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THE PHOSPHOLIPID REQUIREMENT OF THE (Ca^{2+} + Mg^{2+})-ATPase FROM HUMAN PLATELETS

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The phospholipid requirement of the (Ca^{2+} + Mg^{2+})-ATPase present in a membrane fraction from human platelets was studied using various purified phospholipases. Only those phospholipases, which hydrolyse the negatively charged phospholipids, inhibited the (Ca^{2+} + Mg^{2+})-ATPase activity. The ATPase activity could be restored by adding mixed micelles of Triton X-100 and phosphatidylserine or phosphatidylinositol. Micelles with phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine or sphingomyelin could not be used for reconstitution and inhibited the activity of the native enzyme.

Introduction

Platelets are anucleated blood cells which play an important role in the formation of a hemostatic plug and in the coagulation process. They can undergo a number of reactions such as adhesion to various substances, shape change, the release of their granule content and aggregation. Calcium ions play probably a key role in the regulation of platelet function [1,2]. It is demonstrated that a Ca^{2+} ionophore can induce the release of stored substances from the granules, which may result in aggregation [3].

Several membrane fractions from platelets are described which have a Ca^{2+} transport activity similar to that of muscle sarcoplasmic reticulum [4–8] and which exert (Ca^{2+} + Mg^{2+})-ATPase activity [5,8,9]. The (Ca^{2+} + Mg^{2+})-ATPase activity [9] and the Ca^{2+}-uptake [7] is probably to a large extent effected by membranes from the dense tubular system, an intracellular compartment, which ressembles the sarcoplasmic reticulum of muscle [9,10].

(Ca^{2+} + Mg^{2+}-ATPases from sarcoplasmic reticulum or red blood cells require lipids for the maintenance of enzyme activity. Various phospholipids [11] or detergents [12] can be used as lipid source for the enzyme of sarcoplasmic reticulum.

It is known, especially from the work with purified (Ca^{2+} + Mg^{2+})-ATPase from red blood cells, that the phospholipid environment can also be crucial for the action of calmodulin and other activators [13]. Niggli et al. [14] have shown that acidic phospholipids and unsaturated fatty acids can mimic the effect of calmodulin on the purified enzyme. Therefore we decided to investigate the effect of phospholipids on the (Ca^{2+} + Mg^{2+})-ATPase of human platelets. For this purpose we used a membrane fraction, which is already used to study the Ca^{2+} uptake [8], and purified phos-

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PA, phosphatidic acid; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; EGTA, ethyleneglycolbis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

pholipases, which differ in their mode of action towards different phospholipids [15,16].

Materials and Methods

Materials

Buffer A: 100 mM KCl/20 mM Hepes-KOH (pH 8.0). Hepes, Triton X-100, dithiothreitol, bovine albumin (essentially fatty acid free) polyvinyl alcohol, malachite green, ATP (sodium salt) and phospholipids were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were obtained from Merck (Darmstadt, F.R.G.). Phospholipase A_2 from *Naja naja*, phospholipase C from *Bacillus cereus*, phospholipase C from *Clostridium welchii* and sphingomyelinase C from *Staphylococcus aureus* were purified according to Zwaal et al. [17].

Preparation of platelet membranes

Blood was drawn from the antecubital vein of donors. 9 volumes of whole blood were collected in 1 volume of anticoagulant (22 mM citric acid/103 mM trisodium citrate/22 mM NaH_2PO_4/177 mM glucose). The preparation of washed platelets by differential ultracentrifugation, the lysis by ultrasonication and the isolation of the membrane fraction by ultracentrifugation were exactly performed as described earlier [8]. The final membrane pellet was suspended in buffer A and stored at −80°C. The protein concentration was determined according to Bradford [18]. The membrane fraction was characterized as described by Le Peuch et al. [8]. The platelet membranes/whole platelet lysate average ratio was 1.44 for the plasma membrane marker enzyme phosphodiesterase, 0.9 for the lysosomal marker enzyme β-glucuronidase and 0.12 for the cytoplasmic marker enzyme lactate dehydrogenase. The specific radioactivity of ^{125}I-iodinated plasma membrane proteins was 1.62 relative to whole platelets. Furthermore the platelet membranes/whole platelet lysate average ratio for the dense granule marker serotonin was 0.32 and for the α-granule marker β-thromboglobulin 0.58. Serotonin was determined with preparations preloaded for 30 min with ^{14}C-labeled serotonin (CEA, Saclay, France) according to Rendu et al. [19] and β-thromboglobulin with the β-thromboglobulin RIA kit (Amersham, U.K.). The specific activity of the endoplasmic reticulum marker enzyme, the antimycin insensitive NADH-cytochrome *c* reductase, was 9.0 relative to whole platelets and was assayed as described by Tolbert [20].

Measurement of the ATPase activity

Unless indicated otherwise, the ATPase activity was measured by incubating 0.01 ml of membranes, containing 10 μg of protein, in a final reaction mixture of 0.2 ml, containing buffer A, 2 mM $MgCl_2$, 0.5 ml $CaCl_2$, 0.5 mM EGTA and 0.001% Triton X-100. EGTA was not added when the membrane suspension contained 10 mM EGTA (after phospholipase treatment). The reaction was started by adding 0.2 mM ATP and the mixtures were incubated for 15 min at 37°C. The reaction was stopped with 2 ml malachite green reagent, used for the colorimetric determination of P_i [21]. The Mg^{2+}-ATPase activity was calculated by substracting the values obtained in the absence of $MgCl_2$, from those obtained in the presence of $MgCl_2$, without adding $CaCl_2$. The (Ca^{2+} + Mg^{2+})-ATPase activity was calculated by substracting the values obtained without adding $CaCl_2$ from those obtained with added $CaCl_2$. Results are expressed as nmoles P_i liberated per mg protein per min. Free calcium concentrations were calculated as reported by Lotersztajn et al. [22] using apparent association constants of $6.03 \cdot 10^8$, $9.7 \cdot 10^2$, $9.55 \cdot 10^3$ and $4.57 \cdot 10^4$ M^{-1} for CaEGTA, MgEGTA, CaATP and MgATP, respectively. At the used pH 8.0 only the binding of Ca^{2+} and Mg^{2+} by the ligands with four negative charges was considered. The free calcium concentration for the standard assay medium was $0.9 \cdot 10^{-6}$ M.

Phospholipase treatment

The various phospholipases were added to the membranes, containing 1 mg of protein per ml, in buffer A with 5 mM $CaCl_2$ and 0.01% Triton X-100. After incubation for 30 min at 37°C 10 mM EGTA was added. The samples were dialyzed against buffer A with 10 mM EGTA at 4°C for 30 min in a MicroProdicon negative pressure dialysis concentrator (Bio-Molecular Dynamics, Oregon, OR, U.S.A.) using dialysis tubes with a diameter of 0.6 cm (Medicell international Ltd., London,

U.K.). The samples were kept on ice before measurement of the ATPase activity.

Phospholipid extraction from platelet membranes and phospholipid analysis

The platelet membranes were suspended in buffer A with 20 mM EDTA and the phospholipids were extracted according to the method of Bligh and Digher [23]. Phospholipid analysis was carried out with two-dimensional thin-layer chromatography as described by Verkley et al. [24].

Reconstitution experiments

Phospholipids were added to the various mixtures as micelles from lipids and Triton X-100 in a 1 : 2 ratio (w/w). The micelles were prepared by evaporating the solvent from the phospholipid and resuspending the phospholipid in buffer A and Triton X-100. When phospholipid was suspended without Triton X-100, the suspension was sonicated with a Branson sonifier B12 at 50 watt, five times for 1 min.

Results

Characterization of the ATPase activity

Two distinct ATPase activities are present in the platelet membrane fraction: a Mg^{2+}-ATPase and a (Ca^{2+} + Mg^{2+})-ATPase. Under our reaction conditions a (Na^+ + K^+)-ATPase or a mitochondrial ATPase activity could not be detected.

A time-course of the ATPase activity is shown in Fig. 1. At 37°C the reaction rate was proportional with the protein concentration and linear for at least 15 min, when concentrations below 100 μg protein per ml were used. Extra ATP had to be included in the reaction mixture to prevent exhaustion of ATP. The decrease in activity with time (Fig. 1) can be noticed when about 50% of the ATP is hydrolyzed.

The optimal reaction conditions for the (Ca^{2+} + Mg^{2+})-ATPase activity were as follows: pH 7.0–8.0, [KCl] 0–0.2 M and 0.1–2 mg Triton X-100 per mg protein. The stimulation by Triton X-100 was about 2-fold. Higher concentrations inhibited the activity. Addition of reducing agents (dithiothreitol, mercaptoethanol) did not affect the reaction. The K_m values of Mg^{2+} and ATP were 0.08 and 0.01 mM, respectively. The Ca^{2+} dependence is shown in Fig. 2. The stimulation of the ATPase activity was maximal with 0.01–0.1 μM Ca^{2+}. Higher Ca^{2+} concentrations were inhibitory. This inhibition was less pronounced, when more

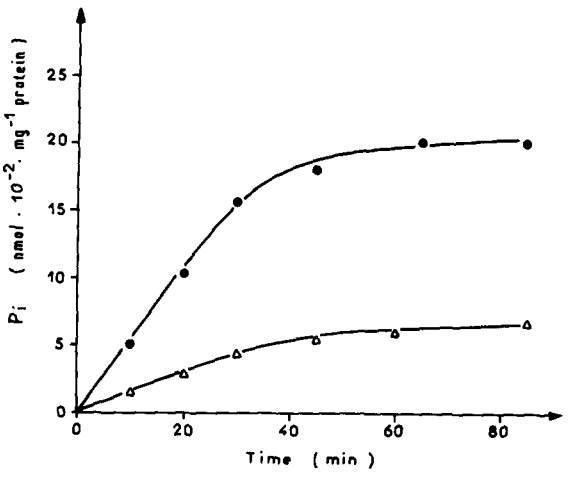

Fig. 1. The (Ca^{2+} + Mg^{2+})-ATPase (●) and Mg^{2+}-ATPase activity (△) in platelet membranes. Membranes, containing 10 μg of protein, were incubated and the amount of P_i was measured after various incubation times. Experimental conditions are given in Materials and Methods.

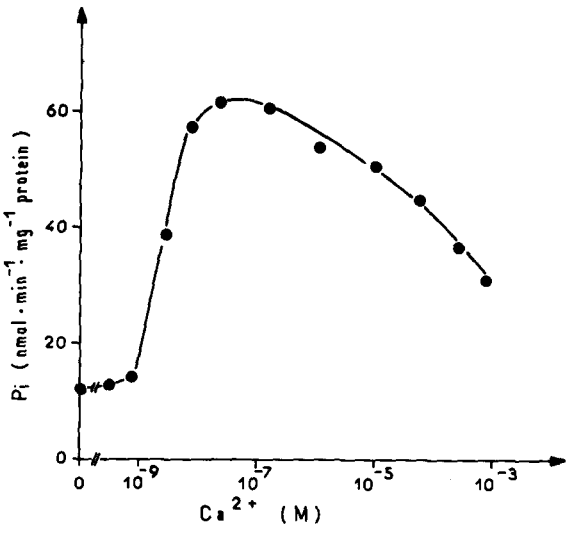

Fig. 2. The effect of Ca^{2+} on the ATPase activity in platelet membranes. Various amounts of $CaCl_2$ were added to the reaction mixture which contained 0.5 mM EGTA. The free Ca^{2+} concentration was calculated as described in Materials and Methods and is plotted against the ATPase activity. Experimental conditions are also given in Materials and Methods.

TABLE I

THE EFFECT OF PHOSPHOLIPASES ON THE (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY

Hydrolysis of phospholipids was performed with 5 I.U of the various phospholipases per mg membrane protein and ATPase activity was assayed after hydrolysis as described in Materials and Methods. Bovine albumin was added, if indicated, at a concentration of 2 mg/ml. Data are the mean of at least two experiments with individual preparations. The number in parentheses refers to the number of experiments from which the S.D. has been calculated. More data on the phospholipid composition are given in Table II.

Phospholipase added	(Ca^{2+} + Mg^{2+})-ATPase activity (nmol $P_i \cdot min^{-1} \cdot mg^{-1}$)	Phospholipid/protein (mg/ml)
None	60 ± 13 (n = 14)	1.0
A_2 *Naja naja*	50 ± 17 (n = 4)	0.23
C *B. cereus*	5 ± 6 (n = 4)	0.27
C *Cl. welchii*	58 ± 11 (n = 4)	0.25
Sphingomyelinase	51	0.8
A_2 *Naja naja* + bovine albumin	0	0.23
C *Cl. welchii* + bovine albumin	60	0.22

Mg^{2+} was included in the reaction mixture (data not shown).

The effect of various phospholipases on the (Ca^{2+} + Mg^{2+})-ATPase activity

Various phospholipases were assayed for their effect on the (Ca^{2+} + Mg^{2+})-ATPase activity (Table I). Sphingomyelinase and phospholipase C from *Cl. welchii* were not affecting the activity, whereas phospholipase C from *B. cereus* was inhibiting strongly. Phospholipase A_2 from *Naja naja* either stimulated, did not affect or inhibited the ATPase activity depending on the reaction conditions. Without Triton X-100 the activity was stimulated (not shown). This stimulation could be mimicked by adding Triton X-100 and was absent when Triton X-100 was included in the reaction mixture. In the presence of bovine serum albumin which binds free fatty acids and lysophospholipids, the split-products of phospholipase A_2, a complete inhibition with phospholipase A_2 was effected. The phospholipid content before and after hydrolysis with phospholipases was also determined (Table II). It seems that the presence of PS and/or PI is essential for the preservation of Ca^{2+}-ATPase activity. Only when those phospholipids were degraded, the ATPase activity was diminished. The split-products of phospholipase A_2 are also preserving ATPase activity. Furthermore, the ATPase activity seems proportional with the content of negatively charged phospholipid as could be demonstrated by degradation with different amounts of phospholipase C from *B. cereus* (Table III).

The effect of various lipids on the (Ca^{2+} + Mg^{2+})-ATPase activity

The effect of lipids on the (Ca^{2+} + Mg^{2+})-ATPase activity was assayed by the addition of mixed micelles of Triton X-100 and lipids to the membrane fraction or by the reconstitution with

TABLE II

THE PHOSPHOLIPID CONTENT AFTER HYDROLYSIS WITH PHOSPHOLIPASES

After hydrolysis of the membrane fraction (2 mg protein) with the various phospholipases, as described in the legend to Table I, phospholipids were extracted and analysed as described under Materials and Methods. Data with phospholipase A_2 and sphingomyelinase are the mean of two experiments with individual preparations. Other data are expressed as mean values ± S.D. The number in parentheses refer to the number of experiments performed with individual membrane preparations. SPH, sphingomyelin.

Phospholipase added	Phospholipid (mg/mg protein)						
	PC	SPH	PS	PI	PE	LysoPC	LysoPE
None (n = 7)	0.36 ± 0.03	0.2 ± 0.03	0.09 ± 0.02	0.03 ± 0.02	0.26 ± 0.02	0	0
A_2 *N. naja*	0	0.2	0	0	0	0.3	0.24
C *B. cereus* (n = 4)	0.02 ± 0.04	0.18 ± 0.02	0.01 ± 0.01	0.0	0.04 ± 0.04	0	0
C *Cl. welchii* (n = 4)	0.04 ± 0.02	0.02 ± 0.06	0.09 ± 0.01	0.04 ± 0.03	0.05 ± 0.02	0	0
Sphingomyelinase	0.3	0.01	0.1	0.03	0.26	0	0

TABLE III

THE $(Ca^{2+} + Mg^{2+})$-ATPase ACTIVITY AND PHOSPHOLIPID CONTENT AFTER HYDROLYSIS WITH VARIOUS AMOUNTS OF PHOSPHOLIPASE C FROM *B. CEREUS*

The phospholipids were analysed as described in the legend to Table II. The ATPase activity was assayed as described in Materials and Methods. The data are the results of a typical experiment. SPH, sphingomyelin.

Phospholipase C *B. cereus* added (I.U. per mg protein)	Phospholipid (mg/mg protein)			$(Ca^{2+} + Mg^{2+})$-ATPase activity (nmol $P_i \cdot min^{-1} \cdot mg^{-1}$)
	SPH	PC + PE	PS + PI	
None	0.19	0.66	0.13	62
0.05	0.20	0.41	0.11	43
0.2	0.17	0.11	0.09	40
1	0.24	0	0.07	20
5	0.20	0	0.02	0
25	0.19	0	0.01	0

lipids of a membrane fraction, depleted of phospholipid with phospholipase C from *B. cereus* (Table IV). Triton X-100 or mixed micelles of Triton X-100 and PC, PE or sphingomyelin inhibited the activity almost completely. Mixed micelles with PA inhibited slightly, whereas mixed micelles with PS or PI stimulated the activity. When we added lipids to a preparation depleted of phospholipids, the ATPase could only be restored completely with PS or PI. The restoration was less pronounced, when more than 2 I.U of phospholipase C from *B. cereus* was used to degrade the phospholipids (not shown). A phospholipid extract of the membrane fraction was less effective in the reconstitution experiments than micelles from pure negatively charged phospholipids. Various free fatty acids ($C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$) were also tested for their effect on the ATPase activity (not shown). They hardly affected the ATPase activity of the native enzyme. On the other hand, like PA, they were not active in restoring the enzyme activity after hydrolysis with phospholipase C from *B. cereus*.

TABLE IV

THE EFFECT OF VARIOUS PHOSPHOLIPIDS ON THE $(Ca^{2+} + Mg^{2+})$-ATPase ACTIVITY

The membrane fraction was depleted from phospholipid with phospholipase C from *B. cereus* (2 I.U. per mg membrane protein). Triton X-100 (1 mg/ml) or Triton X-100 (1 mg/ml) plus phospholipids (0.5 mg/ml) were added to the reaction mixture containing 0.1 mg protein per ml. Experimental conditions are given in Materials and Methods. Data are mean values ± S.D. The numbers in parentheses refer to the number of experiments with individual preparations. SPH, sphingomyelin.

Phospholipids added	$(Ca^{2+} + Mg^{2+})$-ATPase activity (nmol $P_i \cdot min^{-1} \cdot mg^{-1}$) in:	
	Native enzyme ($n = 6$)	Phospholipid-depleted enzyme ($n = 4$)
None	64 ± 12	19 ± 7
Crude extract of platelet membranes	51 ± 18	42 ± 17
None plus Triton X-100, PC or SPH	0	0
PE	14 ± 6	6 ± 8
PS	117 ± 37	109 ± 24
PI	96 ± 22	101 ± 23
PA	50 ± 38	23 ± 20

The phospholipid requirement of the Mg^{2+}-ATPase activity

It is not unlikely that at least part of the Mg^{2+}-ATPase activity might be affected by the same enzyme as the $(Ca^{2+} + Mg^{2+})$-ATPase activity (see Discussion). Therefore we also investigated the phospholipid requirement of the Mg^{2+}-ATPase activity. It resulted that about 50% of this activity could be inhibited with phospholipase C from *B. cereus*, whereas phospholipase C from *Cl. welchii* had no effect. This result suggests the importance of negatively charged phospholipids. When 2 I.U. of phospholipase C from *B. cereus* were used to degrade the phospholipids of a membrane fraction, containing 1 mg of protein, 40% of the

Mg^{2+}-ATPase activity could be inhibited. Restoration of activity was possible with mixed micelles with PS or PI and Triton X-100.

Discussion

The characteristics of the $(Ca^{2+} + Mg^{2+})$-ATPase from platelet membranes are similar to those of the $(Ca^{2+} + Mg^{2+})$-ATPase from sarcoplasmic reticulum and it is demonstrated that antibodies raised against highly purified ATPase from sarcoplasmic reticulum of rabbit muscle cross-reacts with the ATPase of platelet membranes [9]. We have measured the influence of the various reaction components on the ATPase activity, and the apparent K_m values of ATP and Mg^{2+} were similar to those given by Javors et al. [6] for the Ca^{2+} uptake by platelet membrane vesicles. A low affinity binding site for ATP, which is inferred from the kinetics of activity [6,9], could not be demonstrated by us in the presence of Triton X-100. The absence of low-affinity binding of ATP in the presence of detergent is also known from the $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum [25]. The maximal stimulation by Ca^{2+} was already effected at a concentration of 0.01 μM, whereas an apparent K_m value of about 0.1 μM is reported for the Ca^{2+}-uptake [6] and the $(Ca^{2+} + Mg^{2+})$-ATPase activity [9]. This discrepancy in the effect of Ca^{2+} might be explained by the inhibition by Ca^{2+} beyond the maximal activation at 10^{-7} M Ca^{2+} (Fig. 2), which we found in our preparations. The inhibition by Ca^{2+}, which is antagonized by Mg^{2+}, is known from other $(Ca^{2+} + Mg^{2+})$-ATPases and may have implications for the reaction mechanism [26,27]. The influence of Ca^{2+} on the ATPase activity is usually measured in a medium with EGTA and calculated for free Ca^{2+}. It is however not unequivocally established that the Ca-EGTA complex does not have any activity towards the ATPase. It is shown by Berman [28] that the $(Ca^{2+} + Mg^{2+})$-ATPase from the sarcoplasmic reticulum of rabbit muscle is most probably active with CaEGTA. A half maximal stimulation for the Ca^{2+} transport is observed at 19 μM CaEGTA. A possible activity of CaEGTA towards the platelet ATPase might explain the residual ATPase activity, which we found without adding Ca^{2+} and which was also dependent on negatively charged phospholipid. This Mg^{2+}-ATPase activity might be ATPase activity with the complexed Ca^{2+}, which is always present in our buffers (about 5 μM).

Analysis of the phospholipid content of the membrane fraction showed that it contains about 1 mg phospholipid per mg protein. We studied the action of various phospholipases and demonstrated clearly that the neutral phospholipids are not essential for $(Ca^{2+} + Mg^{2+})$-ATPase activity. Sphingomyelinase, which degrades only sphingomyelin, and phospholipase C from *Cl. welchii* which degrades PC, PE and sphingomyelin, did not affect the ATPase activity. The neutral phospholipids were neither effective in restoring the activity after addition of Triton X-100 or after treatment of the enzyme with phospholipase C from *B. cereus* (restoration of activity could only be accomplished with the negatively charged PS or PI). On the other hand phospholipase C from *B. cereus* and phospholipase A_2, which also degrade PS and PI, were potent inhibitors of the ATPase activity. The inhibition by phospholipase C from *B. cereus* could already be demonstrated, when only a small amount of lipid was degraded. This might be an indication for a minimal amount of negatively charged lipid in the environment of the ATPase. It is not likely that the diacylglycerols formed after hydrolysis with phospholipase C from *B. cereus* cause the inhibition, as no inhibition was observed after hydrolysis with phospholipase C from *Cl. welchii*. We conclude that phospholipid is essential for $(Ca^{2+} + Mg^{2+})$-ATPase activity and that micelles with PS or PI can be used as lipid source. The activation of a $(Ca^{2+} + Mg^{2+})$-ATPase by acidic phospholipids is known from the work with red blood cells [13]. The presence of acidic phospholipids is, however, not important for the $(Ca^{2+} + Mg^{2+})$-ATPase activity from sarcoplasmic reticulum; detergents, PC or other neutral phospholipids, on the other hand, are required for activity [11,12]. Until now the different phospholipid requirement seems the only major difference between the $(Ca^{2+} + Mg^{2+})$-ATPase from sarcoplasmic reticulum and platelet membranes.

Since we know the lipid dependency of the $(Ca^{2+} + Mg^{2+})$-ATPase, solubilization of the membrane fraction to achieve a further purification and a subsequent reconstitution of enzyme activity

is feasible. Furthermore a manipulation of the lipid environment might be useful to study the regulation of the $(Ca^{2+} + Mg^{2+})$-ATPase from platelets, as it is known from the $(Ca^{2+} + Mg^{2+})$-ATPase from red blood cells that the effect of various activators, like calmodulin, is dependent on the lipid composition of the enzyme preparation [13].

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