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THE LIPID REQUIREMENT OF THE (Ca^{2+} + Mg^{2+})-ATPase IN THE HUMAN ERYTHROCYTE MEMBRANE, AS STUDIED BY VARIOUS HIGHLY PURIFIED PHOSPHOLIPASES

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Summary

1. When complete hydrolysis of glycerophospholipids and sphingomyelin in the outer membrane leaflet is brought about by treatment of intact red blood cells with phospholipase A_2 and sphingomyelinase C, the (Ca^{2+} + Mg^{2+})-ATPase activity is not affected.

2. Complete hydrolysis of sphingomyelin, by treatment of leaky ghosts with spingomyelinase C, does not lead to an inactivation of the (Ca^{2+} + Mg^{2+})-ATPase.

3. Treatment of ghosts with phospholipase A_2 (from either procine pancreas or *Naja naja* venom), under conditions causing an essentially complete hydrolysis of the total glycerophospholipid fraction of the membrane, results in inactivation of the (Ca^{2+} + Mg^{2+})-ATPase by some 80—85%. The residual activity is lost when the produced lyso-compounds (and fatty acids) are removed by subsequent treatment of the ghosts with bovine serum albumin.

4. The degree of inactivation of the (Ca^{2+} + Mg^{2+})-ATPase, caused by treatment of ghosts with phospholipase C, is directly proportional to the percentage by which the glycerophospholipid fraction in the inner membrane layer is degraded.

5. After essentially complete inactivation of the (Ca^{2+} + Mg^{2+})-ATPase by treatment of ghosts with phospholipase C from *Bacillus cereus*, the enzyme is reactivated by the addition of any of the glycerophospholipids, phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine or lysophosphatidylcholine, but not by addition of sphingomyelin, free fatty acids or the detergent Triton X-100.

6. It is concluded that only the glycerophospholipids in the human erythrocyte membrane are involved in the maintenance of the (Ca^{2+} + Mg^{2+})-ATPase

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activity, and in particular that fraction of these phospholipids located in the inner half of the membrane.

Introduction

The role of lipids in the (Ca^{2+} + Mg^{2+})-ATPase system has been studied extensively during the last decade. For obvious reasons, particular attention has been payed in this respect to the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum, in which no less than some 80% of the protein moiety consists of this enzyme.

Since a recent study has shown that the (Na^+ + K^+)-stimulated ATPase activity of the human erythrocyte membrane depends on its association with phosphatidylserine [1], it became of particular interest to have a more detailed knowledge about the (phospho)lipid(s) involved in the maintenance of the other cation transporting enzyme system present in this membrane, the Mg^{2+}-dependent, Ca^{2+}-activated ATPase [2—7].

The lipid dependence of the (Ca^{2+} + Mg^{2+})-ATPase in the human erythrocyte membrane has been demonstrated recently by Coleman and Bramley [8]. In their studies they have shown a loss of (Ca^{2+} + Mg^{2+})-ATPase activity upon treatment of ghosts with a partially purified preparation of phospholipase C from *Clostridium welchii*, as well as a subsequent reactivation of the enzyme by the addition of phospholipids. However, these investigators did not study systematically which phospholipids are able to reactivate the enzyme and which fail to do so. Moreover, interpretation of their results is complicated by the fact that they have been using an only partially purified phospholipase preparation. It has been extensively argued before [1], that the use of highly purified phospholipases in such studies is of paramount importance. In addition, it should be mentioned that reactivation experiments produce conclusive results only when they are based on preparations which do not show any considerable residual activity.

This paper reports the effects of highly purified phospholipases A_2 (from porcine pancreas and *Naja naja* snake venom), phospholipases C (from *Bacillus cereus* and *Clostridium welchii*) and sphingomyelinase C (from *Staphylococcus aureus*) on the (Ca^{2+} + Mg^{2+})-ATPase of the human erythrocyte membrane.

In addition, reconstitution of this ATPase activity was studied by the addition of various pure phospholipids to ghost preparations previously treated with phospholipase C from *B. cereus*.

Materials and Methods

Preparation of erythrocyte ghosts

Fresh human red blood cells, in acid/citrate/dextrose solution, were obtained from the Swiss Red Cross Blood Bank. The red cells were washed four times with isotonic saline. Ghosts were prepared essentially according to the method of Wolf [9]. The washed cells were haemolyzed at 0—4° C in 10 volumes of 10 mM NaCl, 20 mM sucrose, 20 mM Tris · HCl (pH 7.45). Ghosts were collected by centrifugation at 30 000 × *g* for 15 min in a Sorvall RC-2B refrigerated cen-

trifuge. After several washings with an ice-cold solution containing 1 mM KCl, 1 mM NaCl, 2 mM sucrose and 2 mM Tris · HCl (pH 7.7) (centrifugation at 30 000 × *g* for 15 min and 0–4° C, after each wash), the white ghosts were frozen in an ethanol/dry-ice mixture and stored at −28° C for not longer than 24 h. Fresh ghosts were prepared for each individual experiment.

Phospholipases

Pure phospholipase A_2 from porcine pancreas, prepared as described by de Haas et al. [10], was kindly supplied by Dr. A.J. Slotboom (Utrecht).

Phospholipase A_2 from *N. naja* venom was highly purified by a modification [11] of the procedure of Cremona and Kearney [12].

Phospholipase C from *B. cereus* was prepared as described by Zwaal et al. [11,13,14].

Phospholipase C from *C. welchii* was purified from a lyophilized culture filtrate (purchased as Type I from Sigma), and sphingomyelinase C from log phase cultures of *S. aureus* (strain 269 HH); both purifications were made according to Zwaal et al. [11].

Degradation of the outer phospholipid leaflet in intact erythrocytes

6.0 ml of washed packed cells were mixed with 10 ml 150 mM NaCl, 10 mM $CaCl_2$, 0.25 mM $MgCl_2$ and 50 mM Tris · HCl (pH 7.4). This erythrocyte suspension was incubated at 37° C with gentle shaking. The phospholipases were added as follows: 25 I.U. *N. naja* phospholipase A_2 at zero time, and a second sample of 25 I.U. of this enzyme after 30 min. At t = 60 min, 8 I.U. of sphingomyelinase C were introduced. It should be remembered that the sequence in which these enzymes are added is very important, in order to avoid lysis of the cells [15].

After a total incubation time of 120 min, the enzymatic activities were stopped by addition of 20 ml of a solution containing 150 mM NaCl, 0.2 M sucrose and 5 mM EGTA, adjusted to pH 7.4 with Tris. After centrifugation for 5 min at 2000 × *g*, the cells were washed twice; once with the same solution containing EGTA and a second time without the presence of this chelating agent.

Control cells were subjected to exactly the same treatments, except for the presence of the phospholipases. Finally, white ghosts were prepared from the treated and control cells, as described above.

Treatment of ghosts with phospholipases

All phospholipase incubations were carried out in a shaking water bath at 37° C.

Sphingomyelinase C. White ghosts, derived from 6 ml packed erythrocytes, were suspended in 0.1 M Tris · HCl buffer (pH 7.4), containing 0.25 mM $MgCl_2$. Total incubation volume was 10 ml. For incubation with pure sphingomyelinase C, 7 I.U. of this enzyme were added at t = 0 min and t = 45 min. After a total incubation time of 90 min, the suspension was cooled in ice and 30 ml of an ice-cold solution containing 1 mM KCl, 1 mM NaCl, 2 mM sucrose and 2 mM Tris · HCl (pH 7.4) (henceforth, called "wash buffer") was added. After centrifugation for 10 min at 15 000 × *g* and 0–4° C, the ghosts were

washed twice with 40 ml of the wash buffer.

Phospholipase A_2 from N. naja. The incubations were carried out with the same quantity of ghosts as described for the sphingomyelinase C treatment, suspended in a total volume of 10 ml 0.1 M Tris · HCl (pH 7.4), but containing 10 mM $CaCl_2$, instead of 0.25 mM $MgCl_2$. The incubation was carried out by the addition of 20—30 I.U. of the phospholipase at $t = 0$ min, and a second sample of 20—30 I.U. at $t = 60$ min. After a total incubation time of 120 min, the phospholipase activity was stopped by cooling in ice and the addition of 30 ml wash buffer, containing 6 mM EGTA (pH adjusted at 7.4 with Tris). After centrifugation for 10 min at 15 000 $\times g$ and 0—4°C, the ghosts were washed three more times with 40 ml of the cold wash buffer, not containing EGTA

Phospholipase A_2 from pancreas. The same procedure was followed as described for the incubation with the *N. naja* phospholipase, except for the following modifications. The 0.1 M Tris · HCl incubation buffer contained 5 instead of 10 mM $CaCl_2$. At $t = 0$ min and $t = 60$ min, 40—100 I.U. of pancreatic phospholipase A_2 were added. The concentration of EGTA in the wash buffer, added to terminate the reaction, was 3 mM instead of 6 mM.

In some experiments, the lyso-compounds and fatty acids produced by the phospholipase A_2 treatment were removed by the following procedure. After inhibition of the phospholipase activity by cooling in ice, addition of the wash buffer containing 3 mM EGTA and centrifugation, the ghosts were washed twice with 40 ml cold wash buffer, containing 1% bovine serum albumin (Calbiochem, fatty acid poor, B grade). After each addition, and prior to centrifugation, the suspensions were gently stirred for 5 min. Finally, the bovine serum albumin was removed by three more washes with 40 ml cold wash buffer.

Phospholipase C from B. cereus. The incubation conditions were the same as described for pancreatic phospholipase A_2. The following quantities of phospholipase C were added: 80 I.U. at $t = 0$ min and another 80 I.U. at $t = 60$ min. After 120 min, the incubation mixture was cooled in ice and the phospholipase was inactivated by the addition of 3 ml 50 mM *o*-phenanthroline (E. Merck, Darmstadt) and 30 ml ice-cold wash buffer, containing 3 mM EGTA. The ghosts were subsequently collected by centrifugation as described above. The pellet was washed three times with 40 ml cold wash buffer, without EGTA.

Phospholipase C from C. welchii. The procedure applied for ghost treatments with this phospholipase, subsequent inactivation of the enzyme and washes of the ghosts, were identical to those described for phospholipase C from *B. cereus*. In this case however, 60 I.U. of phospholipase C were added twice to the incubation mixture.

All phospholipase treated ghosts were finally suspended in the wash buffer (total volume 5.0 ml) and frozen and thawed once, prior to the ATPase assay.

As control in all the phospholipase treatments identical samples, without phospholipase present, were run in parallel.

It should be mentioned that the phospholipase incubation conditions, as described above, accomplish an essentially complete hydrolysis of the corresponding substrates present in the membrane. In cases where an incompiete digestion of the substrates was desired, phospholipase quantities and incubation times were reduced correspondingly.

Anhydrous ether extraction

Cholesterol was removed by extraction of lyophilized ghosts with dry ether, as described before [1].

Phospholipid analysis

In order to determine the alterations in lipid composition produced by the action of phospholipases, ghosts were extracted according to Reed et al. [16]. The phospholipids were separated by two-dimensional thin-layer chromatography, using the procedure of Broekhuyse [17], and determined as phosphate after destruction with 70% $HClO_4$ at 200° C by a modification of the procedure of Fiske and Subbarow [18]. Percentage degradation of the different phospholipid classes was calculated as described previously [15,19].

Aqueous lipid dispersions

Phosphatidylserine was purified from pig brain according to the procedure of Sanders [20] and subsequently liberated from Ca^{2+} [21]. Phosphatidylcholine and phosphatidylethanolamine were purified from egg yolk [22,23]. Pure sphingomyelin from bovine brain was purchased from Koch Light. Lysophosphatidylcholine and free fatty acids were prepared from egg lecithin by a method essentially similar to that of Hanahan [24], using pure pancreatic phospholipase A_2. The lyso-compound and free fatty acids were separated by preparative thin-layer chromatography on silica gel H plates in a nitrogen atmosphere, using chloroform/methanol/water (65 : 35 : 5, by vol) as a developing system.

The individual phospholipids appeared to be pure when examined by two-dimensional thin-layer chromatography.

Lipids were dispersed in the wash buffer by ultrasonic radiation (Branson Sonifier B-12) under a nitrogen atmosphere and with cooling in an ice bath. The pH of the dispersions was measured at time intervals of 30 s of sonication, and when necessary readjusted to 7.4. The opalescent dispersions were centrifuged for 5 min at 3000 × *g*, in order to remove large lipid aggregates and small metal particles released from the tip during sonication. The phospholipid concentrations in the dispersions were calculated from phosphorus determinations.

Resuspension of phospholipase C treated ghosts

In the last wash of phospholipase C treated ghosts (see above), the 40 ml suspension was divided into 5 fractions of 8.0 ml. After centrifugation for 10 min at 15 000 × *g*, the pellets were resuspended in either 1.0 ml wash buffer, or 1.0 ml lipid dispersion. These suspensions were kept in ice for 30 min and shaken frequently. Unless otherwise stated, all suspensions were frozen and thawed once, prior to the ATPase incubation.

ATPase assay

(Ca^{2+} + Mg^{2+})-ATPase was assayed by measuring the liberation of inorganic phosphate in the following incubation medium: 110 mM choline-chloride, 30 mM imidazole-chloride, 2 mM $MgCl_2$, 2 mM Mg-ATP, 0.17 mM ouabain, 0.5 mM Ca-EGTA buffer (pH 7.0 at 37° C). Ca^{2+} concentrations were calculated according to Wolf [25]. When Ca^{2+} concentrations above 10^{-5} M were required,

TABLE I

EFFECT OF DEGRADATION OF THE OUTER PHOSPHOLIPID LAYER IN HUMAN ERYTHROCYTES ON THE (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY

(Ca^{2+} + Mg^{2+})-ATPase activity was assayed in ghosts derived from intact erythrocytes, previously treated with phospholipase A_2 and sphingomyelinase C, as described in Materials and Methods. The maximal rate at saturating Ca^{2+} concentrations ($V_{sat.\ Ca}$) and K_{Ca} values were found by plotting activation curves obtained with six different concentrations of Ca^{2+} between $5 \cdot 10^{-7}$ and $2 \cdot 10^{-4}$ M, according to the Lineweaver-Burk plot.

Experiment No.		Percent degradation of phospholipids					(Ca^{2+} + Mg^{2+})-ATPase	
		Total phospholipid	Sphingomyelin	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine	$V_{sat.\ Ca}$ (μmol P_i/h per mg ghost protein)	K_{Ca} ($M \cdot 10^{-6}$)
1	Control	0	0	0	0	0	1.67	4.1
	Treated	48	79	76	25	0	1.67	4.8
2	Control	0	0	0	0	0	1.70	1.8
	Treated	48	80	78	23	0	1.87	1.7
3	Control	0	0	0	0	0	1.62	5.6
	Treated	47	79	78	21	0	1.82	5.3

$CaCl_2$ replaced the Ca-EGTA buffer. The basal Mg^{2+}-ATPase activity was assayed in the above medium, with 0.5 mM Tris-EGTA instead of Ca-EGTA. The incubation mixture contained 0.2 ml ghost suspension (0.3—0.6 mg protein). Total volume was 2.5 ml. After 1 h incubation at 37°C, the reaction was stopped by cooling in ice and the addition of 2.5 ml ice-cold trichloroacetic acid (10%). After filtration, 2.0 ml of the clear supernatant was taken for determination of inorganic phosphate [26]. P_i liberation due to (Ca^{2+} + Mg^{2+})-ATPase activity was obtained by subtraction of Mg^{2+}-ATPase activity from the total P_i liberation. Protein concentrations of ghost samples were determined by the method of Lowry et al. [27].

Unless otherwise stated, the adjustment of solutions to the indicated pH values has been carried out at room temperature.

Results

Treatment of intact erythrocytes with phospholipase A_2 and sphingomyelinase C

Successive tratments of intact cells with phospholipase A_2 from *N. naja* and sphingomyelinase C, under isotonic conditions, result in a considerable non-haemolytic degradation of the phospholipids present in the outer membrane layer. The degradation percentages of the phospholipids, thus obtained in this study (Table I), are esentially identical to those reported earlier [11,15]. As is shown also in Table I, the degradation of the outer phospholipid layer definitely does not result in a decrease of the (Ca^{2+} + Mg^{2+})-ATPase activity. Moreover, this treatment does not affect the affinity of the enzyme system for Ca^{2+} (K_{Ca} in Table I), although some variation in this value is to be noted among the three different membrane preparations.

Treatment of ghosts with sphingomyelinase C

As could be expected already from the foregoing experiments, in which no less than some 80% of the sphingomyelin was degraded without affecting the (Ca^{2+} + Mg^{2+})-ATPase, a complete hydrolysis of this phospholipid has no significant effect either on the specific activity of the enzyme, or on the value of K_{Ca} (Table II). It should be remembered in this context that the pure sphingo-

TABLE II

EFFECT OF SPHINGOMYELINASE C TREATMENT OF GHOSTS ON THE (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY

$V_{sat.\ Ca}$ and K_{Ca} values were calculated as indicated in Table I.

Experiment No.		Percent degradation of phospholipids			(Ca^{2+} + Mg^{2+})-ATPase	
		Total phospholipid	Sphingomyelin	Glycerophospholipids	$V_{sat.\ Ca}$ (μmol P_i/h per mg ghost protein)	K_{Ca} ($M \cdot 10^{-6}$)
1	Control	0	0	0	2.86	2.2
	Treated	25	98	0	3.09	1.8
2	Control	0	0	0	1.56	0.8
	Treated	25	100	0	1.75	0.8

myelinase C from *S. aureus* has an extremely high substrate specificity and, consequently, does not degrade any of the glycerophospholipids [28].

Treatment of ghosts with phospholipases A_2

Treatment of ghosts with phospholipase A_2, either from porcine pancreas or *N. naja*, resulting in an essentially complete degradation of the glycerophospholipids, causes a drastic decrease of the $(Ca^{2+} + Mg^{2+})$-ATPase activity (Fig. 1 and Table III).

Although the K_{Ca} of the ATPase in the treated ghosts is equal to that in the control ghosts (Fig. 1B), already indicating that the activity found after phospholipase A_2 treatment is indeed residual $(Ca^{2+} + Mg^{2+})$-ATPase activity, an experiment was conducted to make sure that the observed residual activity was not (partially) due to a Ca^{2+}-activated ATPase, that is independent of Mg^{2+} [29,30]. Phospholipase A_2 treated, as well as control ghosts, were incubated with the usual series of Ca^{2+} concentrations, but without Mg^{2+} present (Mg-ATP was replaced by Tris-ATP). The inorganic phosphate produced in these incubations did not exceed the blank value.

The lyso-compounds and free fatty acids, produced by the phospholipase A_2 action, remain quantitatively in the membrane, provided that albumin is not included in the incubation medium and wash buffers, as has been the case in these experiments. Removal of the lyso-compounds (and fatty acids) by

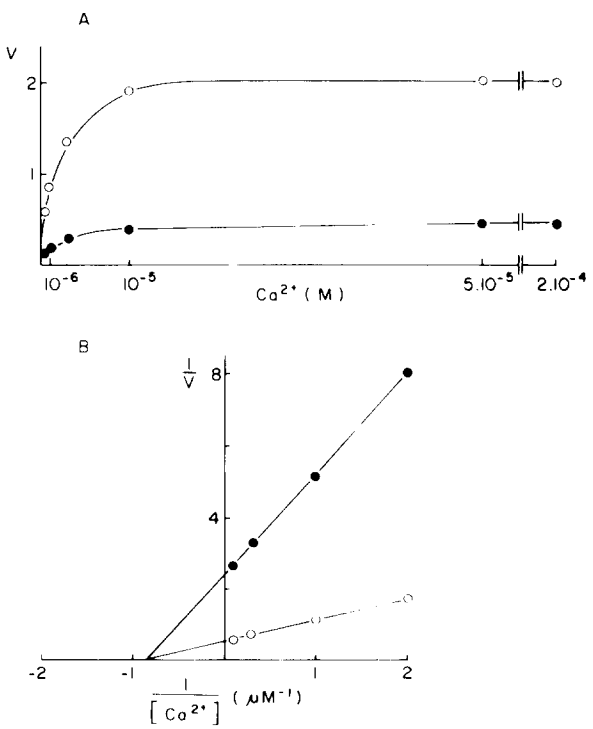

Fig. 1. A. The activation of $(Ca^{2+} + Mg^{2+})$-ATPase by Ca^{2+} in control ghosts (○), and in ghosts treated with *N. naja* phospholipase A_2 (●). B. The corresponding Lineweaver-Burk plot. The reaction rate (V) is expressed in μmol P_i/h per mg ghost protein.

TABLE III

EFFECT OF PHOSPHOLIPASE A_2 TREATMENT OF GHOSTS ON THE $(Ca^{2+} + Mg^{2+})$-ATPase ACTIVITY

Erythrocyte ghosts were treated with phospholipases A_2 from *N. naja* and porcine pancreas, as described in Materials and Methods, without the presence of bovine serum albumin in the incubation medium or the wash buffers.

Expt. No.	Phospholipase A_2	Percent degradation of phospholipids				Relative $V_{sat.\ Ca}$ (% of $V_{sat.\ Ca}$ in control ghosts)
		Sphingomyelin	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine	
1	*N. naja*	0	93	93	91	21
2	*N. naja*	0	99	97	97	21
3	Pancreas	0	99	98	100	16
4	Pancreas	0	99	99	100	14

washing of the phospholipase A_2 treated ghosts with buffer containing 1% albumin (see Materials and Methods), leads to a complete disappearance of the 15—20% residual $(Ca^{2+} + Mg^{2+})$-ATPase activity (Fig. 2). The protein recovery in the phospholipase A_2 treated, 1% albumin washed, ghosts was certainly not lower than that in the control ghosts, which indicates that these treatments

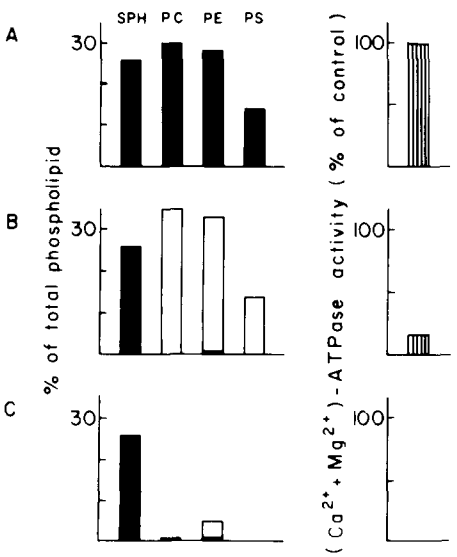

Fig. 2. Phospholipid composition and $(Ca^{2+} + Mg^{2+})$-ATPase activity of (A) native ghosts, (B) ghosts treated with pancreatic phospholipase A_2 and (C) subsequently washed with 1% bovine serum albumin. Identical results were obtained from two independent experiments. Solid and open columns represent intact phospholipids and their lyso-derivatives, respectively. Relative $(Ca^{2+} + Mg^{2+})$-ATPase activities ($V_{sat.Ca}$) have been determined from corresponding Lineweaver-Burk plots. Abbreviations: SPH, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine,; PS, phosphatidylserine.

do not lead to a considerable release of membrane proteins. However, a selective solubilization of the $(Ca^{2+} + Mg^{2+})$-ATPase, although not very likely, cannot be precluded with certainty.

Treatment of ghosts with phospholipases C

It should be remembered that the two phospholipases C used in these studies differ significantly in their substrate specificities. Phospholipase C from *B. cereus* degrades only the glycerophospholipids (lecithin, phosphatidylethanolamine and phosphatidylserine, the first two being the best substrates) in the ghost membrane, leaving the sphingomyelin intact [13,19]. The *C. welchii* phospholipase C has a strong preference for both the choline-containing phospholipids (lecithin and sphingomyelin) [31,32], whereas at sufficiently high enzyme concentrations, also phosphatidylethanolamine can be hydrolyzed completely. Even under such conditions, phosphatidylserine is not attacked by this phospholipase C.

Complete degradation of the glycerophospholipids in red cell ghosts by treatment with *B. cereus* phospholipase C, causes a complete inactivation of the $(Ca^{2+} + Mg^{2+})$-ATPase (Fig. 3B). On the other hand, ghosts treated with pure *C. welchii* phospholipase C, essentially containing phosphatidylserine as the only remaining phospholipid, show a residual $(Ca^{2+} + Mg^{2+})$-ATPase activity of 36% of the control value (Fig. 3C).

It has been demonstrated [1] that the $(Na^+ + K^+)$-stimulated ATPase activity of the human erythrocyte membrane depends exclusively on its association with phosphatidylserine. Nevertheless, treatment of native ghosts with pure *C. welchii* phospholipase C (not affecting phosphatidylserine) results in a con-

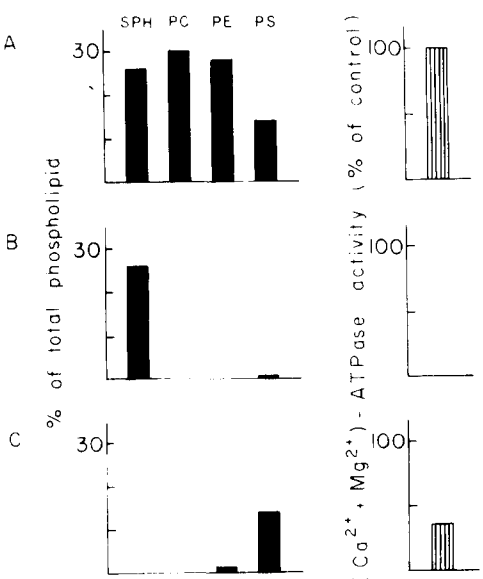

Fig. 3. Phospholipid composition and $(Ca^{2+} + Mg^{2+})$-ATPase activity of (A) native ghosts, (B) ghosts treated with *B. cereus* phospholipase C and (C) ghosts treated with *C. welchii* phospholipase C. See also legend to Fig. 2.

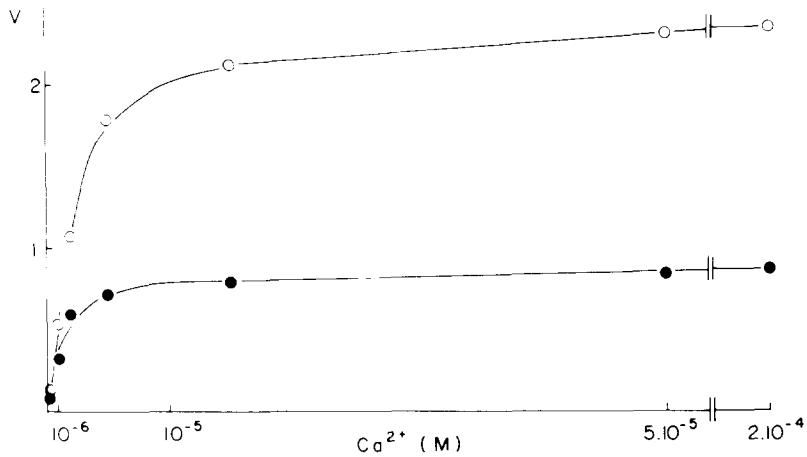

Fig. 4. The activation of $(Ca^{2+} + Mg^{2+})$-ATPase by Ca^{2+} in ghosts subjected to anhydrous ether extraction and subsequent incubation with (●) and without (○) *C. welchii* phospholipase C. The reaction rate (*V*) is expressed in μmol P_i/h per mg ghost protein. The phospholipase C incubation was carried out as described in Materials and Methods, with the omission of the subsequent wash procedures. It appeared, for unknown reasons, that application of the usual wash procedures after this series of ghost treatments leads to a complete loss of $(Ca^{2+} + Mg^{2+})$-ATPase activity, also in the control ghosts. Therefore, the phospholipase reaction was stopped by addition of an equimolar amount of EGTA only. Degradation of substrates was found to be complete at this stage.

siderable loss of this particular ATPase activity. However, when ghosts are freed from their cholesterol by anhydrous ether extraction, prior to the treatment with *C. welchii* phospholipase C, the $(Na^+ + K^+)$-ATPase activity appears to be completely preserved (Roelofsen, B., unpublished). When ghosts are subjected to this sequence of treatments, e.g. lyophilization, dry ether extraction and incubation with *C. welchii* phospholipase C, the maximal rate of the $(Ca^{2+} + Mg^{2+})$-ATPase in the phospholipase C treated ghosts is again found to be some 36% of that of the corresponding control (Fig. 4).

Since it is not possible to remove phospholipase C (or A_2) completely by wash procedures [1], the effects of partial phospholipid degradation were studied by arresting phospholipase C activity by *o*-phenanthroline. From the data presented in Table IV, it is obvious that there is no linear relationship between the residual ATPase activity and the remaining quantity of any of the individual phospholipids or the total phospholipid content of the membrane. Since it has been shown above that a complete degradation of sphingomyelin alone does not affect the ATPase activity, it makes sense to restrict such a comparison to the glycerophospholipids. The relation between residual intact glycerophospholipids and residual ATPase activity is visualized in Fig. 5. The intercept of line A (representing glycerophospholipids in total membrane) with the ordinate is a consequence of (1) the asymmetric distribution of the phospholipids among the two membrane layers [11,15] and (2) the differences in degradation rates of each of the phospholipid classes (see Table IV). A direct proportionality between percent residual ATPase activity and percent residual intact phospholipids (represented by a straight line through the origin with a slope equal to 1) appears to exist only when the glycerophospholipids in the inner membrane layer are considered (line B in Fig. 5).

TABLE IV

RESIDUAL (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY AND RESIDUAL PHOSPHOLIPIDS AFTER TREATMENT OF GHOSTS WITH PHOSPHOLIPASES C

Enzyme concentrations and incubation times were varied in the experiments involving *B. cereus* phospholipase C, from 0.06 I.U. and 30 min in Experiment 11, to 2 × 80 I.U. and 120 min in Experiment 1. Further conditions were identical to those described in Materials and Methods. Percent residual glycerophospholipids in the inner membrane layer was calculated by using the data for phospholipid distribution among the two membrane layers according to Verkleij et al. [15], and assuming that in open ghosts there is no difference in degradation rate for a given substrate, either present in the inner or in the outer layer.

Expt. No.	Phospholipase C	Residual phospholipids							Residual ATPase activity
		Total membrane						Inner membrane layer	
		Sphingomyelin	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine	Total phospholipid	Total glycerophospholipid	Total glycerophospholipid	
		(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
1	*B. cereus*	100	0	0	4	27	<1	<2	<2
2	*B. cereus*	100	1	1	20	30	5	8	11
3	*B. cereus*	100	3	0	66	37	14	21	21
4	*B. cereus*	100	4	0	84	40	18	27	25
5	*C. welchii*	0	0	5	100	16	21	34	35
6	*C. welchii*	0	0	9	100	17	22	36	36
7	*C. welchii*	0	0	14	100	18	24	39	37
8	*B. cereus*	100	8	16	100	48	29	41	43
9	*B. cereus*	100	48	36	100	66	53	58	60
10	*B. cereus*	100	79	73	100	86	81	85	84
11	*B. cereus*	100	88	92	100	94	92	96	95

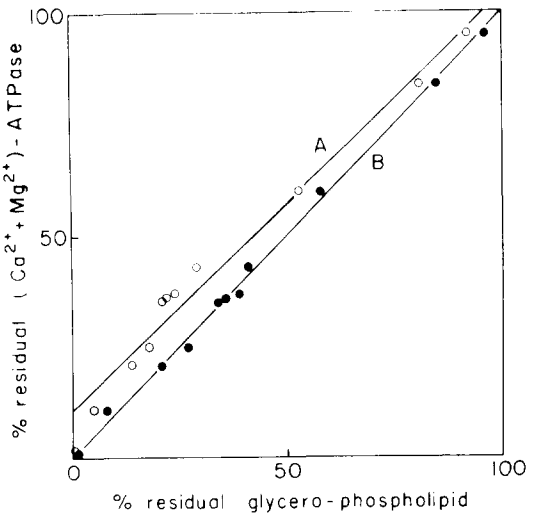

Fig. 5. Percent residual ATPase activity as function of percent residual total glycerophospholipid content of total membrane (line A) and inner leaflet (line B). ○, experimental points from experiments with total membrane; ●, values for inner leaflet calculated on the basis of known distribution of phospholipids between inner and outer membrane layer and assuming that all lipids are equally accessible to phospholipases. A (r = 0.9899) and B (r = 0.9989) are calculated regression lines. Slopes of A and B are not statistically different from each other or from 1. The intercept of A with the ordinate is significant ($P < 0.001$), which demonstrates deviation from direct proportionality between ATPase activity and glycerophospholipid content of total membrane.

Reconstitution of (Ca^{2+} + Mg^{2+})-ATPase activity

Reactivation experiments have been carried out with ghost preparations in which the (Ca^{2+} + Mg^{2+})-ATPase had been inactivated by treatment with phospholipase C from *B. cereus*. The *C. welchii* phospholipase C is not very suitable for such experiments, on account of the considerable residual (Ca^{2+} + Mg^{2+})-ATPase activity, and phospholipases A_2 are unsuitable due to the impossibility to remove or inhibit these enzymes completely.

The phospholipase C (*B. cereus*) inactivated ghosts, in which the residual (Ca^{2+} + Mg^{2+})-ATPase activity ranged from 0—7% of the control value, were resuspended in a series of four dispersions containing different quantities of phospholipid. As shown in Fig. 6, all three of the glycerophospholipids tested brought about a considerable reactivation; phosphatidylserine having the greatest effect. At a ratio of 2.0 μmol of this phospholipid per mg ghost protein, the reactivated activity is more than 90% of that of the control. Application of a mixture of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, in molar ratios of 2 : 5 : 3, respectively, which corresponds with the situation in the inner membrane layer, appeared to be equally effective as phosphatidylserine alone.

Freezing and thawing of the recombined mixtures, prior to the ATPase incubation as described in Materials and Methods, appeared to be essential. When this step was omitted, the reconstituted activity was only some 10% of that observed after freezing and thawing. Identical low activities were found when phospholipase C treated ghosts were frozen and thawed prior to the addition of phospholipids.

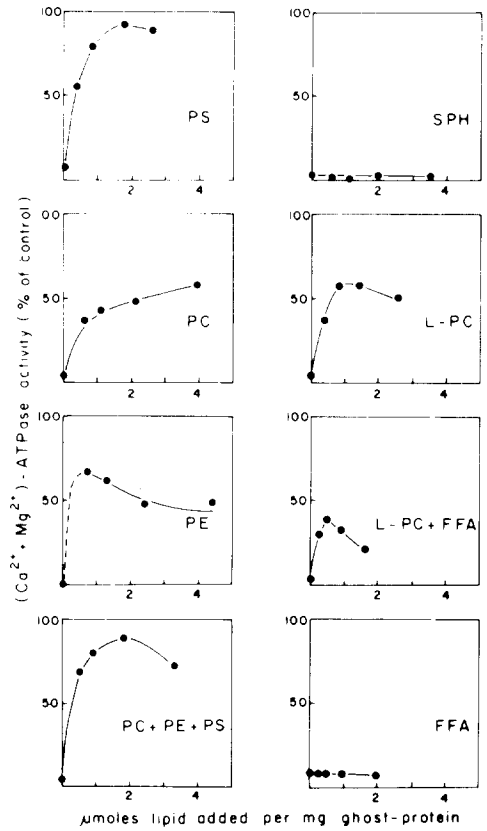

Fig. 6. Reactivation of the (Ca^{2+} + Mg^{2+})-ATPase in phospholipase C (*B. cereus*) treated ghosts by various phospholipids. ATPase activity of each sample was determined by duplicate incubations at $5 \cdot 10^{-5}$ M Ca^{2+}. For further details see text. Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PC + PE + PS, glycerophospholipid mixture at molar ratio's of 2 : 5 : 3, respectively; SPH, sphingomyelin; L-PC, lysophosphatidylcholine; FFA, free fatty acids.

Sphingomyelin was unable to accomplish any reactivation of the (Ca^{2+} + Mg^{2+})-ATPase in the phospholipase C treated ghosts (Fig. 6).

Considerable reactivation of the (Ca^{2+} + Mg^{2+})-ATPase could also be obtained, however, by the addition of lysophosphatidylcholine, whereas free fatty acids (both derived from the same lecithin) appeared to be completely inactive in this respect (Fig. 6). The reactivating capacity of lyso-phosphatidylcholine appeared to be strongly impaired, particularly at higher quantities per mg ghost protein, when this phospholipid was mixed with an equimolar quantity of free fatty acids. The data for the reactivation by lysophosphatidylcholine and lysophosphatidylcholine plus free fatty acids, presented in Fig. 6, are obtained from experiments with different blood specimens, which might make a comparison doubtful. Therefore, an additional experiment was conducted, using one batch of phospholipase C treated ghosts. As can be seen from the results shown in Fig. 7, there is indeed a strong inhibitory effect of the free fatty acids on the reactivation by lysophosphatidylcholine. This inhibition increases as the lipid-protein ratio is elevated.

TABLE V

PROPERTIES OF THE (Ca^{2+} + Mg^{2+})-ATPase, REACTIVATED BY GLYCEROPHOSPHOLIPIDS IN PHOSPHOLIPASE C TREATED GHOSTS

For details, see the text. In experiment No. 3 phosphatidylcholine + phosphatidylethanolamine + phosphatidylserine has been added in the molar ratios 2 : 5 : 3, respectively.

Experiment No.	Phospholipid added		(Ca^{2+} + Mg^{2+})-ATPase	
	Type	μmol per mg protein	$V_{sat.\ Ca}$ (μmol P_i/h per mg ghost protein)	K_{Ca} ($M \cdot 10^{-6}$)
1 Control	—	—	1.74	1.6
Phospholipase C treated	—	—	≈0.08	—
Phospholipase C treated	Phosphatidylserine	2.0	1.60	1.5
2 Control	—	—	2.67	2.0
Phospholipase C treated	—	—	0.00	—
Phospholipase C treated	Phosphatidylcholine	4.2	1.32	1.8
Phospholipase C treated	Phosphatidylethanolamine	1.2	1.52	1.8
3 Control	—	—	1.88	2.5
Phospholipase C treated	—	—	≈0.05	—
Phospholipase C treated	Phosphatidylcholine + phosphatidylethanolamine + phosphatidylserine	1.6	1.78	2.2

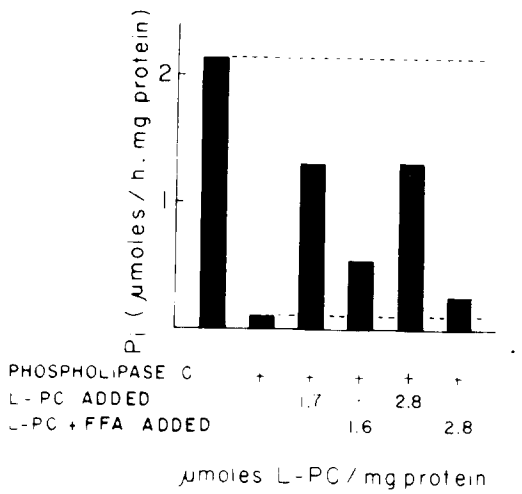

Fig. 7. Reactivation of the $(Ca^{2+} + Mg^{2+})$-ATPase in phospholipase C (*B. cereus*) treated ghosts by lysophosphatidylcholine alone and by lysophosphatidylcholine plus an equimolar quantity of free fatty acids. Lysophosphatidylcholine and free fatty acids were added as mixed liposomes. ATPase incubation conditions and abbreviations are as in Fig. 6.

In order to make sure that the glycerophospholipid reactivated ATPase was indeed the genuine $(Ca^{2+} + Mg^{2+})$-ATPase, the activation by Ca^{2+} of the reconstituted enzyme was compared to that of the native one. Glycerophospholipids were added to phospholipase C treated ghosts in such quantities that an (almost) optimal reactivation of the ATPase could be expected. The thus reconstituted ghosts were incubated at a series of 6 different Ca^{2+} concentrations, ranging from $5 \cdot 10^{-7}$ to $2 \cdot 10^{-4}$ M, so as to allow a determination of the maximal rate of the ATPase and its affinity constant for Ca^{2+}, from Lineweaver-Burk plots. As can be seen in Table V, the K_{Ca} values of the reconstituted $(Ca^{2+} + Mg^{2+})$-ATPase preparations deviate from those of the corresponding controls by not more than some 10%. Moreover, it was observed that the reactivated enzyme preparations do not exhibit any Ca^{2+}-stimulated ATPase activity when Mg^{2+} is omitted.

Finally, it should be mentioned that the detergent Triton X-100 (used by others for the partial purification of the $(Ca^{2+} + Mg^{2+})$-ATPase from erythrocyte ghosts [33,34]), at concentrations of either 0.05, 0.2, 0.4 or 0.8% (w/v), failed to accomplish any reactivation of this enzyme in phospholipase C treated ghosts.

Discussion

Phospholipases have been widely used in studies concerning the lipid requirement of the $(Ca^{2+} + Mg^{2+})$-ATPase in sarcoplasmic reticulum [35—42] and erythrocyte [8,43,44] membranes. Some of these studies also included reactivation of the enzyme by addition of lipids to the phospholipase inactivated preparations. In most cases, however, commercial or only partially purified phospholipase preparations have been used. The uncontrolled

influences that such phospholipase preparations, as well as an inadequate inhibition of the lipase afterwards, may have on the final experimental results, have been extensively discussed before [1].

It is known that the final properties of the $(Ca^{2+} + Mg^{2+})$-ATPase in erythrocyte ghosts are strongly influenced by the procedures used for haemolysis and subsequent washes [45,46]. The procedure according to Wolf [9], used in these studies, resulted in ghosts with a relatively high $(Ca^{2+} + Mg^{2+})$-ATPase activity (1.5—3.0 μmol/h per mg protein) of uniform affinity for Ca^{2+} at all Ca^{2+} concentrations.

The complete preservation of the $(Ca^{2+} + Mg^{2+})$-ATPase activity, after treatment of intact erythrocytes with *N. naja* phospholipase A_2 and sphingomyelinase C (Table I), indicates that the ATPase does not require the presence of the original phospholipids of the outer leaflet and hence the intact phospholipid bilayer.

An involvement of sphingomyelin in the $(Ca^{2+} + Mg^{2+})$-ATPase can be ruled out, because (1) a selective and complete degradation of this phospholipid has no effect on the activity (Table II), (2) ghosts which have been "delipidized" by either *B. cereus* phospholipase C or phospholipase A_2 plus albumin, do not exhibit any residual activity, although they still contain their original quantity of sphingomyelin (Figs. 2 and 3), and (3) sphingomyelin completely fails to reactivate the enzyme in phospholipase C treated ghosts (Fig. 6).

The drastic decrease in $(Ca^{2+} + Mg^{2+})$-ATPase activity, found when the glycerophospholipids are (almost) quantitatively converted into their lyso-derivatives and free fatty acids (Table III), is in contradiction with observations made with sarcoplasmic reticulum membranes. Fiehn and Hasselbach [38] and Meissner and Fleischer [41] even found a stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase in sarcoplasmic reticulum treated with phospholipase A_2. Moreover, The and Hasselbach [39,40], as well as Meissner and Fleischer [41], were able to restore the ATPase activity in phospholipase A_2 treated preparations, not only by lysophosphatidylcholine, but also by free fatty acids. The high purity of the phospholipases A_2 used in our studies, excludes the possibility that the observed decreases in the ATPase activity in treated red cell ghosts is caused by proteolytic activities or other contaminations. The reduction in activity, however, is consistent with the finding that the reactivation of the $(Ca^{2+} + Mg^{2+})$-ATPase in phospholipase C treated ghosts by lysophosphatidylcholine is strongly impaired when free fatty acids are also present, the latter compounds being without effect when added alone (Figs. 6 and 7). Therefore, it may be concluded that the enzyme systems from sarcoplasmic reticulum and erythrocyte membranes behave differently in this respect.

It has been claimed recently by Quist and Roufogalis [43] that phospholipase A_2 treatment affects only the so-called low affinity $(Ca^{2+} + Mg^{2+})$-ATPase in erythrocyte ghosts and not the high affinity one. It should be mentioned that in our experiments the ghost preparations exhibited only the high affinity $(Ca^{2+} + Mg^{2+})$-ATPase activity between $5 \cdot 10^{-7}$ and $2 \cdot 10^{-4}$ M Ca^{2+} and that therefore, it seems impossible to compare the present results with those of Quist and Roufogalis.

Exhaustive hydrolysis of the glycerophospholipids with *B. cereus* phospholipase abolishes the $(Ca^{2+} + Mg^{2+})$-ATPase activity completely (Fig. 3B). On the

other hand, extensive treatment of ghosts with *C. welchii* phospholipase C results in a considerable residual activity (Fig. 3C). It should be emphasized that treatment of ghosts with *C. welchii* phospholipase C results in a degradation of more than 80% of the total phospholipid complement (see Table IV). Recently, Coleman and Bramley [8] have found a complete inactivation of the $(Ca^{2+} + Mg^{2+})$-ATPase already at 50—60% degradation of the total phospholipids by this phospholipase. It is likely that the discrepancy with our results is due to the fact that they applied only a heat treatment to the commercial *C. welchii* preparation according to Sabban et al. [47], which appears to be insufficient to destroy the haemolytic activity effectively [11]. This, in combination with the inadequate procedure used by Coleman and Bramley to inhibit the phospholipase activity, could also be the reason for the failure to reactivate the ATPase when high levels of phospholipase were used.

Treatment of ghosts with *C. welchii* phospholipase C leads to a considerable inhibition of the $(Na^+ + K^+)$-ATPase, unless cholesterol has been (Roelofsen, B., unpublished) removed previously *. Apparently, the situation with regard to the $(Ca^{2+} + Mg^{2+})$-ATPase is quite different in this respect. Removal of cholesterol by anhydrous ether extraction, prior to the *C. welchii* phospholipase C incubation, has no effect on the level of the residual $(Ca^{2+} + Mg^{2+})$-ATPase activity. It should be mentioned in this context that, as is illustrated by the high activity of the control ghosts in Fig. 4, the removal of cholesterol by itself does not lead to an inactivation of the $(Ca^{2+} + Mg^{2+})$-ATPase. This behaviour is similar to what was found by Drabikowski et al. [50] in fragmented sacroplasmic reticulum.

Absence of an interference of cholesterol or diglycerides [8] with the activity allows a comparison between the degree of phospholipid degradation by phospholipase C and the level of residual $(Ca^{2+} + Mg^{2+})$-ATPase activity. A direct proportionality between percent residual ATPase activity and percent residual intact phospholipids exists only when the total glycerophospholipid complement in the inner layer of the membrane is considered. This is in agreement with the observation that the $(Ca^{2+} + Mg^{2+})$-ATPase activity is affected neither by a complete degradation of sphingomyelin, nor by enzymatic hydrolysis of the phospholipids in the outer membrane layer.

Phospholipase C from *B. cereus* degrades phosphatidylcholine and phosphatidylethanolamine more readily than phosphatidylserine, whereas the latter is not affected at all by the *C. welchii* enzyme. This means that the ratio of (phosphatidylserine) : (phosphatidylcholine plus phosphatidylethanolamine) increases at the lower end of line B in Fig. 5, yet there is no deviation from the straight line relationship, which seems to indicate that phosphatidylserine and phosphatidylcholine plus phosphatidylethanolamine are equipotent in maintaining the ATPase activity.

The results from enzymic degradation are supported by the reactivation

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* This may indicate that "free" cholesterol (no longer forming a complex with intact phospholipids) interferes with the phosphatidylserine-protein interaction, which is essential for the $(Na^+ + K^+)$-ATPase activity [1]. Such an inhibitory effect of cholesterol is consistent with findings of others [48,49], and with the fact that a complete reactivation of the $(Na^+ + K^+)$-ATPase in phospholipase C treated ghosts is only achieved when cholesterol has been removed prior to the addition of phosphatidylserine [1].

experiments, which show that the (Ca^{2+} + Mg^{2+})-ATPase in *B. cereus* phospholipase C treated ghosts can be reactivated by each of the three glycerophospholipids, but not by sphingomyelin. The fact that considerable reactivation is observed only when the ghost suspensions are frozen and thawed after the addition of phospholipid could indicate that closed structures are formed when the treated ghost recombines with the added lipid. At present, however, this possibility cannot be more than a speculation.

Summarizing, it can be concluded that the (Ca^{2+} + Mg^{2+})-ATPase of the human erythrocyte membrane is dependent upon the total glycerophospholipid complement of the membrane, and most likely only upon that fraction which forms part of the inner monlayer.

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