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RABBIT PLATELET CALCIUM ATPase DIFFERS FROM THE HUMAN ERYTHROCYTE ($Ca^{2+} + Mg^{2+}$)-ATPase IN ITS RESPONSE TO THREE PURIFIED PHOSPHOLIPASES A_2, EXOGENOUS PHOSPHOLIPIDS AND CALMODULIN

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Human erythrocyte ($Ca^{2+} + Mg^{2+}$)-ATPase and calcium ATPase of rabbit platelets were compared by their responses to a variety of treatments. These included three purified phospholipases A_2 (acidic, neutral and basic) from *Agkistrodon halys blomhoffii*, as well as several phospholipids and lysophospholipids. The erythrocyte enzyme was stimulated 2–3-fold by all three phospholipases with maximal stimulation occurring at different concentrations of the three enzymes. The basic phospholipase was the most potent, followed by the neutral and acidic enzymes in that order. The calcium ATPase activity of the platelet was also stimulated by phospholipase treatment, but only by 10–20%. The stimulatory activity was attributable to hydrolysis of a very small portion of the total membrane phospholipid. Inactivation of the phospholipases by heating or chemical modification with *p*-bromophenacyl bromide abolished their ability to stimulate. Addition of polyphosphoinositides stimulated both ATPases. However, another acidic phospholipid, lysophosphatidic acid, stimulated only the erythrocyte enzyme and failed to affect the platelet calcium ATPase. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) had no effect on either enzyme, while the platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), its lyso compound and lysoPC inhibited both ATPases. Calmodulin stimulated the erythrocyte enzyme, but did not affect the platelet calcium ATPase. These results demonstrate that the protein-lipid interactions operative in the erythrocyte and platelet calcium ATPases are quite different.

Introduction

The biological function of many integral membrane proteins is dependent on lipids [1,2]. The lipid requirements of these proteins frequently are studied with phospholipases. The ($Ca^{2+} + Mg^{2+}$)-ATPase of human erythrocyte membranes has been examined with a wide variety of these phospholipases [2–9], but conclusions have been difficult to make from these experiments. This is due mainly to the complexity of the system. Much of the confusion has now been resolved by reconstitution of purified ATPase into defined lipid environments. The results of these experiments demonstrate a requirement for glycerophospholipids, but no specific requirement for acidic phospholipids [10,11].

Though phospholipases were not very useful in defining the lipid requirement of the erythrocyte

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Abbreviations: PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate.

calcium ATPase, they do have some interesting effects on this enzyme. In our laboratory, treatment of the ATPase with an acidic phospholipase A_2 resulted in a 2-fold activation of the enzyme [6]. In this case, phospholipid hydrolysis was less than 1%, so delipidation had not occurred. Others have seen lesser or greater activation [3,7] and inhibition [4]. These differences appear to be related to the extent of phospholipid hydrolysis, with maximal activation taking place when minimal hydrolysis occurs.

At present, the activation and inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase by phospholipases are not well understood. This study continues to explore the effects of phospholipases on the erythrocyte ATPase and extends these efforts to another calcium ATPase found in rabbit platelet microsomes. Three highly purified phospholipases A_2 from the Japanese mamushi *Agkistrodon halys blomhoffii* have been used [12]. They are referred to as acidic, neutral and basic according to their isoelectric points which are 4.9, 6.9, and 8.7, respectively. In addition, seven phospholipids and calmodulin were tested for their ability to affect the two enzymes.

Materials and Methods

Crude lyophilized venom from *A. halys blomhoffii* was purchased from the Miami Serpentarium (Miami, FL). The acidic (p*I* 4.9), neutral (p*I* 6.9) and basic (p*I* 8.7) phospholipases A_2 were purified by the method of Hanahan et al. [12]. Crystallized bovine serum albumin was from Miles Laboratories, Inc. (Elkhart, IN). *p*-Bromophenacyl bromide was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Polyphosphoinositides were obtained from Sigma Chemical Co. (St. Louis, MO). Phosphatidic acid was from Avanti Polar Lipids (Birmingham, AL). Lysophosphatidic acid was from Serdary Research Laboratories (London, Ontario, Canada). 1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholine and 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine were purchased from Bachem (Torrance, CA). PC and PE were prepared in this laboratory from egg yolk. LysoPC was prepared by phospholipase A_2 treatment of egg PC. Precoated thin-layer chromatography plates (silica-gel) were purchased from Analtech (Newark, DE). Saponin was from Calbiochem (San Diego, CA). All other chemicals and solvents were of the highest analytical grade.

Lipid extraction and analysis. Phospholipids were extracted from erythrocytes and analyzed as reported before [13]. Phospholipids were separated by thin-layer chromatography on precoated silica-gel G plates in a solvent system of chloroform/methanol/ammonia/water (65 : 35 : 2 : 4, v/v). Components were visualized by iodine vapor treatment, and the spots were removed by scraping and assayed for phosphorus by the method of Bartlett [14].

Heat and chemical treatment of phospholipases A_2. Solutions of the purified phospholipases A_2 (i.e., acidic, neutral or basic) containing 1 mg/ml enzyme in 150 mM NaCl/5 mM histidine buffer (pH 7.5) were incubated in a boiling-water bath for various time periods. No lipid substrate or calcium was present during these incubations. Residual enzymatic activity was determined as described previously [13].

In some experiments, purified acidic, neutral and basic phospholipases A_2 from *A. halys blomhoffii* were treated with *p*-bromophenacyl bromide. A 10-fold excess of this chemical was used for each mole of histidine present in the phospholipase A_2. The treatments were carried out at 30 °C for 22 h, after which, the treated phospholipases A_2 were dialyzed extensively against deionized water to remove the unreacted *p*-bromophenacyl bromide. These enzymes did not show any activity when assayed in an ether/methanol system using egg PC as substrate [15] or on human erythrocyte ghost membranes [13].

Erythrocyte membrane preparation. Human blood was withdrawn from the antecubital vein of healthy donors. The plasma and buffy coat were aspirated carefully after centrifugation at $1500 \times g$ for 8 min at 4 °C. This step was repeated three times. Washed erythrocytes were lysed by adding saponin to a 10% cell suspension in 0.155 M NaCl/2 mM EGTA. The final concentration of saponin was 0.1 mg/ml. After stirring for 15 min at room temperature, the cytosol was separated from the membranes using a Millipore Pellicon Cassette System [16,17]. This unit is capable of producing hemoglobin-free erythrocyte membranes from 1 unit of blood in about 1 h by rapid

recirculation of the lysed erythrocytes over 5 sq. ft. of microporous filters. As filtration occurs, more buffer is added to replace the lost volume until the filtrate is clear and membranes are white. Membranes prepared in this way were suspended in 0.155 M NaCl/5 mM histidine (pH 7.6) and concentrated by centrifugation at 27000 × *g* for 1 h at 4°C. After aspiration of the supernatant, the clean white membranes were removed carefully, leaving behind a small pellet of unlysed cells and yellow-colored membranes. The white membranes were diluted with NaCl/histidine (pH 7.6) to one-half the original lysate volume and frozen in aliquots for later use. The protein concentrations of these preparations ranged from 1.2 to 1.5 mg/ml.

Preparation of platelet microsomes. Washed rabbit platelets were prepared as described [18] without addition of tritiated serotonin. Saponin was added to 0.1% and EGTA was added to 2 mM. The platelets were then disrupted by sonication at 4°C in an ultrasonic cell disrupter model W-375 (Heat Systems Ultrasonics, Inc., Plainview, NY) by giving three pulses (power output 100 W) of 15 s each. An interval of 45 s was given between each pulse. Subsequently, the suspension was centrifuged at 800 × *g* for 15 min. The supernatant was recentrifuged at 150000 × *g* at 4°C for 60 min and the microsomal membrane pellet was transferred to a Potter-Elvejhem glass Teflon homogenizer and resuspended by 8–10 strokes of the Teflon pestle. The platelet membranes were exposed to EGTA for about the same length of time as the erythrocyte membranes.

Assay of ATPase activity. The membrane calcium ATPase assay was that described by Hanahan and Ekholm [19]. In a typical enzyme assay, 25–50 μl of a membrane suspension containing 30–60 μg protein was combined with 0.5 ml ATPase cocktail and incubated for 1 h at 44°C. The final concentrations of reagents in the incubation mixture were as follows: 3.6 mM $MgCl_2$, 80 mM NaCl, 33 mM KCl, 0.05 mM $CaCl_2$ and 2.5 mM Na_2ATP buffered with 80 mM histidine (pH 7.6). The reaction was terminated by the addition of 1 ml 0.5% SDS. Color was developed by adding 0.5 ml 2.5% ammonium molybdate in 1.6 M sulfuric acid followed by 0.2 ml 0.25% 1-amino-2-naphthol-4-sulfonic acid, 0.5% sodium sulfite and 14.64% sodium bisulfite. The color was allowed to develop 15 min before the absorbance at 660 nm was determined. Appropriate phosphate standards demonstrated that the absorbance at 660 nm was linear with inorganic phosphate concentration from 0 to 0.5 μmol.

When the effect of acidic, neutral or basic phospholipase A_2 was to be determined, these enzymes were added to the ATPase assay mixture at the concentrations indicated in the figures. 1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine or lysoPC were dissolved in 0.15 M NaCl containing 2.5 mg/ml bovine serum albumin. Polyphosphoinositides, lysophosphatidic acid, PC or PE were dissolved in 5 mM histidine/0.15 M NaCl (pH 7.6) and gently sonicated. Controls without phospholipase contained the same amount (50 μl) of either bovine serum albumin of NaCl/histidine buffer. In the determination of calcium ATPase from platelet microsomes, the assay and incubation mixture were the same as mentioned above, except 1 mM ouabain was present. The amount of platelet microsomal protein ranged fom 30 to 60 μg. The time of incubation for the platelet ATPase was 30 min. Calcium ATPase activity was determined in both cases by subtracting the calcium-independent ATPase activity determined in a separate assay containing 1 mM EGTA instead of calcium.

Results

Effect of phospholipases A_2 on the calcium ATPase of erythrocyte membranes and platelet microsomes

The effect of the three different purified phospholipases A_2 on the $(Ca^{2+} + Mg^{2+})$-ATPase of human erythrocyte membranes was investigated and the data are shown in Fig. 1. All three phospholipases A_2 enhanced ATPase activity by 2–3-fold. For maximum stimulation of the ATPase, a low concentration of the basic enzyme is sufficient. However, for a comparable degree of stimulation, higher concentrations of the neutral enzyme and the acidic enzyme are required. The calcium ATPase from rabbit platelet microsomes is only slightly sensitive to the phospholipases as only a 10–20% increase in the enzymatic activity was observed (Fig. 2). Both ATPases were inhibited when high concentrations of basic phospholipase A_2 were present in the incubation mixture.

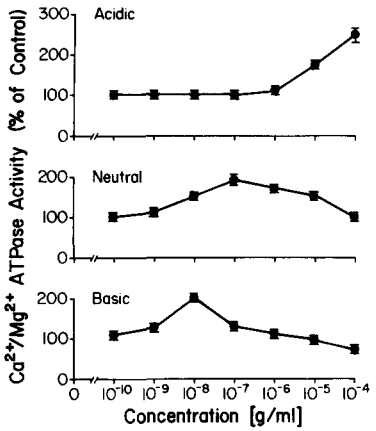

Fig. 1. Stimulation of the human erythrocyte membrane (Ca^{2+} + Mg^{2+})-ATPase by three phospholipases A_2. The final reaction mixture (600 μl) comprised 50 μM $CaCl_2$/3.6 mM $MgCl_2$/80 mM NaCl/33 mM KCl/2.5 mM ATP disodium salt/80 mM histidine buffer (pH 7.6)/35 μg erythrocyte membrane protein. Acidic, neutral and basic phospholipases A_2 were added at indicated concentrations. No preincubation was used. Incubation time was 1 h at 44 °C. Inorganic phosphate was determined as described in Materials and Methods. Values are the mean ± S.D. of three determinations.

Effect of heat-treated and chemically modified phospholipases A_2 on the (Ca^{2+} + Mg^{2+})-ATPase activity of erythrocyte membranes

Heat treatment of acidic, neutral and basic phospholipases A_2, as described in Materials and Methods, greatly reduced their ability to hydrolyze PC in human erythrocyte membranes. After 5 h

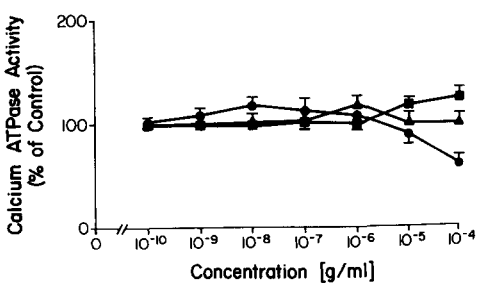

Fig. 2. Effect of acidic, neutral and basic phospholipases A_2 on rabbit platelet calcium ATPase. Conditions were the same as described in Fig. 1, except that 35 μg platelet microsomal protein were used instead of the erythrocyte membrane protein and the incubation time was 30 min at 44 °C. Values are the mean ± S.D. of three determinations. ●——●, Basic phospholipase A_2; ■——■, acidic phospholipase A_2; ▲——▲, neutral phospholipase A_2.

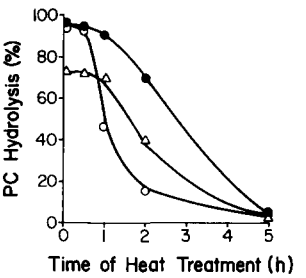

Fig. 3. The ability of heat-treated phospholipases A_2 to hydrolyze phosphatidylcholine of human erythrocyte membranes. Acidic (△——△), neutral (●——●) and basic (○——○) phospholipases A_2 were incubated in a boiling-water bath for different periods of time. These enzymes were then added to erythrocyte ghosts and PC hydrolysis was determined as described in Materials and Methods.

incubation in a boiling-water bath, very little hydrolytic activity remained (Fig. 3). To test whether hydrolytic activity was necessary for the effects on ATPase activity seen in Fig. 1, the heat-treated enzymes were added to erythrocyte ghosts and ATPase activity was measured. Heat treatment generally reduced the ability of the three phos-

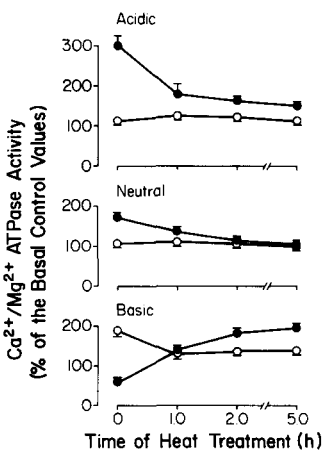

Fig. 4. Effect of heat-treated phospholipases A_2 on human erythrocyte membrane (Ca^{2+} + Mg^{2+})-ATPase. The ATPase assay was the same as in Fig. 1. The phospholipases were inactivated as in Fig. 3. High and low concentrations of each phospholipase were then checked for effects on the ATPase. The acidic enzyme was tested at 100 μg per assay (●——●) or 1.0 μg per assay (○——○). The neutral phospholipase was added at 0.1 μg (○——○) or 1.0 μg (●——●) per assay. The basic enzyme was added at 100 μg (●——●) or 0.01 μg (○——○) per assay. Values are the mean ± S.D. of three determinations.

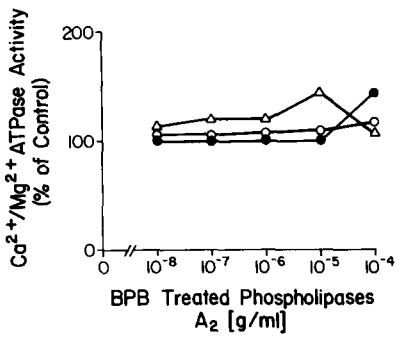

Fig. 5. Effect of *p*-bromophenacyl bromide-inactivated phospholipases A_2 on the $(Ca^{2+} + Mg^{2+})$-ATPase of human erythrocytes. Conditions and incubations were the same as described in Fig. 4, except that the phospholipases were inactivated by chemical modification with *p*-bromophenacyl bromide (BPB) before addition to the incubation mixture. △———△, basic phospholipase A_2; ○———○, neutral phospholipase A_2; ●———●, acidic phospholipase A_2.

pholipases A_2 to stimulate the ATPase, and this reduction was dependent on the time of heat treatment (Fig. 4). The only exception occurred when high concentrations of basic phospholipase A_2 were added. Under these conditions, an increased ATPase activity was observed. This can be atrributed to incomplete inactivation of the enzyme. At these high concentrations, there is enough active phospholipase remaining to cause stimulation. The result is analogous to moving from right to left on the basic phospholipase curve of Fig. 1. Phospholipases A_2 inactivated by *p*-bromophenacyl bromide showed similar effects on the ATPase (Fig. 5). The chemical treatment greatly reduced the ability of the phospholipases A_2 to stimulate the ATPase.

Effect of calmodulin

Platelet and erythrocyte calcium ATPases were assayed as described in the legend to Fig. 1. The platelet enzyme (35 μg microsomal protein) was incubated for only 30 min, since it has a higher specific activity than the erythrocyte enzyme. The erythrocyte ATPase showed a 2-fold increase in activity (from 0.21 to 0.45 μmol ATP hydrolyzed/min per mg protein) in the presence of 10 μg calmodulin. The platelet enzyme showed no change in activity (0.39–0.38 μmol/min per mg) in the presence or absence of calmodulin. The standard

TABLE I

EFFECTS OF PHOSPHOLIPIDS ON HUMAN ERYTHROCYTE $(Ca^{2+} + Mg^{2+})$-ATPase ACTIVITY

The ATPase assay was the same as in Fig. 1. Phospholipids were added as sonicated dispersions at the final concentrations specified. Controls were run without any added phospholipids. Values are the mean ± S.D. of three determinations. AGEPC, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; lysoGEPC, 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine; lysoPA, lysophosphatidic acid; PPI, polyphosphoinositide.

Phospholipid concentration (M)	$(Ca^{2+} + Mg^{2+})$-ATPase activity (μmol P_i/mg protein per h)	% of Control
A. Stimulatory phospholipids		
Control	2.41 ± 0.16	100
PPI		
$3 \cdot 10^{-5}$	2.73 ± 0.10	113
$6 \cdot 10^{-5}$	3.39 ± 0.10	141
$3 \cdot 10^{-4}$	5.56 ± 0.06	231
LysoPA		
$3 \cdot 10^{-5}$	2.94 ± 0.02	122
$6 \cdot 10^{-5}$	3.43 ± 0.04	142
$3 \cdot 10^{-4}$	4.88 ± 0.08	203
B. Inhibitory phospholipids		
Control	2.11 ± 0.28	100
AGEPC		
$3 \cdot 10^{-5}$	2.14 ± 0.10	101
$6 \cdot 10^{-5}$	1.24 ± 0.05	59
$3 \cdot 10^{-4}$	1.08 ± 0.04	52
LysoGEPC		
$3 \cdot 10^{-5}$	2.12 ± 0.04	100
$6 \cdot 10^{-5}$	1.80 ± 0.04	85
$3 \cdot 10^{-4}$	–	0
LysoPC		
$3 \cdot 10^{-5}$	1.73 ± 0.04	82
$6 \cdot 10^{-5}$	0.28 ± 0.07	13
$3 \cdot 10^{-4}$	0.24 ± 0.05	11
C. Inactive phospholipids		
Control	2.41 ± 0.16	100
PC		
$3 \cdot 10^{-5}$	2.37 ± 0.20	98
$6 \cdot 10^{-5}$	2.54 ± 0.09	105
$3 \cdot 10^{-4}$	2.52 ± 0.03	105
PE		
$3 \cdot 10^{-5}$	2.48 ± 0.04	103
$6 \cdot 10^{-5}$	2.57 ± 0.08	107
$3 \cdot 10^{-4}$	2.39 ± 0.12	99

deviations for four determinations were between ± 0.03 and ± 0.04 μmol/min per mg.

Both membrane preparations were treated with EGTA to remove endogenous calmodulin. The platelet membranes were sonicated in the presence of 2 mM EGTA as described in Materials and Methods. Membrane-bound calmodulin, which remains even after EGTA washing, is fairly easily removed from brain microsomes by this procedure [20]. As a control, platelets were sonicated in the absence of EGTA and the results were the same. Calmodulin did not stimulate the platelet calcium ATPase.

TABLE II

EFFECTS OF PHOSPHOLIPIDS ON THE CALCIUM ATPase ACTIVITY OF RABBIT PLATELETS

Conditions were the same in Table I, except that 35 μg platelet microsomal protein were used and the incubation time was 30 min at 44 °C. Values are the mean ± S.D. of three determinations. Abbreviations are the same as in Table I.

Phospholipid concentration (M)	Calcium ATPase activity (μmol P_i/mg protein per h)	% of Control
A. Stimulatory phospholipids		
Control	4.35 ± 0.49	100
PPI		
$3 \cdot 10^{-5}$	5.15 ± 0.08	118
$6 \cdot 10^{-5}$	5.93 ± 0.05	136
$3 \cdot 10^{-4}$	6.91 ± 0.19	159
B. Inhibitory phospholipids		
Control	4.55 ± 0.15	100
AGEPC		
$3 \cdot 10^{-5}$	4.32 ± 0.24	95
$6 \cdot 10^{-5}$	4.63 ± 0.02	102
$3 \cdot 10^{-4}$	2.95 ± 0.10	65
LysoGEPC		
$3 \cdot 10^{-5}$	4.88 ± 0.09	107
$6 \cdot 10^{-5}$	4.61 ± 0.16	101
$3 \cdot 10^{-4}$	1.44 ± 0.09	32
LysoPC		
$3 \cdot 10^{-5}$	4.81 ± 0.14	106
$6 \cdot 10^{-5}$	3.75 ± 0.40	82
$3 \cdot 10^{-4}$	1.31 ± 0.10	29
C. Inactive phospholipids		
Control	4.35 ± 0.49	100
PC		
$3 \cdot 10^{-5}$	4.11 ± 0.14	95
$6 \cdot 10^{-5}$	4.35 ± 0.08	100
$3 \cdot 10^{-4}$	4.32 ± 0.29	99
PE		
$3 \cdot 10^{-5}$	4.31 ± 0.20	99
$6 \cdot 10^{-5}$	4.34 ± 0.17	100
$3 \cdot 10^{-4}$	4.33 ± 0.53	100
LysoPA		
$3 \cdot 10^{-5}$	4.10 ± 0.19	94
$6 \cdot 10^{-5}$	4.27 ± 0.11	98
$3 \cdot 10^{-4}$	4.16 ± 0.20	96

Effect of various phospholipids on the calcium ATPases of erythrocyte membranes and platelet microsomes

The effect of various lipids on the calcium ATPase of erythrocyte membranes and platelet microsomes was investigated. Based on their modulatory effects, the phospholipids were grouped into three different catagories: stimulatory, inactive or inhibitory. Tables IA and IIA give data on phospholipids in the first category. Samples of polyphosphoinositides (a mixture of PI, PI-4-phosphate and PI-4,5-bisphosphate) markedly increased the activity of both ATPases. Lysophosphatidic acid enhanced the erythrocyte membrane ATPase activity but not platelet ATPase activity. The inactive group included PC and PE. They caused no change in the ATPase activity at either low or high concentrations (Tables IC and IIC). The third group which was inhibitory, included 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine and lysoPC, all of which markedly decreased the activity of both ATPases. The inhibition of the ATPase depended upon the applied concentrations of these phospholipids (Tables IB and IIB).

Discussion

Previously, our laboratory has purified three phospholipases A_2 from the same source [12] and examined their behavior toward erythrocyte membranes [13]. These phospholipases displayed specific preferences for different phospholipid substrates in human erythrocytes. They also differed in their ability to cause hemolysis. These properties made them attractive as tools to probe membrane enzyme activities, especially similar activities in different membranes, Here, we have applied them to human erythrocyte (Ca^{2+} + Mg^{2+})-

ATPase and a calcium ATPase from rabbit platelets. Appropriate concentrations of all three phospholipases stimulated ATPase activity and it appeared that this stimulation was dependent on hydrolysis of phospholipids. The addition of exogenous phospholipids also had clear effects on these ATPases. Using the effects of these treatments as a measure of comparison, we find that there is a sharp distinction between the protein-lipid interactions of the rabbit platelet and erythrocyte calcium ATPases.

Acidic, neutral and basic phospholipases A_2 each activated the erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase about 2-fold, but the required concentration varied. This is probably due to differences in the rate of phospholipid hydrolysis, with the basic enzyme being fastest, followed by neutral and acidic, evidence for which has been reported previously [13]. Of course, this assumes that hydrolysis of phospholipids is the cause of the activation, but this is not obvious, since the acidic enzyme caused activation with less than 1% phospholipid hydrolysis [6].

To test this assumption, the phospholipases A_2 were inactivated by heat treatment or chemical modification with *p*-bromophenacyl bromide. Their ability to stimulate the erythrocyte ATPase was reduced and this effect was proportional to the extent of inactivation. Therefore, phospholipid hydrolysis is indeed required for ATPase stimulation.

Based on this result, Fig. 1 may be interpreted in terms of phospholipid hydrolysis. Only a narrow concentration range of phospholipase resulted in ATPase activation. If the activation is caused by phospholipid hydrolysis products, then their concentration is critical. Below this sensitive range, the concentration is too low to cause activation, above it the activation is decreased and in the case of the basic phospholipase A_2, which is the most active phospholipase, inhibition is observed. The peaks of activation appear at different phospholipase concentrations, because the enzymes work at different rates [13]. Such an interpretation would help to explain the widely differing results seen by other investigators.

Ronner et al. [3] saw an initial activation of erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase when ghosts were treated with small amounts of phospholipase A_2 from *Naja naja*. This initial rise was followed by a slow decrease in ATPase activity at higher phospholipase concentrations. The presence of bovine serum albumin in the incubations removed hydrolysis products from the membrane and caused a rapid loss of ATPase activity. Roelofsen and Schatzmann [4] noted a large decrease in ATPase activity when ghosts were exposed to *Naja naja* or porcine pancreas phospholipase A_2. In contrast, Schmalzing and Kutschera [7] observed a large increase in activity while using the *Naja naja* enzyme. We feel these results illustrate a biphasic response to phospholipase treatment. Small amounts of phospholipid hydrolysis result in activation, while more extensive digestion of the membrane lipids causes loss of ATPase activity.

This biphasic response of the $(Ca^{2+} + Mg^{2+})$-ATPase is not restricted to phospholipase A_2 treatment. A similar effect has been noted with oleic acid [7,10,21] and SDS [21]. Together, these results suggest that constraints are imposed on the ATPase by the lipid bilayer, and they are relaxed by limited phospholipid hydrolysis or addition of low levels of oleic acid or SDS. Continued phospholipid hydrolysis or addition of larger amounts of oleic acid or SDS result in loss of activity, perhaps due to denaturation of the ATPase or displacement of required lipids.

The calcium ATPase of rabbit platelet microsomes was also activated by the three phospholipases, but the maximal activation was only about 20%, as compared to 100–150% for the erythrocyte enzyme. This indicates a basic difference between the protein-lipid interactions of the two ATPases. This difference is not surprising, since the platelet enzyme is quite similar to the calcium ATPase of sarcoplasmic reticulum, while the erythrocyte enzyme is not. The sarcoplasmic reticulum and erythrocyte enzymes differ in molecular weight and regulation [22–24]. Antibodies against the sarcoplasmic reticulum ATPase crossreact with the platelet enzyme [25], but not with the erythrocyte enzyme [26]. In addition, antibodies against the erythrocyte ATPase do not crossreact with the sarcoplasmic reticulum enzyme, but they do with proteins from several plasma membrane preparations [26]. This lack of similarity between the platelet and erythrocyte ATPases implies that the platelet enzyme is not a plasma membrane calcium

pump. Rather, it is likely to be a protein of the dense tubular system of the platelet, a system that has been compared to the sarcoplasmic reticulum of muscle [25,27].

Calmodulin had no effect on the calcium ATPase of rabbit platelets. This differs from the human platelet enzyme which was stimulated 1.6-fold by calmodulin [25]. The failure of rabbit platelet calcium ATPase to respond to calmodulin remains unexplained.

When exogenous phospholipids were added to ghosts or platelet micrsomes without delipidation by phospholipase treatment, effects on ATPase were seen. The acidic phospholipids, lysophosphatidic acid and polyphosphoinositides stimulated the erythrocyte ATPase, while only the polyphosphoinositides had any stimulatory effect on the platelet enzyme. Several phospholipids related to 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet-activating factor) inhibited the two ATPases. This was probably due to detergent-like effects, since the concentrations used were above the compounds' critical micellar concentrations, and lysophospholipids can act as detergents.

The results from this study help clarify previously conflicting reports about the effects of phospholipases on the human erythrocyte (Ca^{2+} + Mg^{2+})-ATPase. Activation is probably caused by limited phospholipid hydrolysis, probably less than 1% overall. This fits a general scheme in which a very sensitive (Ca^{2+} + Mg^{2+})-ATPase responds to slight changes in its environment. These changes can be brought about by phospholipases or addition of membrane perturbants such as oleic acid, SDS or phospholipids. Acidic phospholipids activate, including the previously untested lysophosphatidic acid. PC and PE have no effect as seen by Ronner et al. [3], but not Roelofsen and Schatzmann [4], while lysoPC, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine and 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine inhibit, probably through a nonspecific physical effect. These changes do not reflect lipid requirements, but only a response to changes in local environment around the (Ca^{2+} + Mg^{2+})-ATPase.

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