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HYDROLYSIS OF ERYTHROCYTE MEMBRANE PHOSPHOLIPIDS BY A PREPARATION OF PHOSPHOLIPASE C FROM *CLOSTRIDIUM WELCHII*

DEACTIVATION OF (Ca^{2+}, Mg^{2+})-ATPase AND ITS REACTIVATION BY ADDED LIPIDS

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SUMMARY

1. Haemoglobin-free erythrocyte ghosts were prepared in 40 imosM bicarbonate buffer, pH 7.4, containing 1 mM EDTA (40 imosM/1 mM EDTA). The ghost preparation was highly permeable on preparation but partially resealed on incubation in media containing Ca^{2+}.

2. A partially purified preparation of phospholipase C from *Clostridium welchii* caused an increase in observed Mg^{2+}-ATPase activity, reflecting a change in the permeability of the ghosts to substrate. The phospholipase did not decrease Mg^{2+}-ATPase even at the highest levels tested. Mg^{2+}-ATPase activity could therefore be used as a permeability indicator in these experiments.

3. Both (Ca^{2+}, Mg^{2+})- and (Na^+, K^+, Mg^{2+})-ATPase activities of the ghosts were progressively lost as a result of the phospholipid hydrolysis induced by phospholipase C.

4. When a haemolysin in the commercial preparation was destroyed by heat-treatment, deactivation of the (Ca^{2+}, Mg^{2+})-ATPase and (Na^+, K^+, Mg^{2+})-ATPases were still observed but permeability changes were greatly reduced.

5. The products of phospholipase action were not inhibitory to the (Ca^{2+}, Mg^{2+})-ATPase.

6. Lysolecithin brought about a reactivation of the (Ca^{2+}, Mg^{2+})-ATPase which was superimposed upon permeability changes in the preparation.

7. Reactivation of the (Ca^{2+}, Mg^{2+})-ATPase was brought about by a non-lytic, mixed lipid preparation without significant effect upon permeability.

8. Human erythrocyte (Ca^{2+}, Mg^{2+})-ATPase therefore appears to be an enzyme which responds to perturbation of the lipid environment in the membrane and is a "lipid-dependant" enzyme.

Abbreviation: EGTA, ethyleneglycol-bis(aminoethyl)-tetraacetic acid.

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INTRODUCTION

One of the most interesting ways in which the roles of phospholipids in membrane structure and function have been explored has been the treatment of membranes with phospholipases. Modifications induced by such treatments include alterations in the activities of certain membrane-bound enzymes. The (Na^+, K^+, Mg^{2+})-ATPase activities of many membrane preparations have been fairly extensively studied in this respect but studies of the role of lipids in (Ca^{2+}, Mg^{2+})-ATPase activities have been restricted almost exclusively to experiments with sarcoplasmic reticulum preparations.

The present work investigates the effects of manipulation of membrane lipids upon the (Ca^{2+}, Mg^{2+})-ATPase activity, using two different membrane preparations of well-defined properties. Phospholipid hydrolysis was accompanied by a decrease in (Ca^{2+}, Mg^{2+})-ATPase activity; reactivation could be achieved by the addition of lipids.

ATPases can show latency, depending upon the permeability of the ghost preparation. By the use of ghost preparations of known permeability, by the use of haemolytic and haemolysin-inactivated phospholipase preparations and by the use of lytic and non-lytic phospholipids, permeability effects were distinguished from true deactivation-reactivation phenomena.

MATERIALS AND METHODS

Chemicals

Phospholipase C (*Clostridium welchii*, type I) and other fine chemicals were obtained from Sigma Chemical Co., London. Phosphorylcholine was obtained as a Cd^{2+} salt and converted to the free acid with Dowex 50W-X8. This was immediately neutralized with $NaHCO_3$ to pH 7.4 and stored at −20 °C until required.

Lipids

Phosphatidylcholine was prepared from egg yolks by chromatography on alumina [1]. The material gave a single spot on thin-layer chromatography in the same position as authentic phosphatidylcholine.

Lysolecithin (1-acyl-*sn*-3-glycerylphosphorylcholine) was prepared from the phosphatidylcholine by a method essentially that of Kates [2] using *Crotalus adamanteus* venom. The material gave a single spot on thin-layer chromatography in the same position as authentic lysolecithin. The lipid was dissolved in chloroform and stored at −20 °C under N_2.

A fraction of lipids (designated as mixed lipid fraction) derived from ox liver was kindly donated by Dr R. H. Michell. This fraction represents material remaining in solution when 5 vol. ethanol are added to 1 vol. of a chloroform solution of total liver lipids of ox [3] and contains those phospholipids (approx. 75 % phosphatidylcholine, approx. 20 % phosphatidylethanolamine and approx. 5 % sphingomyelin) which are principally the ones hydrolysed by phospholipase C [4], together with neutral lipids (cholesterol, glycerides). This lipid preparation was stored at −20 °C under N_2.

Suspension of lipids for reactivation purposes

Several methods were used for the suspension of the mixed lipid fraction in 0.01 M Tris buffer (pH 7.4): viz. evaporation of an ether solution from above the buffer, dialysis of a deoxycholate-solubilized suspension, and sonication of the dried material into the buffer. Sonication yielded the most effective suspension and a sonication time of 5 min was adequate (the mixture was cooled to 0 °C after each 0.5 min of the sonication). The sonicated lipid suspension was kept at 0 °C and adjusted to a concentration of 5 μmol phospholipid per ml. There was no apparent change in the composition of the mixture due to the sonication.

The chloroform solution of lysolecithin was evaporated and the dried material dispersed with brief sonication in 0.01 M Tris buffer, pH 7.4, to a final concentration of 2 μmol phospholipid per ml, and stored at 0 °C.

Preparation of erythrocyte ghosts

These were prepared from 0^+ human blood by haemolysis and washing in 40 imosM bicarbonate buffer, pH 7.4, containing 1 mM EDTA, as described previously [5]. The final pellet was washed twice with 10 vol. of 40 imosM bicarbonate buffer, pH 7.4, to essentially remove EDTA. The ghost concentration was adjusted to 2 μmol phospholipid per ml and the ghosts were then stored in sealed containers at 0 °C. Ghosts were also prepared and stored at 80 imosM in bicarbonate buffer [6].

Treatment of ghosts with phospholipase C

Phospholipase C was dissolved in 40 or 80 imosM bicarbonate buffer containing 11 mM $CaCl_2$. 0.4 ml phospholipase solution of the appropriate concentration was added to 4 ml of ghost suspension (2 μmol phospholipid per ml). Control incubations contained all components except the phospholipase. The mixtures were incubated at 30 °C for 30 min. Phospholipase action was stopped, except where indicated, by the addition of a slight excess (1.2 mM) ethyleneglycol-bis(aminoethyl)-tetraacetic acid (EGTA). This chelates the Ca^{2+} necessary for the action of the phos-

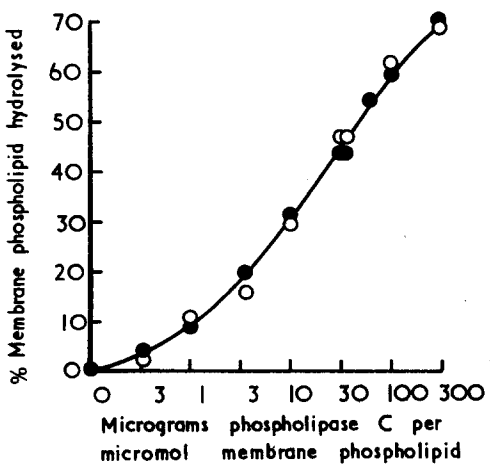

Fig. 1. Extract of phospholipid hydrolysis by increasing concentrations of partially purified phospholipase C. ●, 40 imosM/1 mM EDTA ghosts; ○, 80 imosM ghosts.

pholipase. Ghosts were sedimented at 15 000 × *g* for 10 min, and washed twice in bicarbonate buffer (pH 7.4) of the appropriate osmolarity which contained no Ca^{2+} or EGTA. The extent of phospholipid hydrolysis could be varied from 0 to 70 % by varying the concentration of phospholipase C (Fig. 1).

Heat treatment of phospholipase C

The phospholipase was dissolved at a level of 1 mg/ml in 40 imosM/11 mM Ca^{2+} buffer (or 80 imosM/11 mM Ca^{2+} buffer as appropriate). The solution was heated at 56 °C for 20 min and then cooled to 0 °C. This treatment inactivates a haemolysin often found in commercial *C. welchii* phospholipase C preparations [7]. In our hands, heat treatment also greatly reduced a non-specific phosphatase activity which is sometimes present. The heat-treated material was then diluted with 40 imosM/11 mM Ca^{2+} buffer to yield an appropriate concentration of phospholipase C and 0.1 vol. was added to 1 vol. of ghost suspension.

Basic form of deactivation - reactivation experiments

4 ml of ghost suspension in 40 imosM buffer was added to 0.4 ml of 40 imosM bicarbonate buffer, pH 7.4; 11 mM Ca^{2+}, containing appropriate amounts of phospholipase C. The mixture was incubated at 30 °C for 30 min and then chilled on ice.

For reactivation 2 ml of appropriate lipid in 0.01 M Tris/HCl buffer, pH 7.4, was added and the mixture kept at 0 °C for 30 min. Aliquots of the mixture were then taken for enzyme and lipid determinations.

Lipid extraction, phospholipid and cholesterol determinations

These have been described previously [6]. The extent of phospholipid hydrolysis was calculated relative to a constant feature of membrane composition, in this case cholesterol content. Under the conditions employed for phospholipid hydrolysis and subsequent isolation of the treated ghosts, all the cholesterol of the membranes was sedimented at 15 000 × *g* for 10 min. Cholesterol content has also been used as a reference for other parameters in many of the present experiments, prior to calculation of percentage changes. This corrects for any losses of membrane material.

ATPase activities

Aliquots were removed and treated for ATPase activity in one of the following assays. In early deactivation experiments: Mg^{2+}-ATPase: 100 mM NaCl, 30 mM Tris/HCl, pH 7.4 0.1 mM ouabain, 1 mM EDTA; (Na^+, K^+, Mg^{2+})-ATPase: 100 mM NaCl, 30 mM Tris/HCl, pH 7.4, 30 mM KCl, 1 mM EDTA; (Ca^{2+}, Mg^{2+})-ATPase: 100 mM Tris/HCl, pH 7.4, 1 mM Ca^{2+}. In deactivation -reactivation experiments: Mg^{2+}-ATPase: 100 mM Tris/HCl, pH 7.4, 1 mM EGTA. All incubations contained 3 mM ATP plus 4 mM Mg^{2+} and were carried out in a final volume of 2 ml. Incubation was normally for 2 h at 30 °C and reaction was then halted with 2 ml of cold 15 % trichloroacetic acid. After centrifugation 2 ml of the supernatant was used for determination of inorganic phosphate [8].

(Ca^{2+}, Mg^{2+})- and (Na^+, K^+, Mg^{2+})-ATPase activities were obtained by subtracting the basal Mg^{2+}-ATPase level from the levels obtained in the presence of the appropriate ions. Appropriate controls were carried out lacking enzyme or substrate.

On preparation, 40 imosM: 1 mM EDTA ghosts had Mg^{2+}-ATPase, (Na^+, K^+)-ATPase, and (Ca^{2+}, Mg^{2+})-ATPase levels of approximately 300, 300 and 700 nmol/h per μmol membrane phospholipid respectively. 80-imosM ghosts had levels for all three ATPase activities of approximately 30–50 nmol/h per μmol membrane phospholipid. These ATPase activities are consistent with values reported previously [5, 6]. ATPase activities are reported in the text as percentage of the activity of control ghosts which had been incubated in the absence of phospholipase C.

RESULTS

Phospholipase C treatment of 40 imosM/1 mM EDTA ghosts

As prepared, these ghosts are essentially free from intracellular contaminants and are permeable to small molecules. They have high ATPase activities which are non-latent, since the substrate (Mg-ATP) is able to reach the inside face of the ghost.

Fig. 2 shows the effects of phospholipase action on the ATPase activities of this membrane preparation. The activity of Mg^{2+}-ATPase progressively increased to a maximum at about 20 % hydrolysis of membrane phospholipids (Fig. 2a). The ATPase level reached was about 250 % of the control. Further phospholipase action had little effect on Mg^{2+}-ATPase activity. Both (Na^+, K^+, Mg^{2+})-ATPase (Fig. 2b) and (Ca^{2+}, Mg^{2+})-ATPase (Fig. 2c) showed an initial increase at low levels of phos-

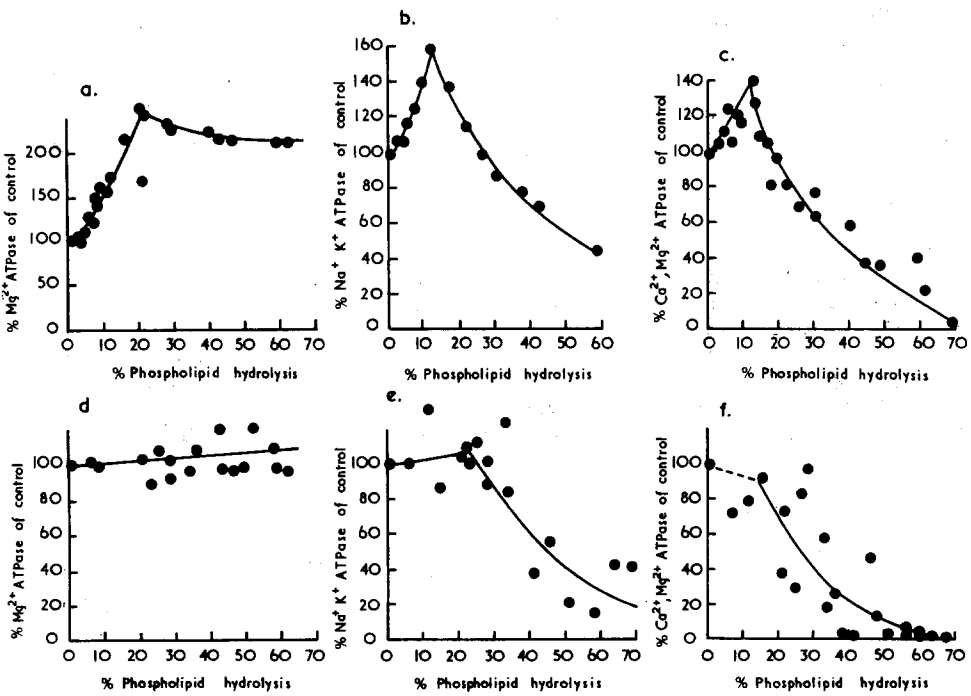

Fig. 2. Action of phospholipase C preparations on ATPase activities in 40 imosM/1 mM EDTA ghosts. For details see Materials and Methods. a, b and c represent experiments using the partially purified phospholipase C preparation. d, c and f represent experiments with this preparation heat treated at 56 °C for 20 min. Data are derived from four separate experiments.

pholipid hydrolysis. The maximum levels of activation achieved were lower than for Mg^{2+}-ATPase however. Further hydrolysis resulted in the progressive loss of both (Na^+, K^+, Mg^{2+})- and (Ca^{2+}, Mg^{2+})-ATPase activities.

Heat treatment of the phospholipase C preparation, in order to reduce its haemolysin activity, modified the response of the membrane ATPase activities. The initial increase at low phospholipid hydrolysis levels shown by all three ATPase activities was absent or less prominent (Figs 2d, 2e, 2f). Mg^{2+}-ATPase activity remained essentially constant (Fig. 2d). However, both (Na^+, K^+, Mg^{2+})-ATPase and (Ca^{2+}, Mg^{2+})-ATPase decreased progressively with increasing phospholipase treatment.

The increases in all three ATPase activities were seen only with haemolysin-containing phospholipase preparations. These increases may have been due to changes in ghost permeability. Before ATPase hydrolysis can occur, the substrate (Mg-ATP) must reach the inner face of the ghost membrane.

C. welchii phospholipase C requires Ca^{2+} for its activity; this Ca^{2+} is present during incubation of ghosts with the phospholipase and may therefore reduce permeability to the ATPase substrate. Experiments where 40 imosM/1 mM EDTA ghosts were incubated for 30 min at 30 °C in the presence of 1 mM Ca^{2+} resulted in ATPase levels which are considerably less (Table I, Expt b) than those of controls incubated without Ca^{2+} (Table I, Expt a). After treatment with sufficient phospholipase to give approximately 15 % hydrolysis of membrane phospholipids, Mg^{2+}-ATPase activity was fully restored, but only when the incubation was stopped with the Ca^{2+} chelator, EGTA (Table I, d). EGTA alone was unable to bring about the restoration of maximal Mg^{2+}-ATPase activity (Table I, Expt c), suggesting that the effects of Ca^{2+} on permeability are exerted from the inner face of the membrane. The effects of Ca^{2+} in reducing permeability, and the subsequent unsealing after treatment with phospho-

TABLE I

EFFECTS OF Ca^{2+} ON APPARENT ATPase LEVELS

Incubation was carried out at 30 °C for 30 min in the presence or absence of Ca^{2+} (1 mM) and phospholipase C, as appropriate. In Expts c and d, the incubation was terminated by 1.2 mol EGTA per mol Ca^{2+}. All ghosts were then diluted, washed twice and resuspended in Ca^{2+}-free buffer (40 imosM bicarbonate, pH 7.4) and sampled for ATPase activities as described in Materials and Methods. In Expt e, phospholipase action was terminated by dilution, washing and resuspension in Ca^{2+}-free buffer. When phospholipase action was stopped with EGTA (d) 17 % hydrolysis of phospholipids occurred. Washing with Ca^{2+}-free buffer alone (e) allowed phospholipid hydrolysis to continue to 28 %.

Expt	Details of treatment			Specific activity (nmol/h/μmol membrane cholesterol)	
	Incubation		Stop and wash EGTA/buffer		
	Ca^{2+}	Phospholipase C		Mg^{2+}-ATPase	(Na^+, K^+)-ATPase
a				310	330
b	+			135	150
c	+		+	155	180
d	+	+	+	300	230
e	+	+		115	153

lipase C followed by EGTA, have been generally confirmed by [^{14}C]sucrose exclusion experiments (Bramley, T. A., unpublished).

Thus the highly permeable 40 imosM/l mM EDTA ghosts had partially resealed on incubation in the presence of Ca^{2+} such that only one half to one third of their ATPase activities were demonstratable. When the permeability barrier was abolished by treatment with a haemolysin-containing phospholipase C (compare Figs 2a and 2d) by lysolecithin (see later) or by conventional detergents (Coleman, R., unpublished), Mg^{2+}-ATPase activities increased by approximately 200–300 %. Mg^{2+}-ATPase activity, therefore, could be used to monitor changes in permeability in these experiments since it was not inactivated by treatment with *Cl. welchii* phospholipase C. Thus a rise in observed Mg^{2+}-ATPase activity indicates increased permeability. A fall in one of the other ATPase activities, unaccompanied by a fall in Mg^{2+}-ATPase, must therefore indicate an inactivation. Both (Na^+, K^+, Mg^{2+})-ATPase and (Ca^{2+}, Mg^{2+})-ATPase were decreased by phospholipase treatment even under conditions of increased permeability.

Effects of phospholipid hydrolysis products on (Ca^{2+}, Mg^{2+})-ATPase activity

A sonicated preparation of diglycerides, isolated from the hydrolysis products of phospholipase C digestion of purified egg yolk lecithin, had no inhibitory action action on (Ca^{2+}, Mg^{2+})-ATPase activity at concentrations equivalent to the amount of membrane phospholipid hydrolysed. Phosphorylcholine was not inhibitory and was not hydrolysed by endogenous phosphatases in sufficient amounts to cause significant contribution of P_i in ATPase determinations.

Fatty acids may be liberated by the action of lipases on the diglycerides produced by phospholipase C action, although human erythrocytes are low in such endogenous lipase activity [9]. Fatty acids have been shown to be strongly inhibitory to certain enzymes [10, 11] but are avidly removed by defatted albumin. The presence of defatted albumin (up to 10 mg/ml) during phospholipase C treatment, however, failed to prevent deactivation of (Ca^{2+}, Mg^{2+})-ATPase.

Reactivation of (Ca^{2+}, Mg^{2+})-ATPase by added lipids

These experiments were carried out in order to see whether the deactivation of the (Ca^{2+}, Mg^{2+})-ATPase by haemolysin-inactivated phospholipase C could be reversed by adding back lipid to the system.

A phospholipase concentration of 12.5 μg/μmol membrane phospholipid was employed in these experiments. This gave 75–80 % loss of (Ca^{2+}, Mg^{2+})-ATPase activity and 35–40 % hydrolysis of membrane phospholipids. It was insufficient however to cause appreciable hydrolysis of the added reactivating lipids. Levels of phospholipase C which gave complete hydrolysis of membrane lipids were not used since they were found to cause hydrolysis of the lipids added for reactivation, thereby nullifying their ability to reactivate.

The addition of the mixed lipid preparation to the phospholipase-treated ghosts brought about marked reactivation of the (Ca^{2+}, Mg^{2+})-ATPase (Fig. 3). This increase cannot be due to an increase in ghost permeability since there was no significant increase in Mg^{2+}-ATPase. It must therefore be due to a genuine reactivation of the (Ca^{2+}, Mg^{2+})-ATPase, demonstrating the lipid dependence [11, 12] of this membrane-bound enzyme in the human erythrocyte.

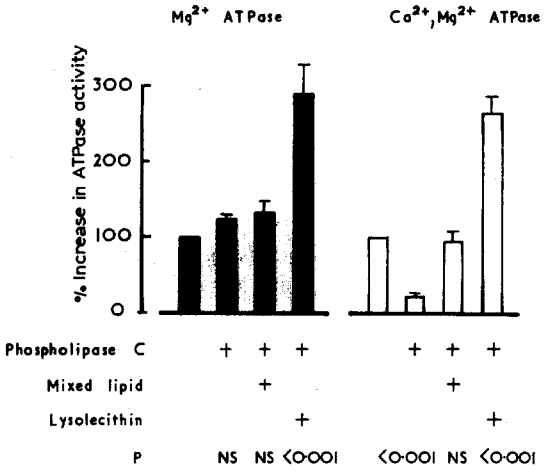

Fig. 3. Effects of mixed lipid and lysolecithin on ATPase activities of phospholipase-treated 40 imosM/l mM EDTA ghosts. 4 ml of ghosts were incubated with heat-treated phospholipase C (12.5 μg/mol membrane phospholipid) for 30 min at 30 °C. 2 ml of mixed lipid (5 μmol/ml) or lysolecithin (2 μmol/ml) in buffer were added and incubated for 30 min at 0 °C. For other details see Materials and Methods. Column heights represent means of 6–9 separate measurements; vertical bars represent S.E. Significance of the difference of means (P) from the appropriate non-phospholipase-treated preparations was calculated using Student's t test. N.S., not significant ($P > 0.1$).

The lytic phospholipid, lysolecithin, brought about a dramatic increase. At the same time there was a marked rise in Mg^{2+}-ATPase (Fig. 3).

Whilst the addition of a large amount of lysolecithin to both treated and untreated ghosts brought about a comparable increase in both Mg^{2+}- and (Ca^{2+}, Mg^{2+})-ATPases, the addition of smaller amounts of lysolecithin had a proportiona-

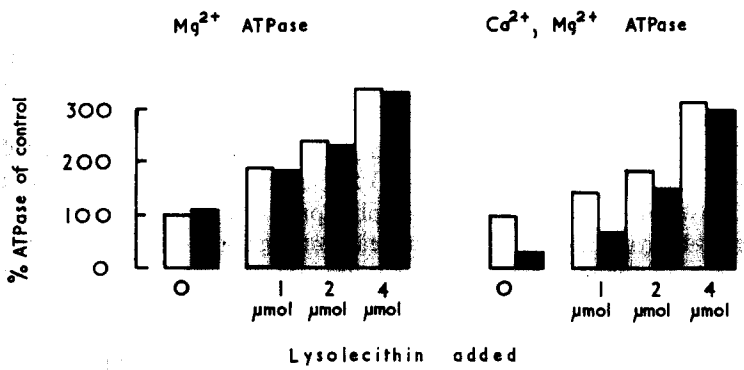

Fig. 4. Effects of lysolecithin concentration on ATPase activities of control and phospholipase-treated 40 imosM/l mM EDTA ghosts. 4 ml of ghosts were incubated with or without heat-treated phospholipase C (12.5 μg/mol membrane phospholipid) at 30 °C for 30 min. 2 ml of sonicated lysolecithin were added containing the amount of lysolecithin shown and incubated for 30 min at 0 °C. For other details see Materials and Methods. Solid columns, phospholipase C treated; open columns, incubated controls.

tely smaller effect on the (Ca^{2+}, Mg^{2+})-ATPase of the phospholipase-treated ghosts (Fig. 4). Thus the activating effects of lysolecithin upon (Ca^{2+}, Mg^{2+})-ATPase are due to reactivation superimposed upon permeability changes.

Phospholipase treatment of 80-imosM ghosts

Ghosts prepared at 80 imosM are considerably less permeable to small molecules than those prepared in 40 imosM/1 mM EDTA. They contain residual haemoglobin and intracellular enzymes and their ATPase activities often represent only about 10–15 % of maximum; removal of latency can often therefore induce increases of the order of 600 % in apparent ATPase levels.

Treatment of 80-imosM ghosts with haemolysin-inactivated phospholipase brought about an increase in Mg^{2+}-ATPase; sonication of the treated material demonstrated however that the majority of the activity was still latent (Fig. 5). (Ca^{2+}, Mg^{2+})-ATPase was apparently lost completely as a result of phospholipase treatment, but brief sonication revealed the presence of latent activity at intermediate levels of hydrolysis. The initial and dramatic loss of (Ca^{2+}, Mg^{2+})-ATPase is therefore probably the result of deactivation of the more permeable proportion of the ghost preparation. It remains to be established whether sealed ghosts are inactivated without resealing or whether sealed ghosts are first unsealed and then deactivated.

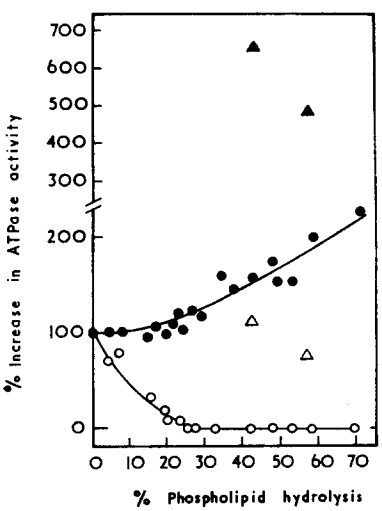

Fig. 5. Action of heat-treated phospholipase C on ATPase activities of 80-imosM ghosts. ●, Mg^{2+}-ATPase; ○, (Ca^{2+}, Mg^{2+})-ATPase. Points are derived from 5 Expts. ▲, Mg^{2+}-ATPase; △, (Ca^{2+}, Mg^{2+})-ATPase, after sonication for 5 s at 0 °C.

DISCUSSION

(Ca^{2+}, Mg^{2+})-ATPase can be deactivated by several widely differing treatments such as low ionic strength, sonication and excess detergent [5, 6]. Deactivation brought about by phospholipase C treatment can therefore indicate little alone about the nature of (Ca^{2+}, Mg^{2+})-ATPase or its membrane environment. Cha et al. [13],

in a study of the relationship of (Ca^{2+}, Mg^{2+})-ATPase to Ca^{2+} uptake in erythrocyte membrane fragments, had briefly observed a deactivation by both phospholipases A and C, but had not attempted to distinguish between denaturation and deactivation of the enzyme.

In the present study it was shown that the products of phospholipase action did not appear to inhibit (Ca^{2+}, Mg^{2+})-ATPase activity nor was the inhibition due to the effect of haemolysin. The reappearance of the ATPase on the addition of lipid demonstrated for the first time that deactivation rather than denaturation had occurred.

A change in membrane permeability may lead to an increase in, or reappearance of, an enzyme activity on the inner surface of the membrane [5, 6, 11]. This was clearly demonstrated in the present experiments by the behaviour of Mg^{2+}-ATPase. The marked increase in activity with the lytic phospholipid, lysolecithin, was due solely to an increase in membrane permeability. Lysolecithin also caused a dramatic increase in (Ca^{2+}, Mg^{2+})-ATPase activity which was due not only to the permeability effect but also to a true reactivation of the deactivated enzyme. A non-lytic phospholipid preparation (of mixed lipids) brought about a reactivation of (Ca^{2+}, Mg^{2+})-ATPase in the absence of a change in permeability. Thus (Ca^{2+}, Mg^{2+})-ATPase of human erythrocyte membranes appears to be a lipid-requiring enzyme (see refs 11, 12 and 14 for general discussions of lipid requirements). It remains to be established which particular chemical and physical lipid properties are essential for reactivation of human erythrocyte (Ca^{2+}, Mg^{2+})-ATPase.

The lipid requirements of the (Ca^{2+}, Mg^{2+})-ATPase of sarcoplasmic reticulum are under study in many laboratories at the present time. This membrane has a very simple protein composition of which the (Ca^{2+}, Mg^{2+})-ATPase is by far the major protein. In addition, the enzyme is outward-facing so that the system is uncomplicated by latency. It is unlikely that the erythrocyte membrane (Ca^{2+}, Mg^{2+})-ATPase will constitute more than a very small proportion of the proteins of this membrane, however. Its specific lipid requirements may therefore be superimposed upon the requirements of other membrane proteins. (Ca^{2+}, Mg^{2+})-ATPase activity has been observed to be increased in membranes as their content of polyphosphoinositide is increased [15].

During the present experiments, a second (Ca^{2+}, Mg^{2+})-ATPase activity was observed which was maximally stimulated by Ca^{2+} at 10^{-6} M. It has not yet been clearly established whether the two activities are reflections of the same enzyme or are two distinct activities; however both appear to be lipid dependent, though show slight differences in detail (Coleman, R. unpublished).

During a study of the lipid requirements of (Na^+, K^+, Mg^{2+})-ATPase in erythrocyte membranes, Roelofsen and Van Deenen [14] observed the deactivation of Mg^{2+}-ATPase by *Bacillus cereus* phospholipase C and its reactivation with added phospholipids. Our own studies have shown that *C. welchii* does not deactivate Mg^{2+}-ATPase. The difference between these two results may be explained by the different substrate specificities of the two types of phospholipase C [4, 14]. In addition, the membrane preparations used were fundamentally different. The preparation used by Roelofsen and Van Deenen [14] had been prepared in CO_2-saturated distilled water, lyophilized, deep frozen, and suspended in very-low-tonicity buffer. These procedures are likely to affect adversely some aspects of the structure and function of the erythro-

cyte membrane; this is borne out by the absence of (Ca^{2+}, Mg^{2+})-ATPase in their preparation. This activity appears to be a sensitive indicator of membrane damage [5, 6].

In both the present and past experiments [5, 6] the properties of ghost preparations have been observed as overall phenomena and no attempt has been made to distinguish different ghost populations within the preparation. It is likely that some of the phenomena observed represent the differential response of permeable and imper meable ghosts in the preparation to the perturbing agent, especially, exemplified in the case of 80-imosM ghosts. The differential behaviour of permeable and impermeable ghosts to *B. cereus* phospholipase C has recently been described [16].

The comparison between haemolysin-containing and haemolysin-inactivated phospholipase C preparations points out an interesting phenomenon in that, in the absence of haemolytic activity, the permeability barrier of the ghosts largely persists despite extensive hydrolysis of membrane phospholipids. This effect can be largely overcome by using a haemolysin-containing preparation, indicating the usefulness of the haemolysin as a tool when dealing with resealed or partly resealed membrane preparations.

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REFERENCES

1 Mangiopane, E. H., Lloyd-Davies, K. A. and Brindley, D. N. (1973) Biochem. J. 134, 103–112
2 Kates, M. (1960) in Lipid Metabolism (Bloch, K., ed.), pp. 165–237, John Wiley, New York
3 Lapetina, E. G. and Michell, R. H. (1973) Biochem. J. 131, 433–443
4 Coleman, R., Finean, J. B., Knutton, S. and Limbrick, A. R. (1970) Biochim. Biophys. Acta 219, 81–92
5 Bramley, T. A. and Coleman, R. (1972) Biochim. Biophys. Acta 290, 219–228
6 Bramley, T. A., Coleman, R. and Finean, J. B. (1971) Biochim. Biophys. Acta 241, 752–769
7 Sabban, E., Laster, Y. and Loyter, A. (1972) Eur. J. Biochem. 28, 373–380
8 Fiske, C. H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–385
9 Michell, R. H., Coleman, R. and Finean, J. B. (1973) Biochim. Biophys. Acta 318, 306–312
10 Cater, B. R. and Hallinan, T. (1971) FEBS Lett. 16, 137–140
11 Coleman, R. (1973) Biochim. Biophys. Acta 300, 1–30
12 Hallinan, T. (1974) Biochem. Soc. Trans. 2, 817–821
13 Cha, Y. C., Shin, B. C. and Lee, K. S. (1971) J. Gen. Physiol. 57, 202–215
14 Roelofsen, B. and van Deenen, L. L. M. (1973) Eur. J. Biochem. 40, 245–257
15 Buckley, J. T. and Hawthorne, J. N. (1972) J. Biol. Chem. 247, 7218–7219
16 Lowe, M. G., Limbrick, A. R. and Finean, J. B. (1973) FEBS Lett. 34, 1–4