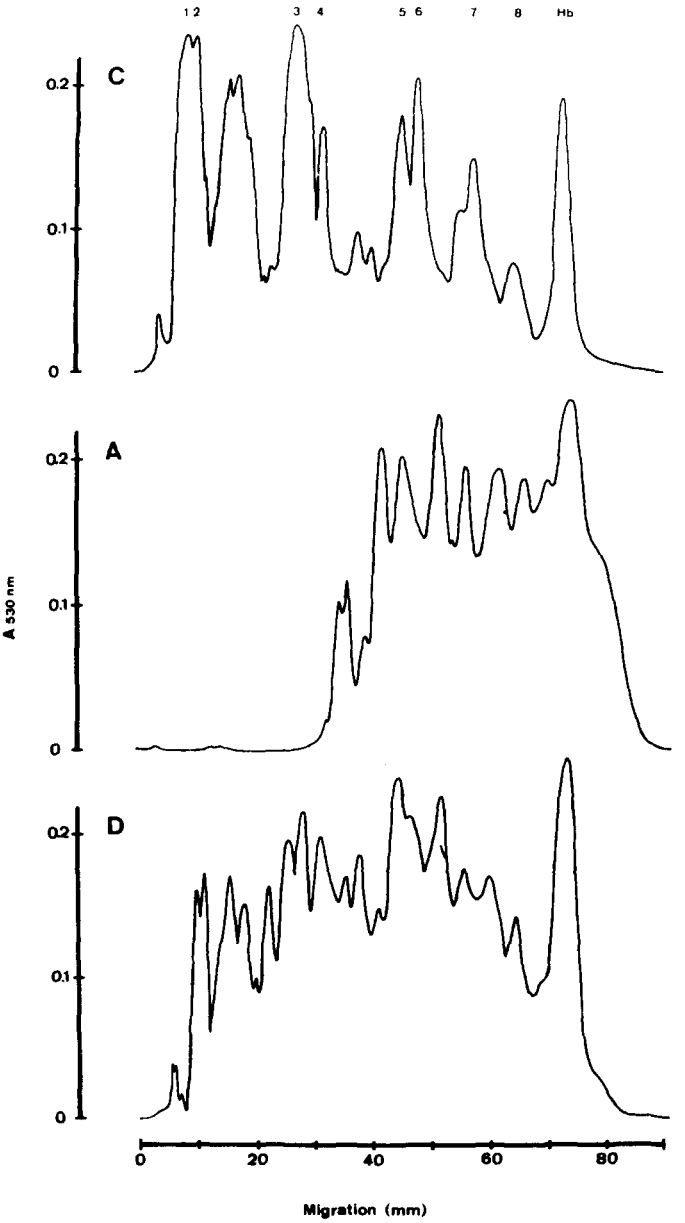

Fig. 5. Densitometer scans of gels C, A and D (nomenclature see Fig. 4). Nomenclature of bands according to Fairbanks et al. [9].

sealed ghosts remained sealed during proteolysis from the outside (no increase of [^{14}C]dextran carboxyl flux).

In a control experiment the possible adsorption of dextran carboxyl to erythrocyte membranes and the release of dextran carboxyl from ghosts upon lysis was tested. In Table I the radioactivities of the different fractions are shown. No significant adsorption of [^{14}C]dextran carboxyl to ghost membranes could be observed.

Upon hemolysis of washed ghosts sealed in presence of [^{14}C]dextran carboxyl 78% of the incorporated radioactivity was released from the ghosts. This control shows that in the experiments with 'leaky' ghosts chymotrypsin could indeed enter the ghosts upon lysis; this is also confirmed by the polyacrylamide gel electrophoresis [4]. In the sealed ghosts bands 1 and 2 (spectrin), which are known to be located on the cytoplasmic side of the membrane [19], were scarcely degraded by chymotrypsin. The 'leaky' ghosts, however, are attacked on both sides of the membrane by the protease. This difference of both preparations is also clearly visible in the densitograms (Fig. 5). However, the phospholipase activities (Table II) of the two membrane preparations show a striking similarity. The phospholipase activity was consistently diminished to the same extent in sealed and 'leaky' ghosts.

The phospholipase could never be totally inactivated by proteases. In similar experiments as the ones described with chymotrypsin, leaky ghosts were incubated with 7 mg of pronase from *streptomyces griseus* for 3 h. All the bands normally visible on polyacrylamide gel electrophoresis were degraded, only one broad band near the front stained with Coomassie blue. But as compared with the control, 18% of the phospholipase activity with phosphatidylcholine as substrate and 10.4% with phosphatidylethanolamine as substrate remained.

The sidedness of the sealed ghosts was assayed by determining acetylcholinesterase activity. The rate of acetyl-thiocholine degradation in presence and absence of Triton X-100 was the same for whole erythrocytes and sealed ghosts. We therefore conclude that the orientation of the membrane in sealed ghosts is the same as in whole erythrocytes, with the acetylcholinesterase facing the exterior.

TABLE II

PHOSPHOLIPASE ACTIVITY IN MEMBRANES AFTER CHYMOTRYPSIN TREATMENT

Phospholipase activity was determined as described in the legend to Fig. 2 and in Materials and Methods. The values given are percentages of the phospholipase activity compared to untreated membranes.

	^{14}C-labelled substrate			
	Phosphatidylcholine		Phosphatidylethanolamine	
Chymotrypsin added (mg)	5.3	21.2	5.3	21.2
Percent of remaining enzyme activity of:				
Sealed ghosts	26.4	21.2	16.9	13.7
Leaky ghosts	37.8	22.8	27.7	16.9

Discussion

Zwaal et al. [2] used erythrocyte membranes without added substrate to assay phospholipase activity towards phosphatidylethanolamine, while Kramer et al. [1] had to use exogenous substrate, because sheep erythrocytes lack phosphatidylcholine. In experiments using 0.2% Triton X-100 ([1], and our experiments), the endogenous and exogenous substrates might not be present in the same form. Grant and Hjertém [20] showed that only 68% of the total erythrocyte membrane proteins are solubilized, using 1% Triton X-100 and a ratio of membrane protein to detergent of 1 : 10. We dealt with even less solubilized membranes, because we used a ratio of membrane protein to detergent of 1 : 1 in our phospholipase assays. On the one hand, the detergent probably forms mixed micelles with the added phospholipid substrate, and on the other hand, it leads to a partial solubilization of the membrane structure. We could not reproduce Zwaal's finding of phosphatidylethanolamine degradation in pig, rat and rabbit erythrocyte membranes, but confirmed a high activity in sheep erythrocyte membranes towards phosphatidylethanolamine.

The pH dependence of phosphatidylcholine [1] and phosphatidylethanolamine degradation (data not shown) is the same, with a broad optimum between pH 6.8 and 8.5. Also the time course of degradation of both substrates is very similar (Fig. 2). The positional specificity is the same for both substrates. Neither Kramer et al. [1] for phosphatidylcholine nor we for phosphatidylethanolamine ever observed radioactive lysocompounds, if we used phospholipids specifically labelled at position 2. These three characteristics, similar for both phospholipid substrates, pointed to only one enzyme responsible for the cleavages.

Condrea et al. [21] postulated a direct inhibitory effect of EDTA and EGTA on the phospholipase from ringhals cobra, beside the calcium-binding capacity of the chelators. This possibility was also considered in our experiments using Ca^{2+}/EGTA buffers. The results of the phospholipase activity in presence of Ca^{2+}/EGTA buffers and in presence of Ca^{2+} as $CaCl_2$ in the range of 10^{-5}—$4 \cdot 10^{-5}$ exclude this possibility, especially for phosphatidylcholine splitting (Fig. 3).

The observation that the rate of phosphatidylethanolamine degradation in presence of Ca^{2+}/EGTA buffers was consistently lower than in the absence of EGTA cannot as yet be explained. It might, however, in part be due to a fact emphasized by Schatzmann [16], namely that free Ca^{2+} concentrations, calculated from the Ca^{2+}/EGTA dissociation constants, are too high, if the ratio of total calcium to total EGTA exceeds 0.9. This is the case in our experiments in the range of 10^{-5}—$5 \cdot 10^{-5}$ M calcium (Fig. 3).

The phospholipase activity at the Ca^{2+} concentrations presented here clearly show the enzyme to be inactive at Ca^{2+} concentrations below 10^{-5} M; a rapid increase in activity against phosphatidylethanolamine and phosphatidylcholine is observed between $5 \cdot 10^{-5}$ and $5 \cdot 10^{-4}$ M Ca^{2+}. If we consider the Ca^{2+} concentrations on both sides of the membrane (less than 10^{-6} M in the erythrocyte and $1.5 \cdot 10^{-3}$ M in the plasma), the phospholipase, if it were facing the inside of the membrane, could be regulated by the amount of calcium influx. This hypothesis leads us to further investigate the sidedness of the enzyme in the membrane.

The experiments comparing sealed and 'leaky' erythrocyte ghosts after treatment with chymotrypsin clearly show the enzyme to be accessible at the exterior of the cell (Table II). If the enzyme was oriented towards the cytoplasm, one would expect inactivation only after proteolysis of leaky ghosts. The fact that in 'leaky' ghosts the phospholipase is less inactivated than in sealed ghosts is probably due to a higher substrate/chymotrypsin ratio in 'leaky' ghosts, where roughly double the membrane surface and the residual hemoglobin act as substrate. This is most evident at low chymotrypsin concentrations (Table II).

From the proteolysis experiments with pronase, where after extensive degradation of all membrane proteins 10% of the initial phospholipase activity remained, we conclude that a part of the enzyme, including the active site, could be imbedded in the hydrophobic part of the lipid bilayer. This hypothesis is supported by experiments leading to the isolation of the phospholipase, where the enzyme was only solubilized with the highly denaturing detergent sodium dodecyl sulfate [4]. The finding that the phospholipase faces the exterior of the cell confirms the results of Zwaal et al. [2], who degraded whole erythrocytes and isolated membranes with pronase, and compared the residual phospholipase activity. We chose sealed and 'leaky' ghosts, as opposed to washed membranes and erythrocytes, for our proteolysis studies for the following reasons: washed membranes and erythrocytes differ in the topology of the membrane, because proteins are stripped from the membranes by the low ionic strength treatment used in their preparation [22—27]. Resealed ghosts and erythrocytes, on the other hand, show no substantial difference in the arrangement of surface proteins, as was shown by Cabantchik et al. [28] with surface labelling and proteolysis of human erythrocytes and resealed ghosts. From the polyacrylamide gel electrophoresis it is not at all possible to discern if the phospholipase is attacked by chymotrypsin or not. The phospholipase is a minor membrane protein and normally not visible on polyacrylamide gel electrophoresis, and any peptides resulting from chymotrypsin degradation would overlap with the phospholipase in the region of its molecular weight, which is approx. 18 500, as Kramer et al. [4] showed. The phospholipase has been isolated in this laboratory in the course of the studies presented here [4].

As far as the degradation of major membrane proteins by chymotrypsin is concerned, we observed that band 3 is not degraded in sealed ghosts (Figs. 4 and 5), but is totally absent on electropherograms of chymotrypsin-treated 'leaky' ghosts. Triplett and Carraway [19] also observed no proteolysis of band 3 in chymotrypsin-treated whole sheep erythrocytes. Band 3 in human erythrocytes shows an inverse behaviour in that it is degraded by chymotrypsin in whole erythrocytes [19,27] and not in inside-out membrane vesicles [27].

The separation of sealed and leaky ghosts on a 43% w/w sucrose cushion was not complete. Therefore, the polyacrylamide gels D and E (Fig. 4) and the densitograms (Fig. 5) show a slight degradation of spectrin, probably from a non-sedimented population of unsealed ghosts, but flux measurements confirm that initially sealed ghosts stayed sealed during chymotrypsin treatment. The differences between the gels A, B and C, D in the low molecular weight region are the result of the extensive breakdown products of the bands 1—4 in the experiments A and B.

The hypothesis that the phospholipase might be regulated by Ca^{2+} influx into the erythrocyte does not hold if the enzyme is oriented towards the exterior. In sheep erythrocyte membranes the phospholipase activity is probably not regulated by the calcium concentration. But the affinity chromatography used in the isolation of the phospholipase [4] shows calcium to be essential for the formation of the enzyme-substrate complex.

The physiological significance of the phospholipase is not yet fully clear. The postulation that the phospholipase is responsible for the low phosphatidylcholine content in ruminants [1] can be criticized, because the enzyme attacks phosphatidylethanolamine and phosphatidylcholine at the same rate, if both substrates are present in the same concentration; this was the case in all the experiments presented here. Under physiological conditions, phosphatidylcholine probably is the main substrate, although the 10% of the total phosphatidylethanolamine which faces the outside of the membrane might also act as substrate. That a discrete phospholipid pool might act as substrate for a phospholipase was shown by Bills et al. [29], who observed phospholipase activity in platelet membranes against a small pool of phosphatidylcholine, previously labelled with externally added [^{14}C]arachidonic acid.

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