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Stimulation of the catalytic cycle of the Ca^{2+} pump of porcine plasma-membranes by negatively charged phospholipids

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The $(Ca^{2+} + Mg^{2+})$-ATPase of the plasma membrane is activated by negatively charged phospholipids. The mechanism of this activation was investigated by studying the effect of negatively charged phospholipids on the steady-state phosphointermediate level and on the *p*-nitrophenylphosphatase activity. Both parameters were differentially affected by different acidic phospholipids. The level of phosphoprotein intermediate was not affected by phosphatidylserine (20% of total phospholipid), but it was increased by 60% by phosphatidylinositol 4-phosphate. Phosphatidylserine increased the *p*-nitrophenylphosphatase activity, whereas phosphatidylinositol 4-phosphate had no significant effect. It is suggested that phosphatidylinositol 4-phosphate mainly affects a reaction step which leads to accelerated formation of the phosphointermediate, whereas the action of phosphatidylserine would affect two reaction steps, one upstream and one downstream of the phosphointermediate.

Introduction

The Ca^{2+}-transporting ATPase of the plasma membrane is an enzyme that extrudes Ca^{2+} out of the cell against a steep electrochemical gradient. It belongs to the so called P-type ATPases, a category of transport enzymes which form a phosphorylated intermediate during the catalytic cycle. The transport reaction is accompanied by a transition between two major conformational states.

A number of studies have shown that the plasma-membrane Ca^{2+}-transporting ATPase can be activated by the Ca^{2+}-calmodulin complex, by self association of ATPase molecules, by partial proteolysis and by negatively charged phospholipids (see Ref. 1 for review). However, little is known on how these agents activate the Ca^{2+} pump. Enyedi et al. [2] postulated the existence of two regulatory domains, one responsible for calmodulin activation and the other one mediating the effect of negatively charged phospholipids. Missiaen et al. [3] have made a detailed analysis of the effect of various negatively charged phospholipids on such kinetic parameters as V_{max}, affinity for Ca^{2+} and Hill coefficient of the Ca^{2+}-stimulated ATPase activity of the purified Ca^{2+} pump from erythrocytes and from smooth-muscle cells. A correlation was found between the number of negative charges on the phospholipid molecules ($PIP_2 > PIP > PI \approx PA \approx PS$) and their potency in stimulating the ATPase activity [3]. The importance of the number of negative charges was more directly established by using fluorescence energy transfer as a measure of molecular interaction between phosphoinositides and the ATPase protein [4]. Wrzosek et al. [5] concluded from circular dichroism and fluorescence measurements that PS induces a change in secondary structure of the ATPase, mainly in its α-helical content. One possible mechanism of action was suggested by Rossi and Rega [6], who observed that PS, as well as calmodulin or partial proteolysis, increases the affinity for ATP at a low-affinity, regulatory site.

In the present paper we have studied the effect of negatively charged phospholipids on the steady-state level of the phosphointermediate and on the *p*-

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Abbreviations: $(Ca^{2+} + Mg^{2+})$-ATPase, $(Ca^{2+} + Mg^{2+})$-activated ATPase; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid; pNPP, *p*-nitrophenyl phosphate; DMSO, dimethylsulfoxide; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EP, phosphointermediate.

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nitrophenylphosphatase (pNPPase) activity, an enzymatic reaction catalysed by the Ca^{2+}-transporting ATPase but differing from the ATPase reaction in several characteristics. These results indicate that at least two reaction steps are involved in the activation of the purified erythrocyte Ca^{2+} pump by negatively charged phospholipids. These reaction steps are differentially affected depending on the phospholipid species resulting either in an acceleration of the phosphoprotein formation or in a faster dephosphorylation.

Materials

Phospho*enol*pyruvate, pyruvate kinase, lactate dehydrogenase, *p*-nitrophenyl phosphate, ATP were obtained from Boehringer (Mannheim, FRG). PC, PS, PIP from bovine brain, PIP_2 from chicken eggs, NADH, neomycin sulfate were all from Sigma Chemical Co. (St. Louis, MO, USA). [γ-^{32}P]ATP was from Amersham International (Amersham, UK).

Methods

Delipidated plasma-membrane Ca^{2+}-transporting ATPase from pig red blood cells was purified using calmodulin affinity chromatography, as described by Missiaen et al. [3], which is a modification of the procedure described by Kosk-Kosicka et al. [7]. The purified ATPase was reactivated by a phospholipid mixture at a ratio of 1 mg phospholipid/mg protein [3] unless otherwise indicated.

The standard assay medium for measuring the (Ca^{2+} + Mg^{2+})-ATPase activity had the following composition: imidazole-HCl (pH 6.9): 30 mM; $MgCl_2$: 6 mM; KCl: 100 mM; EGTA: 0.4 mM; NaN_3: 5 mM; KCl: 20 mM; phospho*enol*pyruvate: 1.5 mM; pyruvate kinase: 40 U/ml; lactate dehydrogenase: 40 U/ml; NADH: 0.26 mM. The ATP concentration is indicated in the figure legends. Ca^{2+} was added to give 10 μM ionized Ca^{2+}. The assay was started by transferring 20 μl of ATPase (about 4 μg protein) to 980 μl assay medium at 37°C and measuring the decrease of the absorbance at 340 nm.

Measurement of the steady-state level of the Ca^{2+}-dependent phosphointermediate was performed at 0°C. The reaction medium contained: imidazole-HCl (pH 6.9): 30 mM; $MgCl_2$: 1 mM; KCl: 100 mM; EGTA: 0.2 mM and 10 μM ionized Ca^{2+}. In control experiments $CaCl_2$ was omitted and 1 mM EGTA was added. The reaction was started by addition of [γ-^{32}P]ATP (final concentration 12 μM) and stopped at different time intervals by 10% TCA containing 0.3% phosphoric acid and 0.5 mM ATP. Since the experiments were done on the purified Ca^{2+}-transporting enzyme (subsequent electrophoresis yielded a single ^{32}P-labelled band at 140 kDa), the level of phosphointermediate was determined by measuring the total radioactivity after filtration on GF/B filters (Whatman, UK) and subsequent washing with stop solution.

Phosphatase activity of Ca^{2+}-transporting ATPase was quantified at 37°C as a *p*-nitrophenylphosphatase activity (pNPPase) by the release of *p*-nitrophenol which was detected spectrophotometrically at 430 nm. The reaction medium had the following composition: Hepes-KOH (pH 7.4): 30 mM; KCl: 100 mM; NaCl: 10 mM; $MgCl_2$: 6 mM; EGTA: 0.4 mM; NaN_3: 5 mM; *p*-nitrophenyl phosphate: 10 mM. For measuring the pNPPase activity in the presence of neomycin a cuvette to which equimolar K_2SO_4 was added instead of the neomycin sulfate was used as a control.

Protein was determined by the bicinchoninic acid (BCA) Protein Assay Reagent kit supplied by Pierce (Rockford, IL, USA). Bovine serum albumin was used as a standard.

The V_{max} and K_m values were calculated from the ATP activation curves using the Enzfitter (Version 1.02) computer program (Elsevier Biosoft). The equation 1 from Rossi and Rega [6] was used to fit the data.

Results

The activation curve of the plasmalemmal Ca^{2+}-transporting ATPase shows a rather complex dependence on the concentration of ATP. Two components can be distinguished in the ATP-activation curve. The high-affinity component would represent the ATP binding to the catalytic site. A further increase of the ATP concentration leads to an augmented ATPase activity by activation at a low affinity 'regulatory' site (see Ref. 8 for review). It is not yet established whether these two components represent different states of the same binding site whereby its affinity for ATP is changed depending on the conformational state of the protein, or whether they are due to the existence of two different binding sites. Fig. 1 shows the ATP-activation curve for the purified and lipid-reactivated Ca^{2+}-transporting ATPase from porcine erythrocytes. This experiment shows that PS and PIP (at 20% of total added phospholipid, the remainder being PC) increased the V_{max} of the high-affinity component by a factor of 1.6 and 2.25, respectively, as compared to pure PC without significantly affecting the K_m. The K_m of the low-affinity component was higher than 5 mM ATP in the presence of PC, and was decreased to 1.8 mM in PS and to 1 mM in PIP. The difference between PS and PIP in the calculated V_{max} of the low-affinity component was not statistically significant. The effect of PS on the purified ATPase from porcine erythrocytes shown in Fig. 1 is very similar to that described by Rossi and Rega [6] on the membrane bound ATPase of human erythrocytes. In addition our results show that PIP exerts a stronger effect than PS.

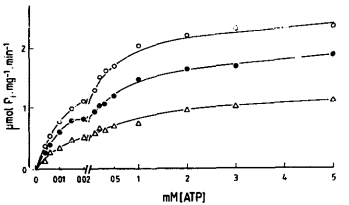


Fig. 1. Effect of negatively charged phospholipids on the Ca^{2+}-stimulated ATPase activity of the erythrocyte plasma-membrane Ca^{2+}-transporting ATPase at different ATP concentrations. The ATPase was reactivated by adding 5 μl of a lipid mixture (5 mg/ml stock) to 100 μl of ATPase (approx. 200 μg/ml). The lipid was either pure PC △ or a mixture of 20% PS (●) or 20% PIP (○) and 80% PC. The total amount of lipid was kept constant. The free Ca^{2+} and Mg^{2+} concentrations were 10 μM and 1.4 mM, respectively. ATP was added in a cumulative way to obtain the indicated concentrations. The experimental points represent the means of 3–5 determinations from two different ATPase preparations. The S.E. ranges between 5 to 10% of the mean.

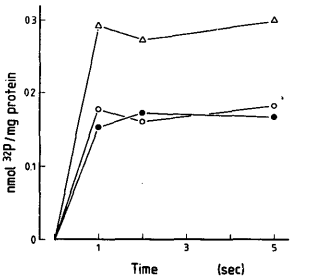
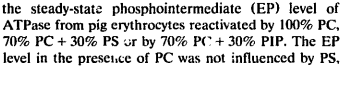


Fig. 2. Effect of the negatively charged lipids on the steady-state level of the phosphoprotein-intermediate of the erythrocyte plasma-membrane Ca^{2+}-transporting ATPase. PIP (△); PS (●); PC (○). The enzyme was preincubated for 10 min at 37°C in 140 μM Ca^{2+} and 2 mM Mg^{2+}. The phosphoprotein-intermediate level was measured at 0°C in the presence of 10 μM Ca^{2+}, 1 mM Mg^{2+} and 12 μM [γ-^{32}P]ATP.

Following the binding of ATP to the E_1 conformer (as illustrated in Scheme I) the γ-phosphate of ATP is transiently transferred to the enzyme. During steady-state ATP hydrolysis, only a small fraction of the ATPase molecules are in the phosphorylated state. This can be ascribed to the fact that the E_2 to E_1 conversion (reaction 6) is very slow and this slow rate results in an accumulation of E_2 conformers [9,10]. Therefore, in the phosphorylation experiments the enzyme was preincubated in conditions which favour the conversion of E_2 to the phosphorylatable state E_1. In this way it could be ensured that within the duration of the experiment the steady state would be reached. The formation of the E_1 conformer is favoured by incubation in the presence of Ca^{2+} and Mg^{2+}, as shown by Adamo et al. [9] and in this conformation, the enzyme is then rapidly phosphorylated by ATP. Fig. 2 shows the steady-state phosphointermediate (EP) level of ATPase from pig erythrocytes reactivated by 100% PC, 70% PC + 30% PS or by 70% PC + 30% PIP. The EP level in the presence of PC was not influenced by PS,



Scheme I. Schematic representation of the catalytic cycle of the Ca^{2+}-transporting ATPase. The cycle includes a transition between two conformational states E_1 and E_2. Additional hypothetical steps which might be involved in the hydrolysis of pNPP in the absence of Ca^{2+} are also given.

whereas PIP increased it by about 60%. Similar results were obtained on the plasmalemmal Ca^{2+}-transporting ATPase purified from pig gastric smooth muscle (data not shown). We have also tested on the latter preparation the effect of two other negatively charged phospholipids, PIP_2 and PA, which also activate the ATPase [3]. PA decreased the steady-state EP level almost to the background value, whereas PIP_2 increased this parameter to approximately the same level as that induced by PIP (data not shown). These results indicate that the interaction of phospholipids with the ATPase may be more complex than previously thought. The observation that some ATPase-activating phospholipids increase the steady-state EP level, whereas others do not affect EP or even decrease it, strongly suggests that more than one reaction step of the ATPase cycle is affected by these phospholipids (see below).

In order to further examine the different effects of various phospholipids we have measured their effect on the *p*-nitrophenylphosphatase (pNPPase) activity. Like other P-type ATPases, the Ca^{2+}-transporting ATPase of the plasma membrane catalyses the hydrolysis of *p*-nitrophenyl phosphate [11]. It has been shown for the Ca^{2+}-transporting ATPase of human erythrocytes that the pNPPase activity is strongly influenced by the experimental conditions, such as the concentration of Ca^{2+} and ATP [11,12]. The pNPPase activity of the purified porcine ATPase under different experimental conditions is summarized in Table I. The highest activity was observed in EGTA and amounted to 321 nmol mg^{-1} min^{-1}. At 100 μM Ca^{2+} the activity de-

creased to 58.4 in the absence of ATP and to 120.4 nmol mg^{-1} min^{-1} in the presence of 0.5 mM ATP. The inhibition of the pNPPase activity by Ca^{2+} and the partial protection against this inhibition by ATP has been described earlier for the ATPase of human erythrocytes by Verma and Penniston [12]. These authors, and also Rega et al. [11] observed that the pNPPase activity of that enzyme requires the presence of calmodulin, while the pNPPase activity of our purified enzyme was not affected by calmodulin. This is in line with the relatively small stimulation of the ATPase activity of the porcine enzyme by calmodulin [3]. Two possible mechanisms for the low calmodulin-sensitivity of Ca^{2+}-transporting ATPase have been described, partial proteolysis and self-association. Partial proteolysis whereby the inhibitory calmodulin-binding domain of the protein is removed [13] could be excluded because the migration distance of the purified ATPase on Western-blotted SDS-polyacrylamide gels was exactly the same as that of freshly prepared membranes presenting calmodulin-stimulated enzyme activity (data not shown). The alternative possibility, self-association of the enzyme involving the calmodulin-binding domain [14,15], is the most likely explanation for the calmodulin-independence. Self-association is favoured by higher enzyme concentrations and it is particularly apparent if the ATPase has been purified in the absence of added phospholipids, as was the case in this study.

The effects of negatively charged phospholipids on the pNPPase activity of the erythrocyte ATPase are summarized in Fig. 3. These experiments were done in the presence of EGTA and in the absence of added Ca^{2+} and ATP. However, the effects of the phospholipids on the pNPPase activity were essentially similar when the activity was measured in the presence of 100 μM Ca^{2+} and 0.5 mM ATP, with or without 10 μg/ml calmodulin (data not shown). The pNPPase activity of the erythrocyte ATPase is stimulated in a dose-dependent way by PS but it is not affected by PIP (Fig. 3).

TABLE I

p-Nitrophenyl phosphatase activity of the Ca^{2+}-transporting ATPase in the presence of different Ca^{2+} concentrations and in the presence and absence of ATP or calmodulin

The ATPase was reactivated by a phospholipid mixture (100% PC) at a ratio of 1 mg phospholipid/mg protein. Results are means ± S.E. of three different determinations.

[Ca^{2+}] (μM)	*p*-Nitrophenylphosphatase (nmol mg^{-1} min^{-1})		
	no ATP present	0.5 mM ATP	0.5 mM ATP + 10 μg/ml calmodulin
0	321.0 ± 25	–	–
10	65.7 ± 15	80.3 ± 17	88.4 ± 11
100	58.4 ± 11	120.4 ± 19	–

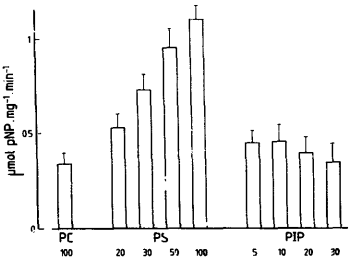


Fig. 3. Effect of negatively charged lipids on the *p*-nitrophenylphosphatase activity of the erythrocyte plasma-membrane Ca^{2+}-transporting ATPase. The pNPPase activity was measured in the presence of EGTA as described in Methods. Phospholipids were added as described for the ATPase measurements. The numbers under the vertical columns represent the amount of negatively charged lipids as a percentage (w/w) of the total amount of lipids. The results are expressed as means ± S.E. of 3–5 determinations from three different ATPase preparations.

Similar results on the effect of PS and PIP were obtained on the plasmalemmal Ca^{2+}-transporting ATPase from pig stomach smooth muscle (data not shown). In this latter preparation, the effect of PA, PI and PIP_2 on the pNPPase activity was studied as well. The effect of PA and PI was very similar to that of PS, while neither PIP nor PIP_2 did exert an effect.

It has been reported by Missiaen et al. [18] that the positively charged compound neomycin inhibits the stimulatory effect of PIP and PIP_2 on the ATPase activity of the plasmalemmal Ca^{2+} pump, while it does not affect the stimulatory effect of PS or PA. A similar behavior was found with respect to the pNPPase activity. Although neomycin decreased the pNPPase activity by about 50%, it did not influence the relative stimulation by PS, expressed as the percentage of the activity in PC (Fig. 4).

The backward reaction of the ATPase whereby inorganic phosphate is transferred to the enzyme is strongly facilitated by adding organic solvents to the reaction medium [19]. This effect is explained by postulating that the phosphate acceptor site resides in a hydrophobic pocket. Organic solvents would facilitate the transfer of the hydrophilic phosphate to the hydrophobic site by reducing the polarity of the medium. Fig. 4 shows that also the pNPPase activity is strongly stimulated by adding 20% (v/v) of the organic solvent DMSO to the reaction medium. In the presence of DMSO there is still a differential effect of PS versus PIP. In this condition the pNPPase activity is no longer stimulated by PS as compared to PC. The activity in the

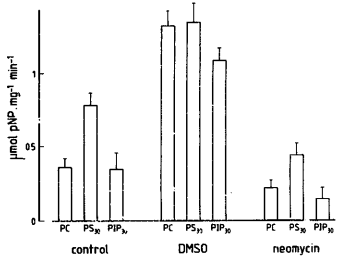


Fig. 4. Effect of the polycationic antibiotic neomycin (5 mM) and of DMSO (20%, v/v) on the pNPPase activity of the erythrocyte plasma-membrane Ca^{2+}-transporting ATPase. The values given with each lipid represent the amount of negatively charged lipids as a percentage of total lipids. In the case of neomycin, controls contained 5 mM K_2SO_4 instead of neomycin sulfate. The values represent the means ± S.E. of four different observations.

presence of PS however remains higher than in the presence of PIP which becomes inhibitory if compared to PC.

Discussion

Although the stimulation of the activity of the plasmalemmal Ca^{2+}-transporting ATPase by negatively charged phospholipids has been described in detail, little is known about their mechanism of action or about the properties of the lipid binding site. It has been shown that there are important quantitative differences in the effects of different negatively charged phospholipids [3]. Lipids possessing a greater number of negative charges are more effective in stimulating the activity of the Ca^{2+} pump. The same correlation was found for binding of the phospholipids to the ATPase protein, as determined by fluorescence energy transfer [4]. Measurement of the effect of negatively charged phospholipids as a function of the Ca^{2+} concentration shows that these compounds increase both the V_{max} and the affinity for Ca^{2+}. Little is known, however, about the site(s) of interaction with the enzyme or about the elementary reaction steps of the ATPase cycle which are influenced by these lipids. It was shown by Rossi and Rega [6] for the membrane-bound ATPase that PS modifies the ATP-activation curve. This curve presents two components, reflecting a high affinity and a low affinity for ATP, respectively. PS increased the V_{max} of the high-affinity component and increased the affinity for ATP at the low-affinity site. Similar results were obtained with our purified, delipidated and reactivated ATPase (Fig. 1). We have found that PIP has a similar effect on the ATP-activation curve but that it is more effective than PS. This result indicates that PS and PIP exert their action on the ATP-dependence of the ATPase by a common mechanism. However, our phosphorylation experiments and pNPPase measurements indicate that additional reaction steps are involved in the action of negatively charged phospholipids on the Ca^{2+}-transport cycle and that these steps are differentially affected by the different phospholipids. Whereas PIP increased the steady-state phosphoprotein level of the purified erythrocyte ATPase, PS did not exert that effect. The observation that in the presence of PIP a larger fraction of the enzyme is in the phosphorylated state indicates therefore that PIP predominantly affects a reaction step which leads to a faster phosphorylation, rather than a reaction favouring dephosphorylation. The phenomenon cannot be due to an inhibition of dephosphorylation because such action would lead to an inhibition of the ATPase activity, whereas the opposite is found. In the Scheme I the most likely site of action of the acidic phospholipids is not on the rate constant of phosphorylation of E_1, but rather on the rate of conversion to the substrate E_1 (reaction 6), because this step implies a conformational change and is much slower than step 1 or 2 [20]. Moreover, a conformational change of the $E_1 \rightarrow E_2$ type very likely depends on the phospholipid microenvironment of the protein since its activation energy may largely result from changed amphipatic properties. This mechanism of action could be responsible for the qualitatively similar effect of PS and PIP on the ATP-activation curve mentioned above (Fig. 1).

Although PS stimulates the turnover rate of the ATPase, it does not increase the steady-state phosphoenzyme level, implying that both the rate of formation and of decomposition of EP are accelerated to a similar extent. This means that PS would not only accelerate reaction 6, but also one of the steps involved in EP decomposition as discussed below.

The reaction steps whereby the Ca^{2+}-transporting ATPase catalyzes the hydrolysis of *p*-nitrophenyl phosphate (pNPP) are not fully understood. The hydrolysis of pNPP by the plasmalemmal ATPase is not accompanied by Ca^{2+} transport, unlike the hydrolysis of pNPP by the Ca^{2+}-transporting ATPase of the sarco(endo) plasmic reticulum. This hydrolysis involves only part of the ATPase reaction-cycle and possibly occurs at the low-affinity ATP-binding site, which is exposed when the enzyme is in the E_2 conformation [17]. Moreover, it probably includes additional reaction steps which are not shared by the ATPase cycle. Therefore, the action of negatively charged phospholipids on the pNPPase is difficult to interpret because effects on the pNPPase activity do not necessarily imply an effect on the ATPase activity. However, it may be indicative that

the negatively charged phospholipids which do not increase the steady-state EP level (PS, PA, PI), increase the pNPPase activity whereas PIP and PIP_2 which increase EP, do not exert such an effect on the pNPPase. These results suggest that part of the effect of the negatively charged phospholipids on the pNPPase activity is mediated by activation of a step which during ATPase cycling results in a faster dephosphorylation of the enzyme. This step may be one that is shared between the ATPase and the pNPPase cycle (reaction 5), or it may be one of the steps favouring a conformation that catalysis pNPPase activity (reactions 3 or 4). PS and PA would mainly use this pathway to stimulate the pNPPase. For PIP and PIP_2, acceleration of the E_2 to E_1 conversion would be relatively more important. The relative importance of the effect on the conversion leading to a faster phosphorylation or on the conversion leading to a faster dephosphorylation would then depend on the kind of phospholipid.

It has been observed by Missiaen et al. [18] that the positively charged compound neomycin inhibits the stimulatory effect of PIP_2 and PIP on the ATPase activity, but that this substance does not inhibit the action of PS. We now report that neomycin did neither affect the stimulation of the pNPPase activity by PS. The selectivity of neomycin to inhibit the action of PIP_2 and PIP but not that of the less negative lipid PS can be explained in at least two ways. It could mean that electrostatic interaction of neomycin with negative charges on the phospholipids is too weak if the phospholipid possesses only one negative charge. Alternatively, it could mean that the effect of PS as opposed to that PIP and PIP_2 is not only due to its different head group, but also to differences in the hydrophobic chains. It should be mentioned in this respect that organic solvents mimic the stimulatory effect of negatively charged phospholipids on the ATPase activity [21]. An additional argument in favour of this hypothesis could be that the effect of PS on the pNPPase activity disappears in the presence of DMSO (Fig. 4). In this interpretation, specificity of the PS effect versus PIP or PIP_2 would imply a considerable difference in the hydrophobic moiety of these phospholipids. However, since it can be expected that the molecular composition of PI will resemble more that of PIP or PIP_2 than that of PS, the observation that the effect of PI from bovine brain is similar to the effect of PS and not to that of PIP or PIP_2 (all from bovine brain) does not support an important role of the fatty-acid composition. We can also mention in this respect that no difference was seen in the effect of PS isolated from bovine brain or from chicken egg (data not shown).

While this study was in progress Rossi and Caride [22] reported on the effect of PS and PI on the pNPPase activity of the Ca^{2+} pump in membranes from human erythrocytes. These authors observed an inhibition of the pNPPase activity by these negatively charged phospholipids. We have no clear explanation for the difference between their results and ours. It could be due to the fact that they used a membrane bound form of the enzyme, while we used in the present study the purified enzyme. Another difference is that we used porcine and not human plasma membranes. Since our preparation differed from that of Rossi and Caride [22] also by its calmodulin-dependence, it can not be excluded that the difference in the pNPPase activation by acidic phospholipids in both preparations could somehow be related to this functional difference. However, the nature of the link, if any, between both properties is not clear at present.

In summary, the present results indicate that the stimulation of the Ca^{2+}-transporting ATPase by negatively charged phospholipids is mediated by the modification of more than one elementary reaction step. One could speculate that one such step is the E_2 to E_1 conversion, leading to a faster phosphorylation, whereas another one would participate in the dephosphorylation. Moreover, the difference in effect between negatively charged phospholipids suggests that some lipids affect mainly the phosphorylation reaction whereas others mainly accelerate the rate of dephosphorylation. Zvaritch et al. [23] have proposed that a lysine-rich region could be a binding site for negatively charged phospholipids. It is an interesting question whether the binding to this region only, possibly involving both hydrophobic and charge interactions, could mediate both the rate of phosphorylation and dephosphorylation and (or) whether a second independent phospholipid binding domain would be involved. An argument in favour for a second binding site besides the lysine-rich region is the observation that also arginine residue(s) play a role in the stimulatory effect of negatively charged phospholipids [24].

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