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Inhibition of the phosphatase activity of the red cell membrane Ca^{2+} pump by acidic phospholipids

Juan P.F.C. Rossi and Ariel J. Caride

Instituto de Química y Fisicoquímica Biológicas (UBA-CONICET), Facultad de Farmacia y Bioquímica, Buenos Aires (Argentina)

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The effect of phospholipids was tested on the *p*-nitrophenylphosphatase activity of the Ca^{2+} pump. Acidic phospholipids like phosphatidylserine and phosphatidylinositol inhibited the phosphatase activity, while neutral phospholipids like phosphatidylcholine did not. This result contrasts sharply with the known activating effect of acidic phospholipids on the Ca^{2+}-ATPase activity of the pump. It is known that the phosphatase activity of the Ca^{2+} pump can be elicited either by calmodulin and Ca^{2+} or by ATP and Ca^{2+}. Unlike calmodulin, acidic phospholipids failed to stimulate the phosphatase activity. Furthermore, calmodulin-activated phosphatase was completely inhibited by acidic phospholipids. Maximal inhibition of the ATP-activated phosphatase was only 70%. Inhibition by acidic phospholipids was non-competitive regarding to calmodulin, suggesting that acidic phospholipids and calmodulin do not bind to the same domain of the pump. The presence of Ca^{2+} was essential for the inhibition, and the apparent affinity for Ca^{2+} for this effect was increased by acidic phospholipids. Results are consistent with the idea that acidic phospholipids stabilize an enzyme–Ca complex lacking phosphatase activity.

Introduction

It is well known that acidic phospholipids increase both the turnover and the apparent affinity of the Ca^{2+}-ATPase for Ca^{2+} [1]. Furthermore, we have reported [2] that the calmodulin antagonist compound 48/80, inhibits the effects of calmodulin or phosphatidylserine with similar apparent affinity. Although Enyedi et al. [3] suggested that the mechanisms by which calmodulin and acidic phospholipids increase the affinity of the Ca^{2+}-ATPase for Ca^{2+} are different, to the best of our knowledge there is no information about how these two modulators interact to activate the Ca^{2+}-ATPase. The calmodulin binding domain has been sequenced and localized on the C-terminal region of the Ca^{2+}-ATPase molecule [4,5]. However, the localization of the domain in which acidic phospholipids interact with the Ca^{2+} pump is still unclear [6].

Previous work from this laboratory showed that, like other E_1,E_2-type ATPases, the Ca^{2+}-ATPase from human red cells displays a phosphatase activity towards *p*-nitrophenylphosphate [7]. This Ca^{2+}-phosphatase activity is expressed only in media with ATP and/or calmodulin [8,9] or in membranes submitted to partial proteolysis with trypsin [10], a treatment that mimics the effect of calmodulin [11]. After partial proteolysis, Ca^{2+} is no longer needed for activation of the phosphatase [10].

With the aim of providing new information about the mechanism by which acidic phospholipids activates the Ca^{2+} pump, in this paper we show the results of experiments designed to test the effect of acidic phospholipids on the hydrolysis of *p*-nitrophenylphosphate catalyzed by the Ca^{2+} pump. Unlike calmodulin, the main effect of acidic phospholipids was to inhibit the phosphatase activity regardless of whether this activity was elicited by calmodulin and Ca^{2+} or by ATP and Ca^{2+}. The results denote that the effects of acidic phospholipids and calmodulin on the Ca^{2+}-ATPase are exerted through different mechanisms. Moreover, the lack of competition between calmodulin and acidic phospholipids for the Ca^{2+}-ATPase suggest that they bind to different domains in the pump.

Materials and Methods

Fresh blood from hematologically normal adults collected on acid/citrate/dextrose solutions was always used. Red cell membranes were prepared following the

Correspondence: J.P.F.C. Rossi, Instituto de Química y Fisicoquímica Biológicas (UBA-CONICET), Facultad de Farmacia y Bioquímica, Junín 956, (1113) Buenos Aires, Argentina.

procedure of Gietzen et al. [12] as follows: 1 vol. of red cells (washed three times with 150 mM NaCl) were lysed in 8 vol. of lysing solution (1 mM EGTA, 15 mM Tris-HCl (pH 7.4)) at 4°C. Membranes were spun down at $17000 \times g$ during 20 min, and washed twice with lysing solution. Then the membranes were suspended in 8 vol. of lysing solution, incubated 15 min at 37°C in this solution and spun down at $17000 \times g$ during 20 min. This step was repeated once. Then membranes were washed with 8 vol. of 15 mM Tris-HCl (pH 7.4), resuspended in 1 vol. of the same solution, and stored at −20°C. This procedure yields membranes devoid of endogenous calmodulin. Calmodulin was purified from bovine brain as described by Kakiuchi et al. [13].

For treatment with chymotrypsin, the membranes were washed and suspended in media containing: 120 mM KCl, 30 mM Tris-HCl (pH 7.40 at 37°C) and 10 μg/ml of N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated α-chymotrypsin (60 units/mg). The mixture was incubated at 4°C and after 2 min chymotrypsin action was terminated by the addition of soybean trypsin inhibitor (final concentration: 200 μg/ml). Control experiments (not shown), indicated that after this treatment the Ca^{2+}-ATPase activity was about 80% of the initial and was insensitive to calmodulin.

Phosphatase activity was measured estimating the release of *p*-nitrophenol from *p*-nitrophenylphosphate [1]. Except otherwise indicated in Results, the incubation media contained: 120 mM KCl, 6.25 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.40 at 37°C), 10 mM *p*-nitrophenylphosphate, 1.0 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid), 0 or 0.5 mM ATP, 0 or 120 nM calmodulin, 1 mM ouabain, 60–80 μg/ml membrane protein and the concentrations of $CaCl_2$ necessary to give the free Ca^{2+} concentrations indicated in the legends of tables and figures. Ca^{2+}-ATPase activity was measured at 37°C in similar media to those used for the estimation of the phosphatase activity, but without the addition of *p*-nitrophenylphosphate and with a final concentration of 2 mM ATP. The concentration of $MgCl_2$ in such media was 3.75 mM. The release of inorganic phosphate from the nucleotide was estimated by a modification of the procedure of Fiske and SubbaRow [14]. To estimate the Ca^{2+}-dependent activities, the activities measured in similar media of those described above, but without the addition of $CaCl_2$ were subtracted. Phospholipid liposomes were obtained by sonicating during 50 s a mixture of 1 mg of pospholipid in 1 ml of the reaction media at 4°C. The procedure was repeated four times more. Membranes were added to this reaction media, mixed and the mixture was preincubated 1 min at 4°C. The reaction was started by transferring the tubes to a 37°C bath. Free Ca^{2+} concentration in the incubation media was measured with an IS-561 Ca^{2+}-electrode [15]. Protein was estimated by the method of Lundahl [16].

Phosphatidylserine, phosphatidylinositol, phosphatidylcholine, *p*-nitrophenylphosphate and α-chymotrypsin were obtained from Sigma (U.S.A.). Salts and reagents were of analytical reagent grade.

Except otherwise indicated, the experiments presented under Results were chosen as representative of two to four experiments. Each of the measurements was performed by triplicate and the individual values did not differ from the mean more than 10%. Equations were adjusted to the experimental results by least-squares non-linear regression, by using the algorithm of Gauss-Newton with optional damping [17]. The concentration variables were assumed to have negligible error and the velocity variable to be homoscedastic. Except otherwise indicated, the equations used to fit the experimental points were chosen among several others on the basis of their best fitting as judged by the minimum standard deviation of the regression. Standard deviation of the regression is the sum of the square errors divided by the number of parameters. Calculations were performed with a microcomputer Epson Equity III + . The program used allows to fit any function with up to two independent variables and up to 15 adjustable parameters and their standard deviations [18].

Results

Comparison of the effects of phospholipids on Ca^{2+}-ATPase and Ca^{2+}-phosphatase activities

Table I allows one to compare the effect of 500 μg/ml of phosphatidylcholine, phosphatidylserine and

TABLE I

Effect of phospholipids on Ca^{2+}-ATPase and Ca^{2+}-phosphatase activities

Phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) was 500 μg/ml of liposomes. Ca^{2+} was 12 μM, except for CaM-phosphatase where Ca^{2+} was 1.5 μM, calmodulin was 120 nM. pNP stands for *p*-nitrophenol. Values are expressed as mean ± S.E. of three independent experiments.

Addition	Ca^{2+}-ATPase (nmol PI/mg protein per min)		Ca^{2+}-phosphatase (nmol pNP/mg protein per min)		
	−CaM	+CaM	+CaM	+ATP	+ATP +CaM
None	2.3 ±0.4	33.3 ±0.8	5.6 ±0.3	3.5 ±0.1	10.7 ±0.5
PC	2.3 ±0.3	33.5 ±0.6	5.7 ±0.1	3.3 ±0.2	10.6 ±0.3
PS	17.8 ±0.9	33.0 ±1.1	1.7 ±0.2	2.9 ±0.1	6.1 ±0.4
PI	12.4 ±0.7	32.3 ±2.0	1.0 ±0.1	1.7 ±0.2	5.1 ±0.3

phosphatidylinositol on Ca^{2+}-ATPase and phosphatase activities. Neither Ca^{2+}-ATPase nor Ca^{2+}-phosphatase was modified by addition of phosphatidylcholine in any of the conditions tested. On the other hand, in the absence of calmodulin addition of acidic phospholipids increased Ca^{2+}-ATPase activity around 7-times, whereas the activity in the presence of calmodulin was not modified by the phospholipids. Table I also shows that phosphatase activities were inhibited by the acidic phospholipids. As has been pointed out, the phosphatase activity of the Ca^{2+} pump can be elicited in two ways: (i) in the presence of calmodulin and low concentration of Ca^{2+} (0.2–5 μM) (CaM-phosphatase) and (ii) in the presence of ATP and Ca^{2+} (1–100 μM) (ATP-phosphatase). Although both phosphatase activities were inhibited, the CaM-phosphatase was the most sensitive to the inhibition by acidic phospholipids. Table I also shows that phosphatidylinositol was more effective in blocking of phosphatase activity than phosphatidylserine, regardless of how this activity was elicited. Nevertheless, we did not find qualitative differences between the mode of action of these two phospholipids, and phosphatidylserine was used in most of the experiments because of its lower cost.

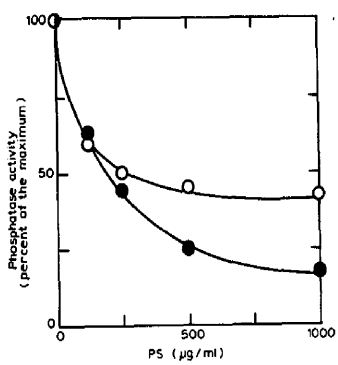

Fig. 1. Effects of increasing concentrations of phosphatidylserine liposomes on CaM-phosphatase and ATP-phosphatase activity of red cell membranes stripped of their endogenous calmodulin and incubated in media containing 2.0 μM Ca^{2+} and 120 nM calmodulin (●) or 12.0 μM Ca^{2+} and 0.5 mM ATP (○). Membrane protein concentration in the assay media was 65 μg/ml. The curves that fit the experimental points represents the equation:

$$v = \frac{V_0}{(1+[PS]/K_i)} + V_r \qquad (1)$$

where V_0 is the fraction of the activity that is sensitive to phosphatidylserine, V_r the activity that is insensitive to inhibition, [PS] the concentration of phosphatidylserine, and K_i the concentration of phosphatidylserine for half-maximal inhibition; with $V_0 = 14.7 \pm 1.1$ nmol *p*-nitrophenol/mg protein per min ((●) 100%); $K_i = 193 \pm 48$ μg/ml and $V_r = 0.12 \pm 0.2$ nmol *p*-nitrophenol/mg protein per min for CaM-phosphatase and $V_0 = 10.4 \pm 0.3$ nmol *p*-nitrophenol/mg protein per min ((○) 100%); $K_i = 66 \pm 8$ μg/ml and $V_r = 6.5 \pm 0.2$ nmol *p*-nitrophenol/mg protein per min for ATP-phosphatase.

Control experiments designed to follow the time-course of activation of the Ca^{2+}-ATPase by acidic phospholipid liposomes under the conditions used for the experiment in Table I, showed that the activity was the highest 1 min after the addition of the liposomes to the assay medium and remained constant after that time. This confirms previous data of Ronner et al. [19] showing that phosphatidylserine added to the assay media rapidly activates the Ca^{2+}-ATPase from red cell membranes partially depleted of their phospholipids. Approximately the same protein concentration was used in all the experiments and no attempt was made to test the effect of acidic phospholipids at different protein concentrations. However, at the concentrations used in all the experiments shown throughout this paper, acidic phospholipids were activators of the Ca^{2+}-ATPase.

Fig. 1 shows the results of an experiment in which the ATP-phosphatase and the CaM-phosphatase activities were measured as a function of increasing concentrations of phosphatidylserine. It can be seen that both CaM-phosphatase activity and ATP-phosphatase activity followed curves that could be fitted by rectangular hyperbolae (Eqn. 1 in the legend of Fig. 1). CaM-phosphatase tended to a value not significantly different from zero, and the K_i for inhibition of this activity was 193 μg/ml. On the other hand, ATP-phosphatase decreased to 30% of the activity in the absence of phosphatidylserine. In this case the value of K_i was 66 μg/ml.

Effect of phosphatidylserine on the velocity versus calmodulin concentration curve of the CaM-phosphatase

Fig. 2 shows the results of an experiment designed to test whether phosphatidylserine and calmodulin interact for the same domain in the Ca^{2+} pump during phosphatase activity. With this purpose, phosphatase activity was measured as a function of calmodulin concentration in the presence of different amounts of phosphatidylserine. It can be seen that calmodulin increased the phosphatase activity from zero to its maximal value along hyperbolic curves, regardless of the concentration of posphatidylserine. Results also show that a similar concentration of calmodulin was required for half-maximum effect, regardless the concentration of phosphatidylserine. Assuming simple Michaelis-Menten kinetics, a plot of the best fitting values of $K_{0.5}$ and apparent V_m from the curves in Fig. 2 (not shown), showed that the values of $K_{0.5}$ were about 25 nM calmodulin and remained unaffected by the phospholipid, while apparent V_m decreased with phosphatidylserine along a rectangular hyperbola. The best fit to the experimental points was obtained with Eqn. 2, in the legend of Fig. 2. This equation implies that the inhibition of the CaM-phosphatase activity by phosphatidylserine is complete and non-competitive with respect to calmodulin. Equations that imply competitive inhibition, or mixed-type inhibi-

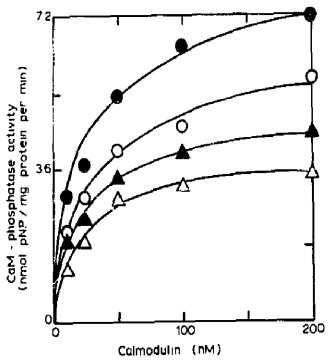

Fig. 2. CaM-phosphatase activity of red cell membranes as a function of the concentration of calmodulin and either 0 (●), 62.5 (○), 125 (▲) and 250 (△) μg/ml of phosphatidylserine, in the presence of 2.0 μM Ca^{2+}. The curves that fit the experimental points represent the equation:

$$v = \frac{V_m}{(1 + K_{CaM}/[CaM])(1 + [PS]/K_i)} \quad (2)$$

where [CaM] is the concentration of calmodulin, K_{CaM} is the [CaM] value for half-maximal activation, V_m is the maximal velocity and the other parameters have the same meaning as in Eqn. 1. The values ± S.E. were: $V_m = 8.0 \pm 0.2$ nmol *p*-nitrophenol/mg protein per min; $K_{CaM} = 25.4 \pm 1.8$ nM and $K_i = 230 \pm 14$ μg/ml.

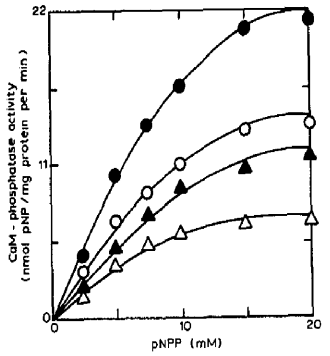

Fig. 3. CaM-phosphatase activity as a function of *p*-nitrophenyl phosphate (pNPP) concentration in media containing 0 (●), 137 (○), 274 (▲) and 550 (△) μg/ml of phosphatidylserine, in the presence of 1.0 μM Ca^{2+}. The following equation was fitted to the whole set of points:

$$v = \frac{V_m}{(1 + K_p/[pNPP])^2 (1 + [PS]/K_i)} \quad (4)$$

where [pNPP] is the concentration of *p*-nitrophenyl phosphate, K_p is the [pNPP] value for half-maximal activation and the other parameters have the meaning as in Eqn. 2. The values ± S.E. were: $V_m = 22.8 \pm 0.7$ nmol *p*-nitrophenol/mg protein per min; $K_p = 3.53 \pm 0.18$ mM and $K_i = 289 \pm 12$ μg/ml.

tion gave poorer fittings. The fact that phosphatidylserine inhibited the CaM-phosphatase in a non-competitive fashion with respect to calmodulin suggests that calmodulin and phosphatidylserine bind to different domains in the Ca^{2+}-ATPase.

Effect of phosphatidylserine on the velocity versus p-nitrophenylphosphate concentration curve of the CaM-phosphatase

Although they differ in their chemical structure, both phosphatidylserine and *p*-nitrophenylphosphate are constituted by a hydrophobic moiety and a phosphate group. So that, at pH 7.4, both are negatively charged. This raises the possibility that phosphatidylserine inhibits the phosphatase activity by displacing *p*-nitrophenylphosphate from its site. To test this, we measured the effects of phosphatidylserine on CaM-phosphatase as a function of *p*-nitrophenylphosphate in the presence of 0, 137, 274 and 550 μg/ml of phosphatidylserine. As previously discussed in extenso (see Ref. 2), the phosphatase activity vs. *p*-nitrophenylphosphate concentration curve is slightly sigmoidal, and can be adequately described by Eqn. 3. Thus, for each phosphatidylserine concentration in Fig. 3, Eqn. 3 was fitted to the data.

$$v = \frac{V_m}{(1 + K_p/[pNPP])^2} \quad (3)$$

Curves with an apparent dissociation constant for *p*-nitrophenylphosphate (K_p) near 3.5 mM and a maximum effect (V_m), which decreased hyperbolically with phosphatidylserine concentration, were obtained. On this basis, Eqn. 4 (in the legend of Fig. 3), corresponding to a non-competitive interaction between *p*-nitrophenylphosphate and phosphatidylserine, was adjusted to the whole set of points. The values of the parameters are given in the legend of Fig. 3. It can be seen that the value of K_i (289 μg/ml) was close to that found in the experiments of Figs. 1 and 2, and that the value of K_p (3.5 mM) was close to that we have reported before [4]. From the results, it is evident that inhibition of the CaM-phosphatase activity by phosphatidylserine is not because of competition of the phospholipid with *p*-nitrophenylphosphate for its site in the Ca^{2+}-ATPase.

Effect of phosphatidylserine and phosphatidylinositol on the response of the CaM-phosphatase activity to Ca^{2+}

In the experiment of Fig. 4 the phosphatase activity of red cell membranes was measured as a function of Ca^{2+} in control media with 120 nM calmodulin, in media with 150 μg phosphatidylserine in the absence of calmodulin, and in media with 120 nM calmodulin and either 150 μg/ml of phosphatidylserine or 150 μg/ml of phosphatidylinositol. As described earlier [4], in media with calmodulin, as Ca^{2+} concentration is in-

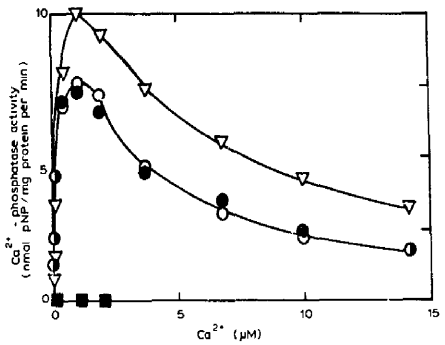

Fig. 4. Ca^{2+}-phosphatase activity as a function of Ca^{2+} concentration in control media (▽) (mean of four independent experiments); in media with 150 μg/ml of phosphatidylserine (○) (mean of three independent experiments); in media with 150 μg/ml of phosphatidylinositol (●) and in media without calmodulin and 150 μg/ml of phosphatidylserine (■) (single experiment). The kinetic parameters obtained for these experiments were shown in Table II.

creased, the phosphatase activity raised, passes through a maximum, and then drops tending to zero. Experimental data were fitted using Eqn. 5.

$$v = \frac{V_m}{\left(1+(K_{Ca}/[Ca^{2+}])^2\right)(1+[Ca^{2+}]/K_{iCa})} \quad (5)$$

where K_{Ca} is the concentration of Ca^{2+} for half-maximal activation; K_{iCa} is the concentration of Ca^{2+} for half-maximal inhibition and V_m has the same meaning as in Eqns. 2 and 4. This empirical equation includes a quadratic term describing the activation of phosphatase activity by Ca^{2+} and a term describing the inhibition at higher concentrations of Ca^{2+}. The values of the kinetic parameters are shown in Table II. In control media, activation by Ca^{2+} took place with high apparent affinity (K_{Ca} near 0.4 μM), and the concentration of Ca^{2+} for half-maximal inhibition was near 10-times higher (K_{iCa} = 4.4 μM). At lower concentrations of Ca^{2+}, the acidic phospholipids affected only marginally the K_{Ca} for activation and did not modify V_m. On the other hand, both phosphatidylserine and phosphatidylinositol significantly decreased the CaM-phosphatase activity by lowering the Ca^{2+} concentration required to inhibit this activity (K_{iCa} = 2.2 μM). It seems, therefore, that the acidic phospholipids inhibit the CaM-phosphatase activity by modulating the affinity for Ca^{2+} of the inhibitory component of the curve.

In Fig. 4 it can also be seen that in the absence of calmodulin, phosphatidylserine did not promote hydrolysis of *p*-nitrophenylphosphate at any of the concentrations of Ca^{2+} assayed (from 0 to 2.5 μM Ca^{2+}).

TABLE II

Kinetic parameters of the effects of acidic phospholipids on CaM-phosphatase activity

The best fitting values and their standard errors were obtained by adjusting Eqn. 5 by non-linear regression to the data in Fig. 4. In comparison with the control ** $P < 0.001$; * $P < 0.01$. PS; phosphatidylserine; PI, phosphatidylinositol; pNP, *p*-nitrophenol.

Addition	V_m (nmol pNP/mg protein per min)	K_{Ca} (μM)	K_i (μM)
None (control)	14.0±1.3	0.382±0.043	4.36±0.37
PS	14.0±1.8	0.332±0.042	2.27±0.56 **
PI	13.4±1.6	0.360±0.039	2.04±0.80 *

Effect of phosphatidylserine on the inhibition by Ca^{2+} of the proteolysed Ca^{2+}-ATPase

We have previously demonstrated that after controlled proteolysis with trypsin of calmodulin-depleted membranes, neither calmodulin nor Ca^{2+} is required for phosphatase activity [10]. In this condition, phosphatase activity is maximum in the absence of Ca^{2+} and is inhibited by micromolar concentrations of Ca^{2+} [10]. Trypsination of the Ca^{2+} pump generates a fragment of 81 kDa that has high affinity for Ca^{2+}, but is still sensitive to acidic phospholipids and a fragment of 75 kDa that is no longer sensitive to acidic phospholipids [3]. Phosphatase activity can be also elicited by treating the membranes with TLCK-treated α-chymotrypsin (our unpublished data). This condition was cho-

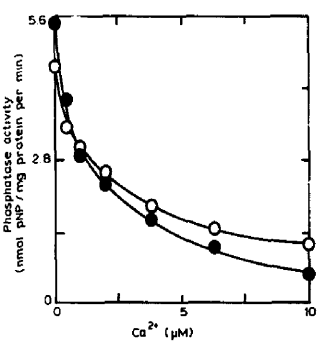

Fig. 5. Phosphatase activity as a function of Ca^{2+} concentration in membranes submitted to controlled proteolysis with TLCK-treated α-chymotrypsin. Incubations were performed in media with (●) and without (○) 150 μg/ml of phosphatidylserine. To the experimental values of both curves the phosphatase activity remnant at 50 μM Ca^{2+} for either condition was subtracted. The following equation was adjusted to the experimental points:

$$v = \frac{V_0}{1+[Ca^{2+}]/K_{iCa}} \quad (6)$$

where the parameters have the same meaning as in Eqns. 1 and 5; with V_0 = 4.7±0.2 nmol *p*-nitrophenol/mg protein per min; K_i = 2.5 ±0.3 μM for the control curve and V_0 = 5.5±0.1 nmol *p*-nitrophenol/mg protein per min and K_i = 1.4±0.1 μM for the curve of the phosphatidylserine-treated membranes.

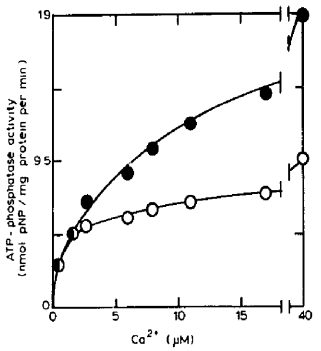

Fig. 6. ATP-phosphatase activity as a function of Ca^{2+} concentration in media with (○) and without (●) 1000 μg/ml of phosphatidylserine. The following equation was adjusted to the experimental values:

$$v = \frac{V_m}{1 + K_{Ca}/[Ca^{2+}]} \quad (7)$$

with $V_m = 22.3 \pm 2.7$ nmol *p*-nitrophenol/mg protein per min; $K_{Ca} = 10.4 \pm 4$ μM for the curve without phosphatidylserine and $V_m = 7.8 \pm 0.7$ nmol *p*-nitrophenol/mg protein per min; $K_{Ca} = 1.0 \pm 0.5$ μM for the curve with phosphatidylserine.

sen to study the effect of phosphatidylserine on the inhibition of the phosphatase activity by Ca^{2+} because chymotryptic proteolysis does not produce fragments insensitive to acidic phospholipids [6].

Fig. 5 shows the results of an experiment in which the phosphatase activity of chymotrypsin-treated membranes was measured as a function of Ca^{2+} concentration in the absence and in the presence of 150 μg/ml of phosphatidylserine. In the presence of phosphatidylserine a small but significant increase in the value of V_0 was observed with respect to the control ($P < 0.001$). Results in Fig. 5 also show that phosphatidylserine increased with high significance ($P < 0.001$) the affinity for Ca^{2+} as inhibitor of the phosphatase. This result agrees with the fact that the acidic phospholipid decreased the K_{iCa} of the CaM-phosphatase activity (Fig. 4). Of course, since there is a slight basic activation of the chymotrypsin-treated enzyme by phosphatidylserine at zero Ca^{2+}, a certain Ca^{2+} concentration must be present for the inhibition to be seen.

Effect of phosphatidylserine during activation of ATP-phosphatase by Ca^{2+}

Fig. 6 shows the ATP-phosphatase activity as a function of increasing Ca^{2+} concentrations. Under control conditions Ca^{2+} activated the phosphatase with $K_{Ca} = 10.4 \pm 4.0$ μM and $V_m = 22.3 \pm 2.7$ nmol of *p*-nitrophenol/mg protein per min. Addition of 1000 μg/ml of phosphatidylserine liposomes decreased the value of K_{Ca} to 1.0 ± 0.5 μM and the value of V_m to 7.8 ± 0.7 nmol of *p*-nitrophenol/mg protein per min. The decrease in K_{Ca} by phosphatidylserine is similar to that described for the Ca^{2+}-ATPase activity. However, the effect of the acidic phospholipid on the V_m is the opposite of what it is described on the V_m of the Ca^{2+}-ATPase [3].

Discussion

Results in this paper show that incubations of red cell membranes with phosphatidylserine as well as phosphatidylinositol inhibited the phosphatase activity of the Ca^{2+} pump. This shows a striking difference in the effect of acidic phospholipids on Ca^{2+}-ATPase and Ca^{2+}-phosphatase activities.

The results showed that CaM-phosphatase activity was completely inhibited by phosphatidylserine. This fact indicates that, regardless of the mechanism of the interaction between the acidic phospholipids and the pump, all the enzyme molecules are affected by the acidic phospholipids.

Results in this paper also show differences on the mode of action of acidic phospholipids and calmodulin on the Ca^{2+} pump. Both activate the pump, increasing its maximal velocity and affinity for Ca^{2+}, but calmodulin, unlike acidic phospholipids, is an activator of the phosphatase activity [9,10]. Furthermore, the inhibition of CaM-phosphatase by phosphatidylserine is non-competitive with regard to calmodulin. These facts strongly suggests that the domain which acidic phospholipids interact with is different from the calmodulin-binding domain. It is worth mentioning that there are differences between the way that calmodulin and acidic phospholipids increase the affinity of the pump for Ca^{2+}, i.e., calmodulin increases the cooperativity of the Ca^{2+} concentration vs. Ca^{2+}-ATPase activity curve, whereas acidic phospholipids decrease the cooperativity of such curve [3].

Acidic phospholipids seem to modulate the inhibition of phosphatase activity exclusively by increasing the affinity for Ca^{2+} at inhibitory sites. This observation was made both for the CaM-activated phosphatase activity and for the chymotrypsin-activated phosphatase activity. In the chymotrypsin-activated phosphatase no inhibition, but a small and significant activation, was observed in the absence of Ca^{2+} whereas inhibition by acidic phospholipids was apparent at Ca^{2+} concentrations higher than 0.5 μM (Fig. 5). Moreover, in the curve of CaM-phosphatase activity vs. Ca^{2+}, the only parameter affected by phosphatidylserine was the K_{iCa}. These results indicate that Ca^{2+} is essential for the inhibition of the phosphatase activity by acidic phospholipids.

In a previous paper we suggested that binding of Ca^{2+} to the transport sites of the pump inhibits either the CaM-phosphatase and the phosphatase activated by proteolysis [10]. The fact that either in the presence of

calmodulin or in membranes treated with chymotrypsin, acidic phospholipids increase the affinity for Ca^{2+} as activator of the ATPase and as inhibitor of the phosphatase (both on the calmodulin-activated and on the chymotrypsin-activated enzyme) by approximately the same proportion (cf. results in this paper and Ref. 6), is additional evidence in favor of such a hypothesis. It is worth to note that phosphatidylserine increased 10-fold the affinity for activation by Ca^{2+} of the ATP-activated phosphatase, as was reported for the Ca^{2+}-ATPase activity [1] and for active Ca^{2+} transport [20] in the absence of calmodulin.

From the experimental evidences shown in this paper and from those of Ref. 3, we can suggest that acidic phospholipids favor a form which is different from that promoted by calmodulin. These enzymic forms differ in the following: (i) the form promoted by calmodulin is able to catalyze the hydrolysis of *p*-nitrophenylphosphate, whereas that promoted by phosphatidylserine is not. (ii) The form promoted by acidic phospholipids has higher affinity for Ca^{2+} than that elicited by calmodulin. Besides, the lack of competition between calmodulin and phosphatidylserine may suggest that there is an enzymic form associated simultaneously with calmodulin and acidic phospholipids. This form would have high affinity and no cooperativity for Ca^{2+}, but it would still be able to catalyze the hydrolysis of *p*-nitrophenylphosphate in the absence of Ca^{2+}.

Although acidic phospholipids were able to inhibit both the CaM-phosphatase and the ATP-phosphatase activities, differences were found on the extent of the inhibition: CaM-phosphatase was completely inhibited by the lipids, but the maximal inhibition produced by phosphatidylserine on the ATP-phosphatase was partial. On analyzing this result, it must be taken into account that, provided Ca^{2+} is present, in the presence of ATP but not in its absence, the enzyme is undergoing the complete cycle of Ca^{2+} transport coupled to ATP hydrolysis. Under this condition, the pump cannot be fully trapped in the enzymic form in which the phosphatase activity is blocked by Ca^{2+} and the acidic phospholipids.

The fact that acidic phospholipids are activators of the hydrolysis of ATP by the Ca^{2+} pump, and inhibitors of the hydrolysis of *p*-nitrophenylphosphate catalyzed by the same system, suggests that at least one step of the hydrolysis cycle of *p*-nitrophenylphosphate is not common with the hydrolysis cycle for ATP.

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