Biochem. J. (1989) 263, 687–694 (Printed in Great Britain)

Phospholipid–protein interactions of the plasma-membrane Ca^{2+}-transporting ATPase

Evidence for a tissue-dependent functional difference

Ludwig MISSIAEN,* Luc RAEYMAEKERS, Frank WUYTACK, Mathias VROLIX, Humbert DE SMEDT and Rik CASTEELS

Department of Physiology, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

The aim of the present work was to investigate the stimulation of the plasma-membrane Ca^{2+}-transporting ATPase by negatively charged phospholipids. The Ca^{2+}-transporting ATPase was purified from pig stomach smooth muscle and from pig erythrocytes, and was reactivated with phosphatidylcholine (PC) in the presence and absence of negatively charged phospholipids. The substitution of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidic acid (PA) or phosphatidylserine (PS) for PC induced profound changes in the $V_{max.}$, the $K_{0.5}$ and the Hill coefficient of the Ca^{2+}-activation curves for both ATPases. Low concentrations of each of the negatively charged phospholipids increased the $V_{max.}$, but high ratios of PIP, PIP_2 or PA to PC decreased this parameter. PI, PA and PS increased the $V_{max.}$ of the erythrocyte enzyme to a larger extent than that of the smooth-muscle enzyme. This difference was less pronounced for PIP and absent for PIP_2. PI (> 20% PC substituted), PIP, PIP_2, PA and PS all increased the affinity of the two Ca^{2+}-transporting ATPases for Ca^{2+} in the following order of potency: $PIP_2 > PIP > PI \approx PS \approx PA$. PI, PA and PS increased the Ca^{2+} affinity of the smooth-muscle enzyme more than that of the erythrocyte enzyme; this difference was less pronounced for PIP and absent for PIP_2. Even in the presence of calmodulin, all of the negatively charged phospholipids were still able to increase the $V_{max.}$ of the erythrocyte enzyme, whereas only PIP and PIP_2 increased the affinity for Ca^{2+}. The effect of PI at low concentrations (< 20%) on the erythrocyte enzyme was peculiar in that it caused a decrease in the Ca^{2+} affinity instead of an increase. This effect was not observed for the smooth-muscle enzyme. All of the negatively charged phospholipids slightly increased the Hill coefficient for Ca^{2+} of both ATPases, and this effect was additive to that of calmodulin. The stimulation of the erythrocyte enzyme exhibited positive co-operativity towards PI and PIP, whereas that of the smooth-muscle enzyme did not. It is concluded (1) that there is a correlation between the number of negative charges on the phospholipids ($PIP_2 > PIP > PA \approx PI \approx PS$) and the magnitude of their effect on the $V_{max.}$ and the $K_{0.5}$ for Ca^{2+}, and (2) that the action of the lipids on the smooth-muscle enzyme differs from that on the erythrocyte enzyme, indicating that these two Ca^{2+}-transporting ATPases are not the same.

INTRODUCTION

The Ca^{2+}-transporting ATPase of the plasma membrane is an enzyme that can extrude Ca^{2+} out of the cell against the large electrochemical gradient for Ca^{2+} across the plasma membrane. This Ca^{2+}-transporting ATPase of the plasma membrane is activated by the Ca^{2+}–calmodulin complex (Jarrett & Penniston, 1977; Gopinath & Vincenzi, 1977), by partial proteolysis (Enyedi *et al.*, 1980) and by negatively charged phospholipids (Niggli *et al.*, 1981; Choquette *et al.*, 1984; Enyedi *et al.*, 1987).

There are at least two observations indicating that the stimulatory effect exerted by the lipids of the phosphatidylinositol cycle on the plasma-membrane Ca^{2+}-transporting ATPase activity might have physiological implications. The first one is the finding by Vrolix *et al.* (1988) that the stimulation of the Ca^{2+}-transporting ATPase by cyclic GMP-dependent protein kinase can be mediated by the generation of phosphatidylinositol 4-phosphate (PIP) from phosphatidylinositol (PI) by activation of a PI kinase. The second observation is that the decreased Ca^{2+}-transporting ATPase activity induced by Ca^{2+}-mobilizing agonists (Missiaen *et al.*, 1988), is accompanied by a reduction in phosphatidylinositol 4,5-bisphosphate (PIP_2) and PIP in the plasma membrane (Berridge, 1988).

Niggli *et al.* (1981) reported on the effect of PI, phosphatidic acid (PA) and phosphatidylserine (PS) on the Ca^{2+}-transporting ATPase of human erythrocytes, and Choquette *et al.* (1984) studied its modification by different concentrations of PI, PIP_2 and PA. Enyedi *et al.* (1987) only studied one phospholipid (PIP) at one concentration to examine the interaction of negatively charged lipids with the ATPase. The aim of the present

Abbreviations used: $(Ca^{2+} + Mg^{2+})$-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.

* To whom correspondence and reprint requests should be sent, at the following address: Laboratory of Physiology, Katholieke Universiteit Leuven, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium.

study is to analyse in detail how different concentrations of the physiologically important negatively charged phospholipids (PI, PIP, PIP_2, PA and PS) affect the $V_{max.}$, the affinity for Ca^{2+}, and the co-operativity for Ca^{2+} of the plasma-membrane Ca^{2+}-transporting ATPase of two different cell types.

EXPERIMENTAL

Purification of the ATPase

The plasma-membrane Ca^{2+}-transporting ATPase was prepared from the antral part of pig stomach smooth muscle and from pig erythrocytes using calmodulin-affinity chromatography. This method was the same as that used by Vrolix *et al.* (1988), which itself was a slight modification of the procedure described by Kosk-Kosicka *et al.* (1986). This procedure allowed purification of the enzyme in the absence of phospholipids. The ATPase preparation was solubilized and purified in the same buffers as described by Wuytack *et al.* (1981), except that 20% (v/v) glycerol was added. The ATPase preparation was finally concentrated in Centriprep 10-Concentrator tubes (Amicon) to a final concentration of about 200 μg/ml, and stored at −75 °C in an ATPase buffer of the following composition: Hepes (pH 7.4), 20 mM; KCl, 130 mM; $MgCl_2$, 1 mM; K-EGTA, 2 mM; Triton X-100, 0.05% and glycerol, 20% (v/v).

Reactivation of the enzyme

The ATPases purified as described above did not show any detectable Ca^{2+}-stimulated Mg^{2+}-ATPase activity, but the ATPase could be reactivated with phospholipids. This stimulation of the ATPase by the phospholipids was dose-dependent and reached a plateau at 100 mol/mol of ATPase. We have therefore used in all experiments a ratio of total lipid to ATPase of 140–210 mol/mol. This ratio was obtained by adding 6 μl of a lipid mixture to 100 μl of ATPase, followed by vigorous vortexing of this mixture for 10 s.

Lipid mixtures were made from stock solutions of PC, PS, PI, PIP, PIP_2 and PA in chloroform/methanol (19:1, v/v). The solvent was evaporated under a stream of N_2, and the lipids were redissolved in the ATPase buffer and sonicated for 3 × 10 s. The final lipid concentration was 5 mg/ml.

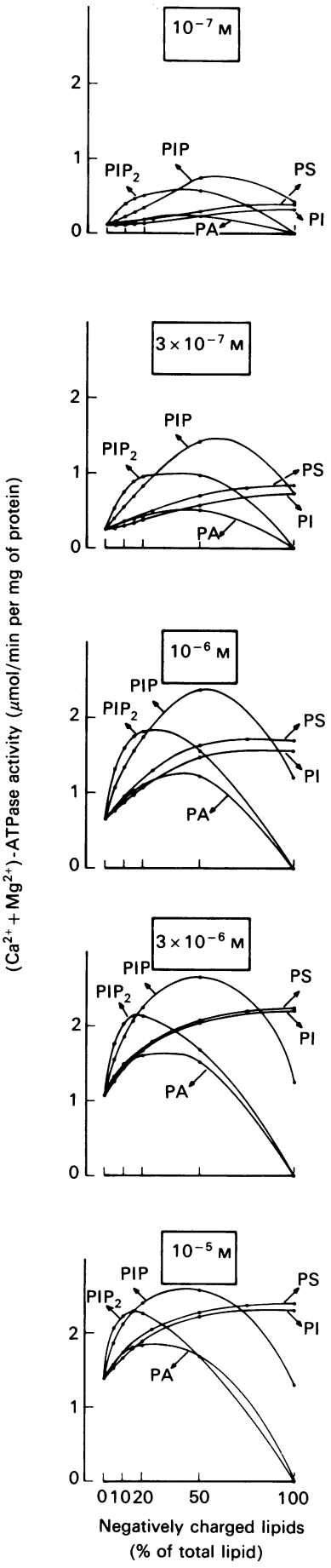

Fig. 1. Effect of the negatively charged lipids on the specific activity of the $(Ca^{2+}+Mg^{2+})$-ATPase of the smooth-muscle plasma-membrane Ca^{2+}-transporting ATPase at different Ca^{2+} concentrations

The ATPase was activated by adding 6 μl of lipid mixture (5 mg/ml stock) to 100 μl of ATPase (approx. 200 μg/ml). The lipid mixtures contained PC plus negatively charged lipids but the total amount of lipid (w/w) was kept constant. The abscissa of the curves gives the negatively charged lipids as a percentage of the total amount of lipids. The ordinate represents the specific activity of the $(Ca^{2+}+Mg^{2+})$-ATPase (μmol/min per mg of protein). The curves in the different panels were obtained at different free Ca^{2+} concentrations. The experimental points represent the mean of 15 determinations (except at point 0% on the abscissa, which is the mean of 32 determinations). The S.E.M. ranged from 5–10% of the mean.

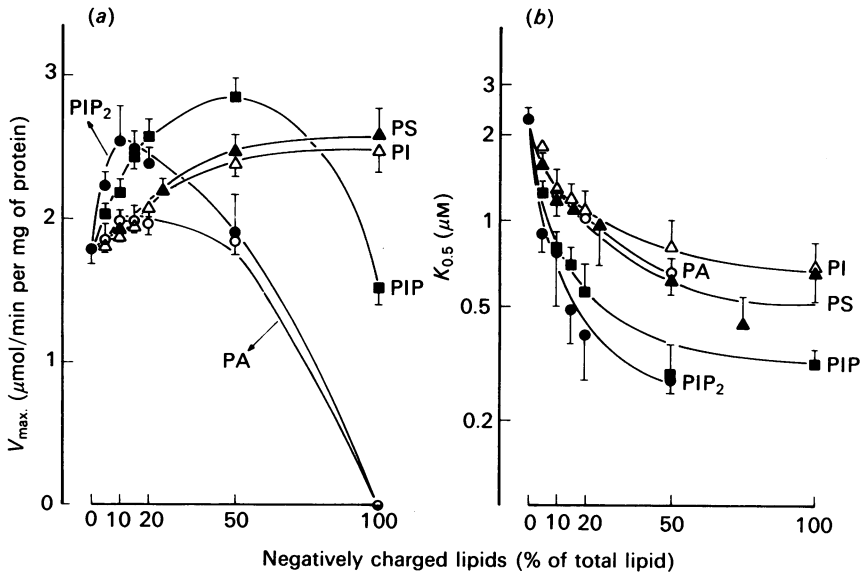

Fig. 2. Effect of negatively charged lipids on the calculated kinetic parameters of the smooth-muscle plasma-membrane Ca^{2+}-transporting ATPase

The abscissa represents the negatively charged lipids as a percentage (w/w) of the total amount of lipid (as in Fig. 1). The ordinate is (*a*) the $V_{max.}$ (μmol/min per mg of protein) or (*b*) the $K_{0.5}$ for Ca^{2+} (μM-Ca^{2+}) calculated by the Enzfitter computer program, from the Ca^{2+}-activation curves of the Ca^{2+}-transporting ATPase. The values represent the means ± S.E.M. for the numbers of observations given in the legend to Fig. 1. ●, PIP_2; ■, PIP; ▲, PS; ○, PA; △, PI.

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

ATPase (20 μl; 4 μg of Ca^{2+}-transporting ATPase) was added to 980 μl of assay medium of the following composition: phosphoenolpyruvate, 1.5 mM; pyruvate kinase, 40 units/ml; lactate dehydrogenase, 40 units/ml; imidazole/HCl (pH 6.9), 30 mM; $MgCl_2$, 5.4 mM; KCl, 100 mM; K-EGTA, 0.5 mM; ATP, 5 mM; NaN_3, 5 mM and NADH, 0.26 mM. The temperature was 35 °C. The free Mg^{2+} concn. was 1 mM. Ca^{2+} was added in a cumulative way to obtain free Ca^{2+} concentrations of 0.10, 0.31, 1.02, 3.23, 10.35 and 50.45 μM. In preliminary experiments, it was ascertained that under these experimental conditions the ATPase activity at any given Ca^{2+} concentration was the same for Ca^{2+} concentrations obtained in a cumulative way or obtained by adding the calculated amount of Ca^{2+} at once to the medium. When calmodulin was added, it was always used at a saturating concentration of 0.6 μM. The ATPase activity was determined by measuring the decrease in absorbance at 340 nm. The inclusion in the ATPase assay of Triton X-100 at 0.005% did not affect the $(Ca^{2+}+Mg^{2+})$-ATPase activity. However, because higher concentrations of the detergent exerted an inhibitory effect, Triton X-100 was omitted during the assay of the $(Ca^{2+}+Mg^{2+})$-ATPase activity. Protein concentrations were measured by the method of Lowry *et al.* (1951), using serum albumin as a standard.

Analysis of the results

From the Ca^{2+}-activation curves, the $V_{max.}$, the $K_{0.5}$ for Ca^{2+} and the Hill coefficient for Ca^{2+} were calculated using the Enzfitter (version 1.03) computer program (Elsevier Biosoft). The Hill equation of that program was used to fit the data. The weighting of the individual points was inversely proportional to their standard error. The results are expressed as means ± S.E.M.

Materials

Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase and ATP were obtained from Boehringer, Mannheim, Germany. NADH, PIP (from bovine brain, 98% pure, product No. P-9638), PIP_2 (from bovine brain, 98% pure, product No. P-9763), PA (from egg yolk lecithin, 98% pure, product No. P-9511), PC (from frozen egg yolk, 99% pure, product No. P-4279) and PS (P-8518) were all from Sigma Chemical Co., St. Louis, MO, U.S.A. PI was from Lipid Products (Redhill, Surrey, England). Calmodulin was prepared from bovine brain according to Gopalakrishna & Anderson (1982).

RESULTS

Effects of phospholipids on the smooth-muscle plasma-membrane Ca^{2+}-transporting ATPase

Fig. 1 illustrates the effects of the negatively charged phospholipids on the activity of the $(Ca^{2+}+Mg^{2+})$-ATPase from smooth muscle at different Ca^{2+} concentrations. PI and PS stimulated the $(Ca^{2+}+Mg^{2+})$-ATPase activity in a concentration-dependent manner. PIP, PIP_2 and PA stimulated the $(Ca^{2+}+Mg^{2+})$-ATPase activity at low concentrations, while they were inhibitory at high concentrations. The stimulation by the different lipids was more pronounced at the lower Ca^{2+} concentrations. The effects of the phospholipids on the Ca^{2+}-activation curves of the ATPase will be described in terms of their effects on the $V_{max.}$, the $K_{0.5}$ and the Hill coefficient for Ca^{2+}. These results are summarized in Fig. 2 and Table 1.

Activation of the purified enzyme in the absence of negatively charged lipids (100% PC), gave a $V_{max.}$ of 1.79 ± 0.08 μmol/min per mg of protein ($n = 32$), a $K_{0.5}$ for Ca^{2+} of 2.28 ± 0.21 μM ($n = 32$) and a Hill coefficient for Ca^{2+} of 0.87 ± 0.05 ($n = 32$).

Table 1. Effect of negatively charged lipids on the Hill coefficient for Ca^{2+} of the smooth-muscle plasma-membrane Ca^{2+}-transporting ATPase

The lipid-mixtures contained 100% PC or 80% PC plus 20% of the indicated negatively charged lipids. The values represent the means ± S.E.M. for the number of observations given in parentheses.

Phospholipid	Hill coefficient
PC	0.87±0.05 (32)
PI	1.19±0.09 (15)
PIP	1.11±0.11 (15)
PIP_2	1.03±0.15 (15)
PS	1.13±0.10 (15)
PA	1.03±0.10 (15)

A partial replacement of the PC by negatively charged lipids increased the $V_{max.}$ of the Ca^{2+}-activation curve (Fig. 2*a*). PI and PS increased the $V_{max.}$ in a similar fashion. This increase in the $V_{max.}$ became more pronounced as the proportion of negatively charged phospholipids increased. The maximal increase was 1.39-fold for PI and 1.45-fold for PS. Low concentrations of PIP, PIP_2 and PA increased the $V_{max.}$ while high concentrations inhibited the enzyme. The inhibition became obvious if PC was substituted by more than 50% PIP, 25% PA or 10% PIP_2. The effect of PIP, PIP_2 and PA could therefore be described as a balance between a stimulatory and an inhibitory action. The maximal increase of the $V_{max.}$ caused by PIP was 1.6-fold, by PIP_2, 1.42-fold and by PA, 1.12-fold. In the low concentration range, the order of potency of stimulation was $PIP_2 > PIP > PI \approx PS \approx PA$.

The effects of partial substitution of PC by negatively charged lipids on the affinity of the ATPase for Ca^{2+} are illustrated in Fig. 2 (*b*). Each of the phospholipids decreased the $K_{0.5}$ for Ca^{2+}. PIP_2 was in this respect the most effective compound, with PIP the next most effective. PI, PA and PS were about equally effective. It should be pointed out that the effects of the lipids increased with increasing concentration. Unlike the concentration dependency of the $V_{max.}$, these curves did not present a maximum followed by a decrease at higher concentrations of the lipid. The affinity at 50% PIP_2 was higher than at 10% PIP_2, although the $V_{max.}$ at 50% PIP_2 was lower than at 10%.

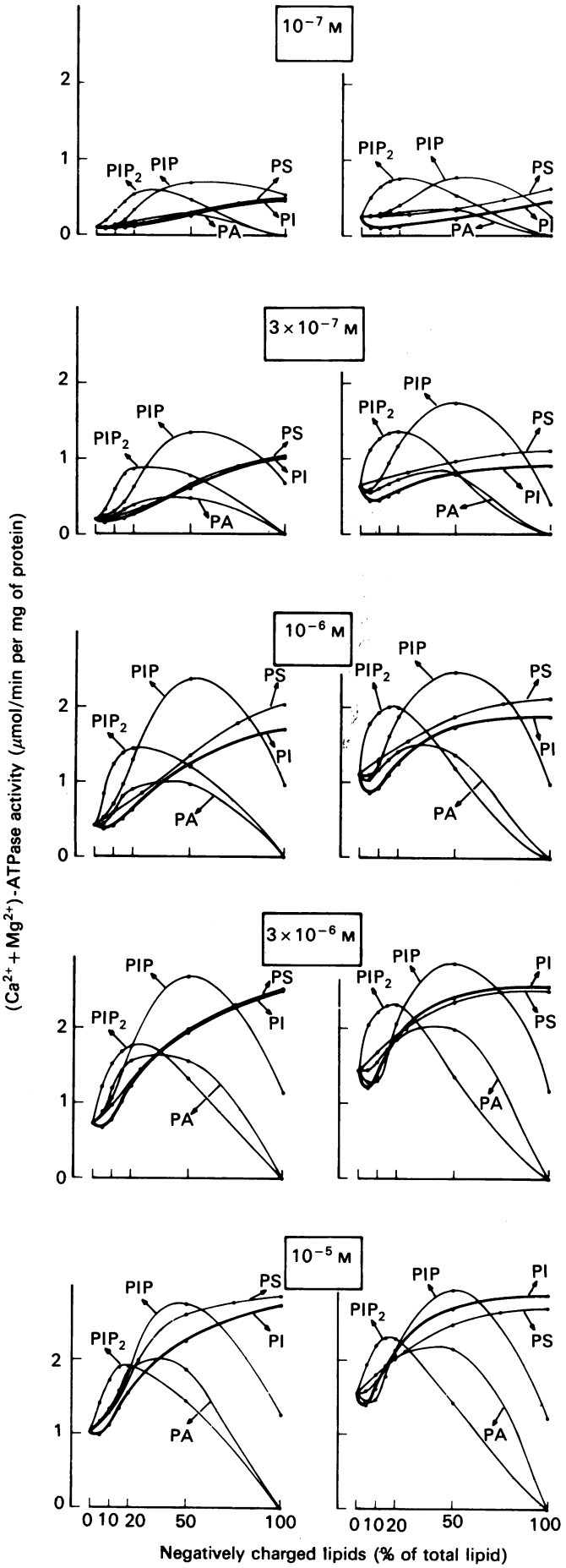

Fig. 3. Effect of the negatively charged lipids on the specific activity of the $(Ca^{2+}+Mg^{2+})$-ATPase of the erythrocyte plasma-membrane Ca^{2+}-transporting ATPase at different Ca^{2+} concentrations

The experiments are similar to those described in Fig. 1 and were carried out on the $(Ca^{2+}+Mg^{2+})$-ATPase from pig erythrocyte plasma membranes. The left panels represent the data obtained in the absence of calmodulin; those on the right were obtained in the presence of 0.6 μM-calmodulin. The experimental points represent the means of 13 determinations [except the point at 0% on the abscissa, which is the mean of 37 determinations (calmodulin absent) or 52 determinations (calmodulin present)]. The S.E.M. ranges between 5 and 10% of the mean. The activation curves for PI are represented by the bold line.

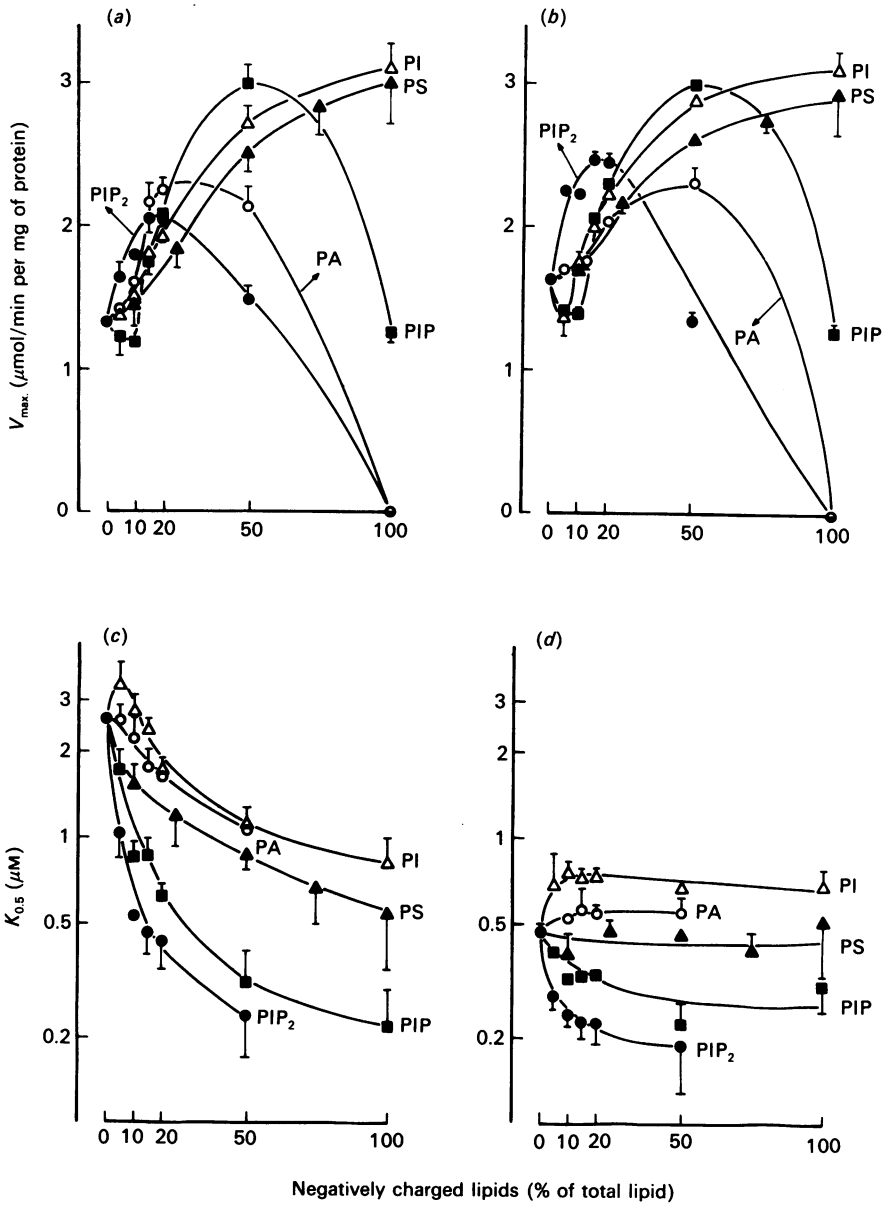

Fig. 4. Effect of negatively charged lipids on the calculated kinetic parameters of the erythrocyte plasma-membrane Ca^{2+}-transporting ATPase

The experiments with the erythrocyte Ca^{2+}-transporting ATPase were done by the same procedure as that described for the smooth-muscle enzyme (Fig. 2). Panels (*a*) and (*c*) were obtained in the absence of calmodulin, panels (*b*) and (*d*) in the presence of 0.6 μM-calmodulin. The effect of the lipids on the $V_{max.}$ (μmol/min per mg of protein) (*a*, *b*) and on the $K_{0.5}$ for Ca^{2+}(μM-Ca^{2+}) (*c*, *d*) are shown. The values represent the means $\pm$ S.E.M. for the numbers of observations given in the legend to Fig. 3. Symbols: ●, PIP_2; ■, PIP, ▲, PS; ○, PA; △, PI.

Table 1 represents the effects of the phospholipids on the Hill coefficient of the activation curves for Ca^{2+}. The negatively charged lipids induced a slight increase in the co-operativity, which was however small in the case of PIP_2 and PA. This co-operativity decreased again at the highest levels of the phospholipids (results not shown).

Effects of the phospholipids on the erythrocyte Ca^{2+}-transporting ATPase

Fig. 3 illustrates the effect of the negatively charged phospholipids on the activity of the erythrocyte $(Ca^{2+}+Mg^{2+})$-ATPase as a function of the Ca^{2+} concentration, in both the presence and absence of calmodulin. PS stimulated the $(Ca^{2+}+Mg^{2+})$-ATPase activity in a concentration-dependent manner. Low concentrations of PIP_2 and PA also stimulated this $(Ca^{2+}+Mg^{2+})$-ATPase activity, but high concentrations of those lipids exhibited an inhibitory action. The effects of PI and PIP were more complex. In the absence of calmodulin, low concentrations of PI did not enhance the $(Ca^{2+}+Mg^{2+})$-ATPase activity and they even inhibited the enzyme if calmodulin was present. PI only increased the $(Ca^{2+}+Mg^{2+})$-ATPase activity at higher concentrations of this lipid (50% PI at 0.1 μM-Ca^{2+}, 12% PI at 10 μM-Ca^{2+}). Low concentrations of PIP (< 10%) inhibited the $(Ca^{2+}+Mg^{2+})$-ATPase activity in

Table 2. Effect of negatively charged lipids on the Hill coefficient for Ca^{2+} of the erythrocyte plasma-membrane Ca^{2+}-transporting ATPase

The lipid-mixtures contained 100% PC or 80% PC plus 20% of the indicated negatively charged lipids. The Hill coefficients were determined in the absence and in the presence of 0.6 μM-calmodulin. The values represent the means ± S.E.M. for the numbers of observations given in parentheses.

Phospholipid	Hill coefficient	
	Calmodulin absent	Calmodulin present
PC	0.79 ± 0.04 (37)	1.04 ± 0.03 (52)
PI	1.00 ± 0.06 (13)	1.17 ± 0.03 (13)
PIP	0.94 ± 0.04 (13)	1.36 ± 0.02 (13)
PIP_2	0.86 ± 0.08 (13)	0.97 ± 0.07 (13)
PS	0.96 ± 0.10 (13)	1.28 ± 0.05 (13)
PA	0.87 ± 0.07 (13)	1.11 ± 0.04 (13)

the presence of calmodulin, while higher concentrations exerted an action which was similar to that of PIP_2 and PA, although it was more pronounced. The stimulatory effect of the specified lipids increased with decreasing Ca^{2+} concentrations.

Fig. 4 and Table 2 summarize the kinetic parameters calculated from the above data. In the presence of 100% PC, 0.6 μM-calmodulin increased the $V_{max.}$ from 1.33 ± 0.04 ($n = 37$) to 1.64 ± 0.02 ($n = 52$) μmol/min per mg of protein. Calmodulin decreased the $K_{0.5}$ for Ca^{2+} from 2.62 ± 0.15 ($n = 37$) to 0.46 ± 0.03 μM-Ca^{2+} ($n = 52$), and it increased the Hill coefficient from 0.79 ± 0.04 ($n = 37$) to 1.04 ± 0.03 ($n = 52$).

The effects of increasing amounts of negatively charged lipids on the $V_{max.}$ of the enzyme are illustrated in both the absence (Fig. 4*a*) and presence (Fig. 4*b*) of calmodulin. PI and PS increased the $V_{max.}$ similarly along a sigmoidal curve. PI maximally increased the $V_{max.}$ by 2.33-fold, and PS by 2.25-fold. The effect of PIP was peculiar because it was slightly inhibitory at low concentrations whereas it stimulated the enzyme at high concentrations. High concentrations of PIP, PIP_2 and PA (PIP higher than 50%, PA higher than 35% and PIP_2 higher than 20%) inhibited the erythrocyte enzyme as was observed for the smooth-muscle enzyme. The increase in the $V_{max.}$ at the maximal effective concentration was 2.23-fold for PIP, 1.56-fold for PIP_2 and 1.63-fold for PA. In the low concentration range, PIP_2 was again the lipid with the most pronounced effect on the $V_{max.}$.

In the absence of negatively charged lipids (100% PC), 0.6 μM-calmodulin only slightly increased the $V_{max.}$ of the Ca^{2+}-activation curve from 1.33 ± 0.04 ($n = 37$) to 1.64 ± 0.02 ($n = 52$) μmol/min per mg of protein. This effect disappeared on substituting a large fraction of PC by negatively charged lipids. In the presence of calmodulin, low concentrations of PI and PIP slightly decreased the $V_{max.}$ of the ATPase.

The effects of the negatively charged lipids on the Ca^{2+} affinity of the Ca^{2+}-transporting ATPase are illustrated in Fig. 4, both in the absence (Fig. 4*c*) and in the presence (Fig. 4*d*) of calmodulin. In the absence of calmodulin, each of the lipids decreased the $K_{0.5}$ for Ca^{2+}. PI (at concentrations above 20%), PS and PA were about equally effective. PIP_2 was the most effective phospholipid, with PIP next. Unlike the curves describing the effect of the negatively charged phospholipids on the $V_{max.}$ the $K_{0.5}$ curves did not present a maximum effect followed by a decrease, e.g. at 100% PIP the affinity for Ca^{2+} was higher than at 50%, while the $V_{max.}$ was 0.58-fold lower. The effect of low concentrations of PI (< 20%) was characterized by a decrease of the affinity of the enzyme for Ca^{2+}. This finding confirms the observations made by Niggli *et al.* (1981) and by Choquette *et al.* (1984).

Calmodulin (0.6 μM) decreased the $K_{0.5}$ for Ca^{2+} from 2.62 ± 0.15 μM ($n = 37$) to 0.46 ± 0.03 μM-Ca^{2+} ($n = 52$). In the presence of calmodulin, PI, PA and PS did not decrease the $K_{0.5}$ for Ca^{2+} any more, while PIP and especially PIP_2 further increased the affinity for Ca^{2+}. A peculiar finding is that PI decreased the affinity for Ca^{2+} both in the presence and in the absence of calmodulin. In the absence of calmodulin, the PI content had to be increased to over 20% before an increase in the affinity could be observed.

Table 2 shows the effects of the lipids on the Hill coefficient for Ca^{2+}. In the absence of calmodulin and in the presence of 100% PC, a slight negative co-operativity was observed (Hill coefficient of 0.79). PI, PIP and PS, and to a lesser extent PIP_2 and PA, slightly increased the Hill coefficient. This effect disappeared at maximal concentrations of the lipids (results not shown). Calmodulin (0.6 μM) increased the Hill coefficient at 100% PC to 1.04, and low concentrations of PI, PIP and PS further slightly increased the Hill coefficient. The positive co-operativity disappeared again at higher concentrations of these lipids (results not shown).

DISCUSSION

General effects of the acidic lipids

The present experiments show that the negatively charged lipids increased the $V_{max.}$ and the affinity for Ca^{2+}, and slightly increased the co-operativity for Ca^{2+}, of the plasma-membrane Ca^{2+}-transporting ATPase from erythrocytes and from stomach smooth muscle. The combined effects on the $V_{max.}$, the $K_{0.5}$ and the co-operativity for Ca^{2+} result in a more pronounced stimulation of the specific activity of the ($Ca^{2+} + Mg^{2+}$)-ATPase at low free Ca^{2+} concentrations than at high concentrations. The relative potency of the negatively charged phospholipids in stimulating the ($Ca^{2+} + Mg^{2+}$)-ATPase resembled the sequence of those lipids according to the number of negative charges at physiological pH ($PIP_2 > PIP > PA > PI \approx PS > PC$).

Effect of the lipids on the $V_{max.}$

The effect of the negatively charged lipids on the $V_{max.}$ of both ATPases was much more complex than their effect on the affinity for Ca^{2+}. The trend of the negatively charged lipids to increase the $V_{max.}$ was counterbalanced by two effects: (1) high concentrations of PIP and especially of PIP_2 and PA reduced the $V_{max.}$ for both ATPases, and (2) very low concentrations of PI reduced the $V_{max.}$ in the absence of calmodulin, and PI and PIP exerted a similar action in the presence of calmodulin. The second effect was only observed for the erythrocyte enzyme. The inhibition of the ATPase activity by high concentrations of PIP, PIP_2 and PA may be related to the

requirement for a minimal amount of neutral phospholipids for the activity of the ATPase. If an important fraction of PC is replaced by PIP, PIP_2 or PA, the latter phospholipids may compete for such critical sites and thereby reduce the ATPase activity (Nelson & Hanahan, 1985).

Effects of the lipids on the $K_{0.5}$ for Ca^{2+}

All of the negatively charged lipids increased the affinity of the enzymes for Ca^{2+}. Only low concentrations of PI did not increase the affinity of the erythrocyte ATPase. The relative efficiency of the different lipids was $PIP_2 > PIP > PI \approx PA \approx PS$. This sequence was the same as that found for the effects on the $V_{max.}$ of the ATPase from smooth muscle. A comparison with the $V_{max.}$ of the erythrocyte enzyme is difficult because of the complexity of the curves (Fig. 4*a*). Vrolix *et al.* (1988) noted that PIP was more effective than PIP_2 in stimulating the ATPase of pig stomach at 0.3 μM-Ca^{2+}. This discrepancy with our results may be related to the fact that in their study, PS was the bulk lipid, whereas in the present study PC was always the main lipid. We have observed in our experiments a clear correlation between the number of negative charges and the efficiency of the lipids in increasing the affinity of the enzyme for Ca^{2+}. A decrease in the $K_{0.5}$ for Ca^{2+} of the human erythrocyte Ca^{2+}-transporting ATPase has already been reported in this presence of PIP_2 and PA (Choquette *et al.*, 1984). In that study, PIP_2 was also more effective than PA. An increased affinity of the enzyme in the presence of PIP has also been reported for the human erythrocyte Ca^{2+}-transporting ATPase (Enyedi *et al.*, 1987).

Effects of the lipids on the Hill coefficient for Ca^{2+}

PI, PIP and PS, and to a lesser extent PIP_2 and PA, induced a slight increase in the Hill coefficient of the Ca^{2+}-activation curves. The effect was observed both in the absence and the presence of calmodulin, indicating that the lipids did not exert this effect by interacting with calmodulin. In contrast, Enyedi *et al.* (1987) reported that PIP abolished the calmodulin-induced positive co-operativity of the human erythrocyte Ca^{2+}-transporting ATPase. However, this finding is not in contradiction with our results. It can be explained by the high concentration of PIP (higher than 10 mg of PIP/mg of ATPase) used in Enyedi's experiments. We also observed that the highest concentrations of PIP which we used prevented the co-operative effect of calmodulin.

Differences between the smooth-muscle and the erythrocyte Ca^{2+}-transporting ATPases

Several observations indicate that the erythrocyte plasma-membrane Ca^{2+}-transporting ATPase functionally differs from the smooth-muscle plasma-membrane Ca^{2+}-transporting ATPase. (1) The increase in the $V_{max.}$ caused by PI, PA and PS was more pronounced for the erythrocyte enzyme than for the smooth-muscle enzyme. This difference between the two ATPases was less pronounced with PIP and non-existent with PIP_2. (2) The increase in the $V_{max.}$ of the erythrocyte Ca^{2+}-transporting ATPase induced by PI and PIP exhibited a strong positive co-operativity towards these lipids, while the stimulation of the stomach enzyme did not show such co-operative behaviour. The Hill coefficients for activation of the smooth-muscle $(Ca^{2+}+Mg^{2+})$-ATPase at 1 μM-Ca^{2+} by PI and PIP were respectively 1.18 ± 0.10 ($n = 15$) and 0.97 ± 0.12 ($n = 15$). In contrast, the stimulation of the erythrocyte Ca^{2+}-transporting ATPase by PI and PIP exhibited a strong co-operativity towards these lipids [Hill coefficients for the activation curves by PI and PIP of respectively 1.97 ± 0.26 ($n = 13$) and 2.12 ± 0.14 ($n = 13$)]. A comparison between the co-operative behaviour of stimulation of the enzyme by PIP_2 and PA has not been performed, because the inhibition at low concentrations of these lipids precluded detailed analysis of the $(Ca^{2+}+Mg^{2+})$-ATPase as a function of the lipid concentration. The difference in co-operativity for PS was less pronounced [Hill coefficients of 0.99 ± 0.19 ($n = 15$) for the muscle enzyme and 1.20 ± 0.13 ($n = 13$) for the erythrocyte enzyme]. The Hill coefficient for activation of the ATPase by the lipids was independent of the free Ca^{2+} concentration. (3) The decrease in the $K_{0.5}$ for Ca^{2+} caused by PI, PA and PS was more pronounced for the smooth-muscle enzyme. The difference between the two enzymes was hardly detectable with PIP, and was absent with PIP_2. (4) Low concentrations of PI reduced the Ca^{2+} affinity of the erythrocyte enzyme, but not that of the smooth-muscle enzyme. (5) The erythrocyte enzyme was stimulated to a greater extent by calmodulin than was the smooth-muscle enzyme. (6) The erythrocyte enzyme was 20 times less sensitive to an inhibition by AlF_4^- than was the smooth-muscle enzyme (Missiaen *et al.*, 1989).

As both plasma-membrane Ca^{2+} pumps were similarly prepared and finely reactivated by an identical mixture of phospholipids, these differences might be ascribed to isoform diversity of the plasma-membrane Ca^{2+} pumps. Up until now, three different cDNAs encoding a plasma-membrane Ca^{2+} pump have been cloned and sequenced (Shull & Greeb, 1988; Verma *et al.*, 1988). Shull & Greeb (1988) described two distinct isoforms of the plasma-membrane Ca^{2+} pump in rat brain, and Verma *et al.* (1988) detected a third isoform in a human teratoma cell line. Only limited information on the amino acid sequence of the erythrocyte plasma-membrane Ca^{2+} pump is available (James *et al.*, 1987, 1988; Filoteo *et al.*, 1987), and the primary structure of the smooth-muscle Ca^{2+} pump is still completely unknown. However, in view of the isoform diversity already known, it is very possible that smooth muscle and erythrocytes express two distinct isoforms of the plasma-membrane Ca^{2+} pump. Such difference could explain the discrepancy in their functional modification by calmodulin, negatively charged phospholipids and AlF_4^-.

Conclusions

The Ca^{2+}-transporting ATPases of plasmalemma of smooth-muscle cells and erythrocytes are stimulated by negatively charged lipids, and these ATPases behave differently in this respect. It has to be determined whether the relationship between the effectiveness in stimulating the ATPase and the number of negative charges on the lipid is purely coincidental, or whether it is causally related. Further experiments are also needed to discriminate between different modes of activation. One possibility is that binding of the lipids to the ATPase is mainly determined by their hydrophobic moiety, while the hydrophilic head group could be the site for the

functional modification of the transport enzyme. Alternatively, the head group could also determine the binding of the lipid to the ATPase.

This work was supported by the FGWO, Belgium.

REFERENCES

Berridge, M. (1988) Proc. R. Soc. London B **234**, 359–378
Choquette, D., Hakim, G., Filoteo, A. G., Plishker, G. A., Bostwick, J. R. & Penniston, J. T. (1984) Biochem. Biophys. Res. Commun. **125**, 908–915
Enyedi, A., Sarkadi, B., Szasz, I., Bot, G. & Gardos, G. (1980) Cell Calcium **1**, 299–310
Enyedi, A., Flura, M., Sarkadi, B., Gardos, G. & Carafoli, E. (1987) J. Biol. Chem. **262**, 6425–6430
Filoteo, A. G., Gorski, J. P. & Penniston, J. T. (1987) J. Biol. Chem. **262**, 6526–6530
Gopalakrishna, A. & Anderson, W. B. (1982) Biochem. Biophys. Res. Commun. **104**, 830–836
Gopinath, R. M. & Vincenzi, F. F. (1977) Biochem. Biophys. Res. Commun. **77**, 1203–1209
James, P., Zvaritch, E. I., Shakhparanov, M. I., Penniston, J. T. & Carafoli, E. (1987) Biochem. Biophys. Res. Commun. **149**, 7–12

Received 9 March 1989/5 June 1989; accepted 14 June 1989

James, P., Maeda, M., Fisher, R., Verma, A. K., Krebs, J., Penniston, J. T. & Carafoli, E. (1988) J. Biol. Chem. **263**, 2905–2910
Jarrett, H. W. & Penniston, J. T. (1977) Biochem. Biophys. Res. Commun. **77**, 1210–1216
Kosk-Kosicka, D., Scaillet, S. & Inesi, G. (1986) J. Biol. Chem. **261**, 3333–3338
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Missiaen, L., Kanmura, Y., Wuytack, F. & Casteels, R. (1988) Biochem. Biophys. Res. Commun. **150**, 681–686
Missiaen, L., Wuytack, F., De Smedt, H., Amant, F. & Casteels, R. (1989) Biochem. J. **261**, 655–660
Nelson, D. R. & Hanahan, D. J. (1985) Arch. Biochem. Biophys. **236**, 720–730
Niggli, V., Adunyah, E. S. & Carafoli, E. (1981) J. Biol. Chem. **256**, 8588–8592
Shull, G. E. & Greeb, J. (1988) J. Biol. Chem. **263**, 8646–8657
Verma, A. K., Filoteo, A. G., Stanford, D. R., Wieben, E. D., Penniston, J. T., Strehler, E. E., Fischer, R., Heim, R., Vogel, G., Mathews, S., Strehler-Page, M.-A., James, P., Vorherr, T., Krebs, J. & Carafoli, E. (1988) J. Biol. Chem. **263**, 14152–14159
Vrolix, M., Raeymaekers, L., Wuytack, F., Hofmann, F. & Casteels, R. (1988) Biochem. J. **255**, 855–863
Wuytack, F., De Schutter, G. & Casteels, R. (1981) FEBS Lett. **129**, 297–300