Pre-steady-state phosphorylation and dephosphorylation of detergent-purified plasma-membrane Ca^{2+}-ATPase

Luis M. BREDESTON and Alcides F. REGA[1]

Instituto de Química y Fisicoquímica Biológicas (Universidad de Buenos Aires-Consejo Nacional de Investigaciones Científicas y Técnicas), Facultad de Farmacia y Bioquímica, Junín 956, 1113 Buenos Aires, Argentina

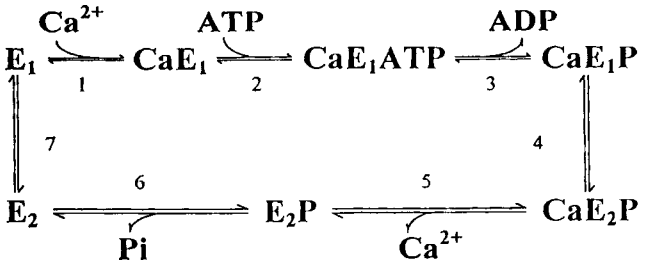
Pre-steady-state phosphorylation and dephosphorylation of purified and phospholipid-depleted plasma-membrane Ca^{2+}-ATPase (PMCA) solubilized in the detergent polyoxyethylene 10 lauryl ether were studied at 25 °C. The time course of phosphorylation with ATP of the enzyme associated with Ca^{2+}, probably the true phosphorylation reaction, showed a fast phase (k_{app} near 400 s^{-1}) followed by a slow phase (k_{app} = 23 s^{-1}). With asolectin or acidic phosphatidylinositol, the concentration of phosphoenzyme (EP) increased at as high a rate as before, passed through a maximum at 4 ms and stabilized at a steady level that was approx. half that without lipids. Calmodulin (CaM) did not change the rate of the fast phase, accelerated the slow phase (k_{app} = 93 s^{-1}) and increased [EP] with small changes in the shape of the time course. Dephosphorylation was slow (k_{app} = 30 s^{-1}) and insensitive to CaM. Asolectin accelerated dephosphorylation, which followed biexponential kinetics with fast (k_{app} = 220 s^{-1}) and slow (k_{app} = 20 s^{-1}) components. CaM stimulated the fast component by nearly 50 %. The results show that the behaviour of the PMCA is complex, and suggest that acidic phospholipids and CaM activate PMCA through different mechanisms. Acceleration of dephosphorylation seems relevant during activation of the PMCA by acidic phospholipids.

Key words: calmodulin, Ca^{2+} pump, Ca^{2+} transport, phospholipids.

INTRODUCTION

It is well established that the plasma-membrane Ca^{2+}-ATPase (PMCA) couples the hydrolysis of ATP to the extrusion of Ca^{2+} from the cytosol across the plasma membrane of most cells [1]. With up to 50 μM ATP, the hydrolysis of the nucleotide by the PMCA is believed to proceed along the series of reactions shown in Scheme 1 [1,2]. The resting enzyme is a mixture of conformers, E_1 and E_2. Because Ca^{2+} binds tightly to the transport site on E_1 and weakly on E_2, in the presence of Ca^{2+} at micromolar concentrations the equilibrium is shifted towards CaE_1.

We have reported results of experiments on the PMCA in native membranes from red blood cells, designed to study the reactions in Scheme 1 under pre-steady-state conditions by means of a rapid chemical quenching technique [3–5]. This preparation retains the enzyme in its natural environment, a condition particularly important due to the high dependency of the PMCA activity on lipids [6,7]. Since PMCA in red blood cell membranes accounts for 0.1–0.2 % by mass of the total membrane protein [1], very concentrated and viscous membrane suspensions had to be used. Although this seems not to impede rapid equilibration of the PMCA with its ligands, the membranes add large amounts of material that retain radioactivity from [γ-^{32}P]ATP.



Scheme 1 Consecutive catalytic cycle of the PMCA

Affinity chromatography on a calmodulin (CaM)–agarose column provides purified PMCA solubilized in detergent [8]. Such an enzyme preparation is ideal for characterizing lipids and CaM action because: (1) it is almost free of contaminating proteins and the radioactivity that it incorporates from [γ-^{32}P]ATP is largely phosphoenzyme (EP); (2) its specific enzymic activity is approx. 400-fold higher than that of the fragmented membranes; (3) it is free of CaM which usually contaminates membrane preparations; and (4) the quality of its accompanying lipids can be controlled. Several pieces of evidence give support to the idea that at low concentration, the PMCA solubilized in detergent is a monomer of low specific activity in contrast with the enzyme at concentrations higher than 10 μg/ml, which behaves as a dimer of high specific activity [9,10]. We have reported [11] that adequate concentrations of asolectin and the detergent polyoxyethylene 10 lauryl ether ($C_{12}E_{10}$), like those used for the experiments reported in the present study, make the PMCA exhibit constant high-specific activity independent of its concentration. Whether the enzyme is monomeric or dimeric under such conditions remains an open question.

There is only one report [2] on the kinetics of pre-steady-state phosphorylation of PMCA purified from human red blood cell membranes and solubilized in detergent. A pronounced EP overshoot with three times the level of EP at steady-state is observed.

In the present study we report results of experiments with solubilized PMCA purified from pig red blood cells (pPMCA) on

Abbreviations used: $C_{12}E_{10}$, polyoxyethylene 10 lauryl ether; CaM, calmodulin; EP, phosphoenzyme; PMCA, plasma-membrane Ca^{2+}-ATPase; pPMCA, PMCA from pig red blood cells.

[1] To whom correspondence should be addressed (e-mail rega@qb.ffyb.uba.ar).

the formation and splitting of EP under pre-steady-state conditions. We found at first that the formation of EP in the detergent-purified pPMCA was faster than in the fragmented membranes, making the measurements difficult to resolve. This persuaded us to lower the temperature from 37 °C, as in the experiments with membranes, to 25 °C. Results showed that in some aspects the kinetic behaviour of the detergent-purified pPMCA differed from that of the enzyme in the membrane. Nevertheless, the preparation allowed us to detect properties of the PMCA that could be of help in understanding its reaction mechanism and its modulation by Ca^{2+}, phospholipids and CaM.

EXPERIMENTAL

Red blood cell membranes depleted of CaM were prepared from pig red blood cells by a modification [12] of the procedure of Gietzen et al. [13]. pPMCA was isolated by affinity chromatography on a CaM–agarose column as described by Penniston et al. [8] with $C_{12}E_{10}$, K-Mops and asolectin replacing Triton X-100, Mops and phosphatidylcholine respectively [14], and 20 % (w/v) glycerol added to all media [15]. The elution buffer contained 20 mM K-Mops (pH 7.4 at 4 °C), 130 mM KCl, 1 mM $MgCl_2$, 0.5 mg/ml $C_{12}E_{10}$, 1 mg/ml asolectin, 2 mM dithiothreitol, 20 % (w/v) glycerol and 1 mM EGTA. Phospholipid-depleted pPMCA was obtained by the same procedure except that asolectin was absent from all the media and the CaM–agarose column with the enzyme bound was washed with 30 column volumes of the washing buffer. No measurements were made concerning the amount of phospholipids that could have remained with the enzyme. Nevertheless, assuming that the activity of the soluble enzyme was fully dependent on asolectin, as judged by the ATPase activity in the absence and presence of asolectin, delipidation might have been close to 80 %. Protein concentration was measured by the method of Lowry et al. [16] after the protein was precipitated using deoxycholate and trichloroacetic acid as described by Peterson [17]. BSA was used as a standard.

Phosphorylation from [γ-^{32}P]ATP was carried out at 25 °C in the rapid mixing apparatus adapted for chemical quenching (Intermekron AB, Uppsala, Sweden) based on the design by Mardh and Zetterqvist [18]. In a typical experiment one of the syringes contained 15–20 μg of pPMCA, 140 μg of $C_{12}E_{10}$, 0 or 66 μg of asolectin and 20 % (w/v) glycerol in 1 ml of buffer consisting of 50 mM Tris/HCl (pH 7.4 at 25 °C), 0.5 mM EGTA, 100 mM KCl, 0.5 mM $MgCl_2$ and variable concentrations of free Ca^{2+}. The other syringe contained 40 μM [γ-^{32}P]ATP in 1 ml of the same buffer. The solution of radioactive ATP was filtered through Millipore filters (type HAWP; 0.45 μm pore size) 1 h before use. The enzyme in the buffer was preincubated at 25 °C for at least 10 min before phosphorylation. The reaction was started by mixing the content of the two syringes, and was ended by collecting the mixture in 9 ml of a solution of 8.5 % (w/v) trichloroacetic acid, 10 mM ATP and 50 mM H_3PO_4 at 0 °C. The EP in the denaturing solution was collected by vacuum filtration, adding the mixture carefully drop-by-drop on to the centre of a Millipore filter (type HAWP; 0.45 μm pore size). The residue on the filters was washed five times with 10 ml of 7 % (w/v) trichloroacetic acid/50 mM H_3PO_4. The filters were subsequently dried and the radioactivity was measured by scintillation counting in 3 ml of 0.4 % (w/v) 2,5-diphenyloxazole and 0.02 % (w/v) 1,3-bis-2-(5-phenyloxazole) in toluene. A blank was prepared by measuring the radioactivity incorporated in the enzyme in medium with no $CaCl_2$. The value of the blank did not vary among experiments, was independent of the reaction time, and was subtracted from the EP measured in the presence of Ca^{2+}. Dephosphorylation was measured at 25 °C using three syringes and two mixing chambers as described previously [5]. EP was formed in the lines between the first and the second mixing chambers and was chased in the second mixing chamber with buffer containing enough unlabelled ATP to give a final concentration of 300 μM. Free Ca^{2+} concentration was estimated with a Ca^{2+}-selective electrode.

We have shown [11] that in media containing 70 μg/ml $C_{12}E_{10}$, either asolectin or phosphatidylcholine (at 25–35 μg/ml) make the PMCA exhibit maximum Ca^{2+}-ATPase specific activity at concentrations between 1 and 10 μg/ml. During the present study, we measured [EP] formed at 20 μM ATP as a function of increasing concentrations of either asolectin or phosphatidylinositol (0–66 μg/ml) in reaction media containing 70 μg/ml $C_{12}E_{10}$. [EP] initially decreased and then remained constant between 25 and 40 μg/ml asolectin or 15 and 33 μg/ml phosphatidylinositol. After this, all reaction media contained 33 μg/ml phospholipid and 70 μg/ml $C_{12}E_{10}$/ml (final concentrations).

[γ-^{32}P]ATP was prepared by the method of Glynn and Chappell [19] except that no unlabelled orthophosphate was added to the incubation medium. Carrier-free [^{32}P]H_3PO_4 was provided by NEN Life Science Products (Boston, MA, U.S.A.). Soya bean phosphatidylcholine Type IV-S (asolectin), L-α-phosphatidylinositol (approx. 99 %), L-α-phosphatidylethanolamine Type V, $C_{12}E_{10}$, CaM–agarose, CaM, and enzymes and cofactors for the synthesis of [γ-^{32}P]ATP were obtained from Sigma. Salts and reagents were of analytical reagent grade.

RESULTS

Phosphorylation of phospholipid-depleted pPMCA

Phospholipid-depleted pPMCA was preincubated in medium containing 150 μM Ca^{2+} and 0.5 mM Mg^{2+} in the presence and absence of 1.9 μM CaM, and then phosphorylated in the same medium containing 20 μM [γ-^{32}P]ATP at 25 °C. Results in Figure 1 show that the time course of phosphorylation of the phospholipid-depleted pPMCA was biphasic with a component that increased very rapidly with a k_{app} of 425 s^{-1} during the first 4 ms of the reaction, followed by a slow component (k_{app} = 23 s^{-1}). CaM did not change the rate, but increased the size of the fast component. At the same time, CaM increased the rate constant of the slow component from 23 to 93 s^{-1}. As a consequence, [EP] at steady-state was higher with CaM.

Phosphorylation of pPMCA purified in the presence of asolectin

pPMCA purified in the presence of asolectin was preincubated as before, in the absence and presence of 1.9 μM CaM, and then phosphorylated in the same medium for various lengths of time (see Figure 2). Without CaM, the concentration of EP increased very rapidly, reached a maximum of 760 pmol/mg of protein at 4 ms, decreased slightly and rose again with less velocity to a steady-state level close to 740 pmol/mg of protein. As a consequence, an EP overshoot appeared. CaM did not change the shape of the phosphorylation curve but almost doubled the level of EP all along. From the initial velocity and the level of EP at 4 ms in Figure 2, a k_{app} of 400 s^{-1} was estimated for the first component in the presence and absence of CaM. This value was almost equal to the rate constant of the rapid components of the time courses shown in Figure 1.

Addition of asolectin during preincubation, made the time course of phosphorylation of the phospholipid-depleted pPMCA used during the experiment in Figure 1 equal to that shown in

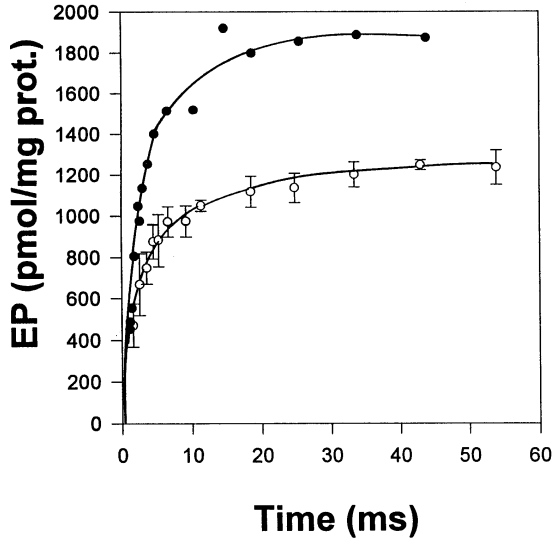


Figure 1 Time course of phosphorylation of phospholipid-depleted pPMCA after preincubation with 150 μM Ca^{2+} and 0.5 mM Mg^{2+} in the absence (○) and presence (●) of 1.9 μM CaM

The concentration of Ca^{2+} in the reaction medium was 150 μM. The curves represent [EP] = $[EP_r](\exp -k_r t) + [EP_s](\exp -k_s t)$, where t is time, and $[EP_r]$ and $[EP_s]$ are the maximum concentrations of EP in the rapid (r) and the slow (s) components and which were 805 and 575 pmol/mg of protein respectively without CaM, and 1430 and 375 pmol/mg of protein respectively with CaM. k_r and k_s represent the rate constants of the rapid (r) and the slow (s) components and were 425 and 23 s^{-1} repectively without CaM, and 375 and 93 s^{-1} respectively with CaM. Kinetic parameters were obtained by fitting the equation to the data using Sigma Plot for Windows. Vertical bars show the S.D. for three experiments. The experimental points of the curve with CaM belong to two experiments with different reaction times. prot., protein.

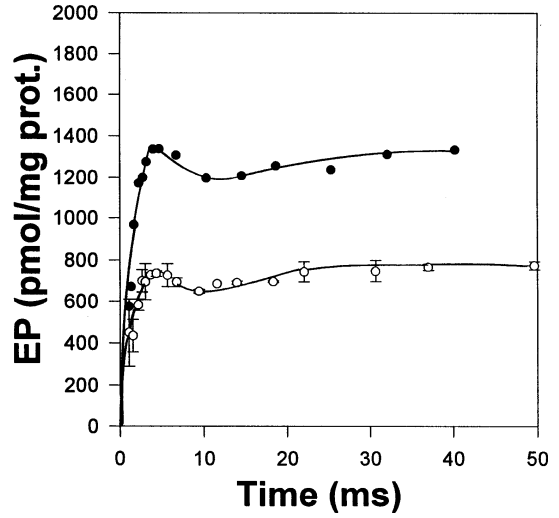


Figure 2 Time course of phosphorylation of pPMCA purified in the presence of asolectin after preincubation with 150 μM Ca^{2+} and 0.5 mM Mg^{2+} in the absence (○) and presence (●) of 1.9 μM CaM

The concentration of Ca^{2+} in the reaction media was 150 μM. The curves were drawn by eye. The time course curve without CaM is the mean of three experiments, with vertical bars representing S.D., and is representative of six experiments performed with enzyme isolated from three different red blood cell batches. The experimental points of the curve with CaM are the means of two experiments with different reaction times. prot., protein.

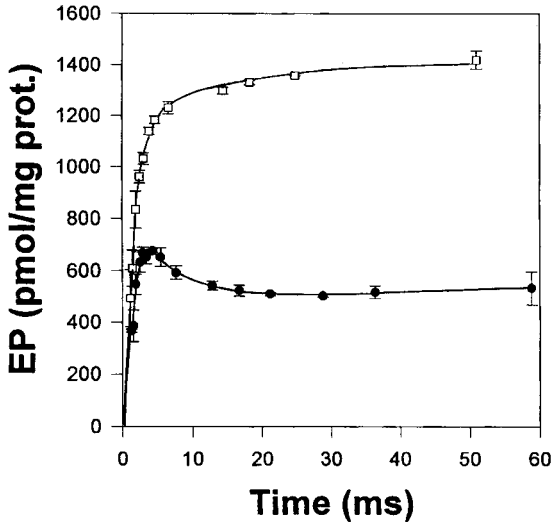


Figure 3 Time course of phosphorylation of phospholipid-depleted pPMCA in reaction media containing either phosphatidylethanolamine (□) or phosphatidylinositol (●) at 33 μg/ml

Vertical bars show S.D. for two experiments. The curve with phosphatidylinositol was drawn by eye. The curve with phosphatidylethanolamine represents [EP] = $[EP_r](\exp -k_r t) + [EP_s](\exp -k_s t)$ where $[EP_r]$ and $[EP_s]$ were 1282 and 85 pmol/mg respectively, and k_r and k_s were 510 and 4 s^{-1} respectively. The symbols are as described in Figure 1. prot., protein.

Figure 2 for the enzyme purified with asolectin (results not shown). This observation served to demonstrate that differences between the time courses of phosphorylation from Figures 1 and 2 were attributable to asolectin rather than to a permanent change due to deterioration of the enzyme by delipidation.

Effect of phosphatidylethanolamine and phosphatidylinositol

Asolectin is a mixture of near equal proportions of the neutral phospholipids phosphatidylethanolamine and phosphatidylcholine, and the acidic phosphatidylinositol. We looked at the consequences of replacing asolectin with pure preparations of either phosphatidylethanolamine or phosphatidylinositol on the phosphorylation of the phospholipid-depleted enzyme. Figure 3 shows that phosphatidylinositol, but not phosphatidylethanolamine, lowered the level of EP at steady-state and induced the appearance of the EP overshoot, making the behaviour of the enzyme close to that with asolectin shown in Figure 2. These results allow us to conclude that the negatively charged phosphatidylinositol replaced asolectin in modifying phosphorylation kinetics. Phosphatidylserine mimicked phosphatidylinositol (results not shown), suggesting that the effects were not specifically due to the latter but rather to negatively charged phospholipids. Furthermore, results in Figure 3 suggest that the effects of asolectin were due to interaction of its phosphatidylinositol component with the pPMCA rather than to non-specific alterations of physical parameters of the enzyme/detergent micelles by the phospholipids. The lack of the recovery of the level of EP with phosphatidylinositol as observed between 20 and 30 ms with asolectin, as seen in Figure 2, could perhaps be due to the absence of neutral phospholipids in pure phosphatidylinositol.

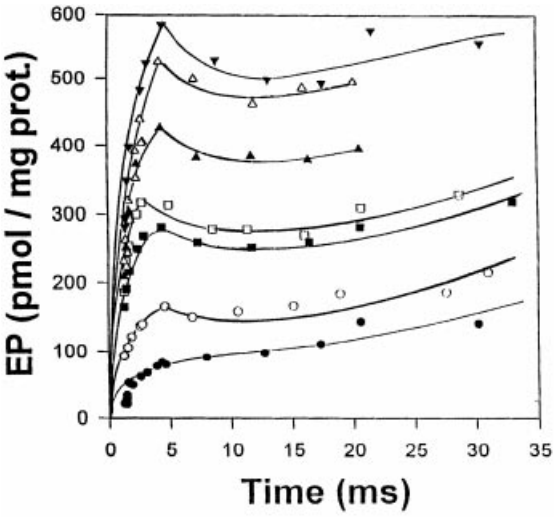


Figure 4 Time courses of phosphorylation of pPMCA purified in the presence of asolectin after preincubation in medium with increasing concentrations of Ca^{2+}

The enzyme was preincubated in medium containing 0 (●), 1.5 (○), 15 (■), 30 (□), 40 (▲), 100 (Δ), and 170 (▼) μM Ca^{2+} for 10 min and then phosphorylated with 20 μM [γ-^{32}P]ATP in the presence of 150 μM Ca^{2+}. The concentrations of $CaCl_2$ in the buffer with the radioactive ATP (syringe II) were those necessary to make the concentration of Ca^{2+} 150 μM in the reaction medium at the start of phosphorylation. The curves were drawn by eye. prot., protein.

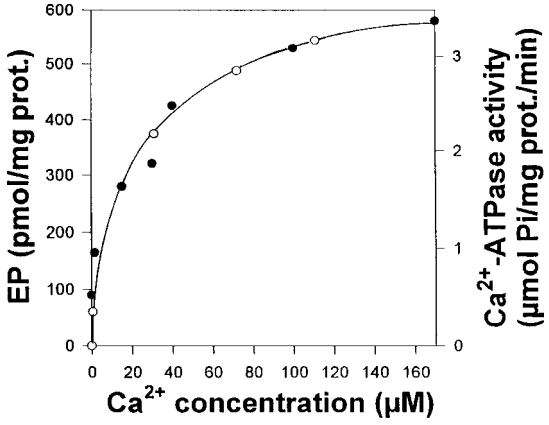


Figure 5 [EP] after 4 ms of phosphorylation (●) and Ca^{2+}-ATPase activity (○) of pPMCA measured in media of identical composition at 25 °C

The values of [EP] are those in Table 1. ATPase activity was measured at 25 °C from the release of [^{32}P]P_i from [γ-^{32}P]ATP in 0.3 ml of reaction medium containing 50 mM Tris/HCl (pH 7.4 at 25 °C), 20 μg/ml pPMCA, 100 mM KC1, 0.5 mM $MgCl_2$, 0.5 mM EGTA, 70 μg/ml $C_{12}E_{10}$, 33 μg/ml asolectin, 10% (w/v) glycerol, 20 μM [γ-^{32}P]ATP and enough $CaCl_2$ to give concentrations of free Ca^{2+} close to those in Table 1. The concentration of free Ca^{2+} was measured with a Ca^{2+}-selective electrode. During measurements of ATPase activity the concentration of P_i in the incubation medium increased linearly with time. The curve was drawn by eye. prot., protein.

Ca^{2+} requirement

Ca^{2+} at the transport site stabilizes the E_1 conformer that catalyses phosphorylation [4]. Hence, knowing the requirements for Ca^{2+} during preincubation could help to find the changes in the enzyme that led to the time courses of phosphorylation shown up to this point. This was tested by phosphorylating pPMCA purified in the presence of asolectin, that had been preincubated in media with Ca^{2+} at various concentrations (0–170 μM), under identical conditions in the presence of 150 μM Ca^{2+}. Results in Figure 4 show that, except that of pPMCA preincubated in the absence of Ca^{2+}, all time courses exhibited the overshoot near 4 ms. Regardless of the conditions of preincubation, all the curves tended to a steady level of EP close to 600 pmol/mg of protein (results not shown) proving that at steady-state, the behaviour of the pPMCA was independent of the conditions during preincubation.

Table 1 shows kinetic parameters from the curves in Figure 4. Both the concentration of EP at 4 ms and v_o increased with increasing Ca^{2+} concentration. In contrast k_{app} values increased from 195 s^{-1} in the absence of Ca^{2+} to a mean value of 420 s^{-1} for all of the concentrations of Ca^{2+} tested. This suggests that the differences among the curves in Figure 4 should be ascribed to the number of enzyme units ready to undergo phosphorylation at the end of preincubation.

Figure 5 shows the concentration of EP as a function of the concentration of Ca^{2+} (data taken from Table 1). The concentration of EP at 4 ms increased along a hyperbolic curve with $K_{0.5}$ near 20 μM Ca^{2+}. This value was close to that of 15 μM reported by us for half-maximum acceleration of phosphorylation after preincubation of pPMCA in native membranes [4]. Nevertheless, and since there is no report on the apparent affinity of the detergent-purified pPMCA for Ca^{2+}, we measured ATPase activity at increasing concentrations of Ca^{2+} in the preincubation medium used for the experiment in Figure 4. Results in Figure 5 show that the points representing the activity fall on the [EP] curve, as expected if Ca^{2+} combined with high affinity to a single class of site(s) to increase both [EP] at 4 ms and ATPase activity. This allowed us to conclude that combination

Table 1 Effects of the concentration of Ca^{2+} during preincubation on kinetic parameters of the phosphorylation reaction

The values were taken from the curves in Figure 4. v_0 corresponds to the slope of the initial part of the curves, and k_{app} is the ratio between v_0 and the concentration of EP at 4 ms.

Ca^{2+} concentration (μM)	EP concentration at 4 ms (pmol/mg of protein)	v_0 (pmol/ms per mg of protein)	k_{app} (s^{-1})
0	90	17.5	195
1.5	164	68	415
15	280	120	428
30	320	152	475
40	426	178	418
100	530	200	377
170	580	240	414

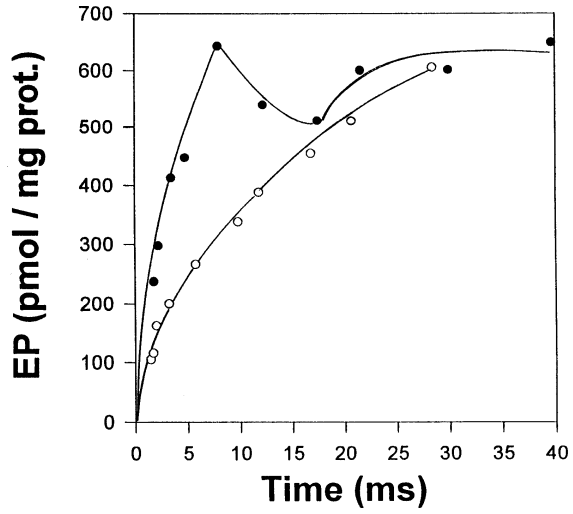


Figure 6 Effects of ATP during preincubation on phosphorylation of pPMCA purified in the presence of asolectin

After preincubation with 150 μM Ca^{2+}, 1 ml of the pPMCA solution in syringe I was mixed with 1 ml of 40 μM ATP from syringe II in the first mixing chamber. The mixture was allowed to react for 40 ms in the lines to the second mixing chamber where it was mixed with 1 ml of 20 μM [γ-^{32}P]ATP from syringe III to form radioactive EP (○). The control experiment (●) was performed in the same way except that ATP was omitted from the solution in syringe II, and syringe III contained 60 μM [γ-^{32}P]ATP. The concentration of Ca^{2+} in the reaction media was 150 μM. The points represent the results of one experiment and the curves were drawn by eye. prot., protein.

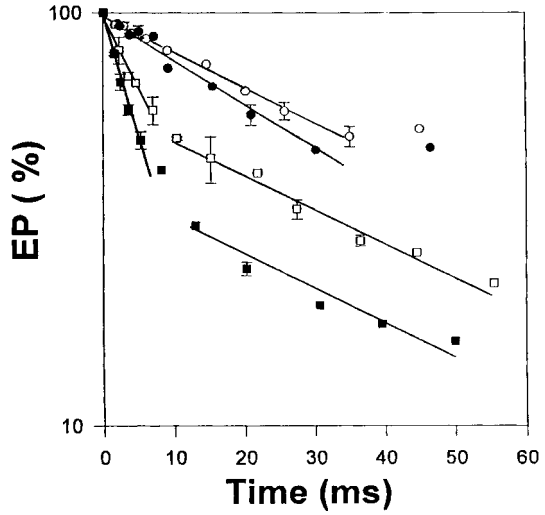


Figure 7 Time course of dephosphorylation of EP

The Figure is a semi-log plot of the data as a percentage of [EP] at 0 ms. Phospholipid-depleted pPMCA preincubated with Ca^{2+} in syringe I was mixed in the first mixing chamber with the radioactive ATP in syringe II. Phosphorylation with [γ-^{32}P]ATP lasted 60 ms and took place in the lines to the second mixing chamber, where phosphorylation was terminated and dephosphorylation started by the addition of 900 μM ATP from syringe III. The procedure was applied in medium with no additions (○), with 1.9 μM CaM (●), with 33 μg/ml asolectin (□), and with 33 μg/ml asolectin + 1.9 μM CaM (■). The concentration of Ca^{2+} was 150 μM. [EP] at 0 ms was 1340, 2410, 803, and 1308 pmol/mg of protein with either no additions, CaM, asolectin, or asolectin + CaM respectively. The experimental points belong to two experiments with different reaction times. Vertical bars show S.D. of points which coincided in reaction time. The curves were drawn by eye.

of Ca^{2+} at the transport site during preincubation was the first event leading to the time courses of phosphorylation of the pPMCA as in Figures 1 and 2.

Effect of ATP

If biphasic time courses of phosphorylation depended on CaE_1 they should not be apparent in enzyme that was cycling during preincubation, because accumulation of CaE_1 requires the enzyme to be at rest. To test this point, an experiment was performed in which ATP was added to pPMCA preincubated with Ca^{2+}, allowed to react for 40 ms, and then phosphorylated with [γ-^{32}P]ATP. A control experiment was run in parallel in the same way except that unlabelled ATP was omitted. Results in Figure 6 show that the enzyme preincubated with Ca^{2+} plus unlabelled ATP exhibited simple phosphorylation kinetics when [γ-^{32}P]ATP was subsequently added, confirming that in the cycling enzyme the fast initial phase of phosphorylation was absent. In contrast, the enzyme preincubated in ATP-free medium exhibited a biphasic time course of phosphorylation.

Dephosphorylation of pPMCA: effects of asolectin and CaM

Since asolectin lowered the level of EP at steady-state with a relatively small change in the rate of the initial phosphorylation of the phospholipid-depleted pPMCA, it seemed right to think that it could have acted on dephosphorylation. Results in Figure 7 show that the EP of phospholipid-depleted pPMCA dephosphorylated along an exponential curve with $k_{app} = 30\ s^{-1}$. Except for a 33 % increase in the k_{app} to 40 s^{-1}, CaM did not change the kinetics of the reaction. The EP prepared in the presence of asolectin dephosphorylated with biphasic kinetics (Figure 7), with a fast component of 410 pmol/mg of protein and $k_{app} = 220\ s^{-1}$ and a slow component of 390 pmol/mg of protein and $k_{app} = 20\ s^{-1}$. Under these conditions, CaM increased the size and the rate constant of the fast component. The rate of the slow component with asolectin was independent of CaM.

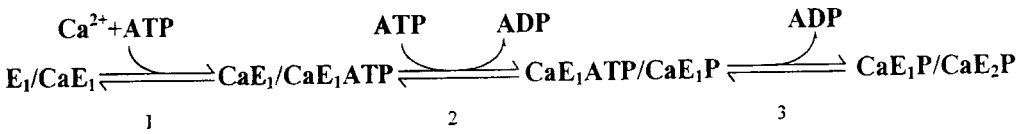
DISCUSSION

The present study describes the kinetics of phosphorylation and dephosphorylation of the pPMCA purified and solubilized in detergent. The results give information on how Ca^{2+}, CaM and acidic phospholipids affect the partial reactions during ATP hydrolysis. It is important to relate these findings to those observed with PMCA in native membranes and other P-type ATPases to understand the mechanisms of enzyme activity.

After preincubation with Ca^{2+}, phosphorylation of the phospholipid-depleted pPMCA displayed biphasic kinetics, with a fast phase followed by a slow phase. The time course of phosphorylation was unlike that of enzyme in native membranes which displays monoexponential kinetics with $k_{app} = 115\ s^{-1}$ at 37 °C [4], but very close to that of the Ca^{2+}-ATPase of sarcoplasmic reticulum in 400 mM KCl (see Figure 6 in Mahaney et al. [20]).

Effects of phospholipids

Results showed that neutral phospholipids were without effect, whereas negatively charged phospholipids dramatically changed the behaviour of the phospholipid-depleted pPMCA. Either phosphatidylinositol or the phosphatidylinositol-containing asolectin made the enzyme phosphorylate along a time course with an overshoot at 4 ms and half the level of EP attained without the lipids at steady-state. This result is at variance with the reported finding that phosphatidylinositol increases the level of



Scheme 2 Dimer model for the PMCA

Each subunit accounts for 50% of the enzyme. Strong interactions between heterologous states create stable species. The right-hand subunit is one step ahead of the left-hand subunit in carrying out the consecutive reactions.

EP at steady-state [21]. On the other hand, the time course was very similar to that of the Ca^{2+}-ATPase of sarcoplasmic reticulum at relatively high Ca^{2+} concentrations [22–24]. The k_{app} of phosphorylation had the same value regardless of phospholipids, allowing us to conclude that the phosphorylation reaction of the pPMCA is independent of phospholipids. In contrast asolectin increased the k_{app} for dephosphorylation by 7-fold. Acceleration of EP decay will increase turnover of the enzyme and explain activation of the Ca^{2+}-ATPase by acidic phospholipids.

Effects of CaM

CaM did not change the rate constant of fast phosphorylation and, in contrast with and regardless of acidic phospholipids, accelerated the slow phosphorylation and increased [EP] at steady-state. A higher [EP] must lead to a higher rate of ATP hydrolysis.

Unlike phospholipids, CaM did not accelerate dephosphorylation of the lipid-depleted pPMCA. Unexpectedly, when asolectin was present, CaM increased the rate and the size of the fast component of dephosphorylation. There are two possible explanations: one will be given below and is based on alternative reaction mechanisms such as that shown in Scheme 2, and the other is based on what is known about the mechanisms of action of CaM and acidic phospholipids on the PMCA, as follows. CaM activates PMCA by interacting only with the C-terminal regulatory region of the enzyme, whereas acidic phospholipids modulate enzyme activity by combining at the same C-terminal region and at a region between the putative transmembrane domains 2 and 3 simultaneously [25,26]. The persistence of slow dephosphorylation in the presence of asolectin suggests that under the conditions used during the experiment shown in Figure 7 the acidic phospholipids of asolectin may not have occupied the C-terminal site in some of the enzyme units. Because of this, it would be reasonable to expect further effects of CaM in the presence of asolectin following association of CaM with these enzyme units. If this were so, it could be that the pPMCA with phospholipids and CaM bound simultaneously catalysed dephosphorylation more effectively, increasing the rate and the size of the fast component. Moreover, a higher [EP] plus the further acceleration of dephosphorylation reported in the present study may contribute to the stimulation of ATPase activity by CaM at limiting concentrations of acidic phospholipids (as shown in Figure 8 in Filoteo et al. [25] and in Figure 8 in Brodin et al. [26]).

Possible mechanisms

More than one interpretation of the data is possible. (1) As proposed by Jencks [27] for sarcoplasmic reticulum, in a consecutive catalytic cycle, such as that in Scheme 1, the fast phase of phosphorylation represents phosphorylation of CaE_1 according to reactions 2 and 3. If this were so, and assuming that association of CaE_1 with ATP was very fast, the values of approx. 400 s^{-1} reported in the present study should be close to the actual rate constant of phosphorylation of pPMCA. On addition of ATP to pPMCA preincubated with Ca^{2+}, two events might take place simultaneously: (i) a transient fast phosphorylation of CaE_1 leading to maximum concentration of EP at 4 ms, and (ii) a slower phosphorylation limited by the rate of formation of CaE_1 in the reaction medium leading to a maximum steady-concentration of EP after approx. 30 ms. The experimental time courses of phosphorylation shown in the present study should be the sum of these two partial time courses. Since slow phosphorylation depended on CaE_1 formed in the reaction medium, CaM may have acted before reaction 3 of Scheme 1, most probably by accelerating the shifting of E_2 into E_1. To give rise to the overshoot of EP, the rate of the decay of the rapid component should be higher than the rate of formation of the slow component, a condition attained with asolectin. Although such a mechanism based on accumulation of CaE_1 accounts for the biphasic increase in EP, it fails to explain the behaviour of the enzyme at rest during preincubation. In fact, one should expect submillimolar concentrations of Ca^{2+} to be enough to achieve stoichiometric conversion of the enzyme into CaE_1 during preincubation. The [EP] of the fast component without CaM in Figure 1 represented 60% of [EP] maximum. (2) As mentioned in the Introduction section, under certain conditions the PMCA solubilized in detergent behaves as a dimer. Although it has not yet gained wide acceptance, the reaction mechanism proposed by Ikemoto and Nelson [28] and Froehlich and his co-workers [29], to explain the complex kinetic behaviour of the Ca^{2+}-ATPase [20,29] and of the Na^+/K^+-ATPase [29,30], provides a way of interpreting the data for a dimeric enzyme. Accordingly, the sequence of reactions during phosphorylation of the pPMCA could be as in Scheme 2. During preincubation E_1/CaE_1 is produced. Reactions 1 and 2 are fast and produce phosphorylation of 50% of the enzyme. Reaction 3 is slower, produces a fully phosphorylated enzyme and is responsible for the slow phosphorylation phase. In accordance with the authors above, in a dimeric enzyme full dephosphorylation implies sequential liberation of P_i from different subunits. In the absence of asolectin both subunits dephosphorylate slowly. Acceleration of dephosphorylation of one of the subunits by asolectin, could provide an explanation for the biphasic decomposition of EP. It has been reported that superstoichiometric concentrations of CaM prevent oligomerization of the PMCA [10,31–33]. This being so, the fact that during phosphorylation and dephosphorylation CaM at 15-fold higher molar concentration increased the amplitude of the fast component, is in keeping with a dimer model, such as that in Scheme 2. Nevertheless, it should be noted here, the difficulty in explaining the activation of the ATPase activity by asolectin on the basis of this model which couples the slow to the fast dephosphorylation. In fact, a cycling mechanism, coupling a fast reaction to a slow one, will slow the former reaction and hence the turnover rate of the enzyme. One way to overcome this difficulty is to suppose that, after

the first cycle of reaction, asolectin lowered the energy of interaction between protomers, making the enzyme able to react according to Scheme 1. Results shown in Figure 6 agree with this view. (3) Human red blood cell membranes contain two PMCA isoforms, of which one is more abundant [34]. If the pPMCA consisted of equal amounts of two isoforms, one capable of rapid phosphorylation and, with asolectin, rapid dephosphorylation, in contrast with the other that phosphorylated and dephosphorylated more slowly, this could give rise to the results observed. Unfortunately, it is not known if pPMCA consists of equal amounts of two isoforms bearing the catalytic properties mentioned above.

This work was supported by grants from the Secretaría de Ciencia y Técnica, Argentina, the Universidad de Buenos Aires, Argentina, and the Ministerio de Salud, Argentina. L. M. B. is a recipient of a fellowship from the Universidad de Buenos Aires.

REFERENCES

1 Rega, A. F. and Garrahan, P. J. (1986) The Ca^{2+} Pump of Plasma Membranes, pp. 77–90, CRC Press Inc., Boca Raton, FL

2 Kosk-Kosicka, D., Scaillet, S. and Inesi, G. (1986) The partial reactions in the catalytic cycle of the calcium-dependent adenosine triphosphatase purified from erythrocyte membranes. J. Biol. Chem. **261**, 3333–3338

3 Adamo, H. P., Rega, A. F. and Garrahan, P. J. (1988) Pre-steady-state phosphorylation of the human red cell Ca^{2+}-ATPase. J. Biol. Chem. **263**, 17548–17554

4 Adamo, H. P., Rega, A. F. and Garrahan, P. J. (1990) The E_2–E_1 transition of the Ca^{2+}-ATPase from plasma membranes studied by phosphorylation. J. Biol. Chem. **265**, 3789–3792

5 Herscher, C. J., Rega, A. F. and Garrahan, P. J. (1994) The dephosphorylation reaction of the Ca^{2+}-ATPase from plasma membranes. J. Biol. Chem. **269**, 10400–10406

6 Ronner, P., Gazzotti, P. and Carafoli, E. (1977) A lipid requirement for the ($Ca^{2+} + Mg^{2+}$)-activated ATPase of erythrocyte membranes. Arch. Biochem. Biophys. **179**, 578–583

7 Niggli, V., Adunyah, E. S., Penniston, J. T. and Carafoli, E. (1981) Purified ($Ca^{2+} - Mg^{2+}$)-ATPase of the erythrocyte membrane. Reconstitution and effect of calmodulin and phospholipids. J. Biol. Chem. **256**, 395–401

8 Penniston, J. T., Filoteo, A. G., McDonough, C. S. and Carafoli, E. (1988) Purification, reconstitution, and regulation of plasma membrane Ca^{2+}-pumps. Methods Enzymol. **157**, 340–351

9 Kosk-Kosicka, D. and Bzdega, T. (1988) Activation of the erythrocyte Ca^{2+}-ATPase by either self-association or interaction with calmodulin. J. Biol. Chem. **263**, 18184–18189

10 Sackett, D. L. and Kosk-Kosicka, D. (1996) The active species of plasma membrane Ca^{2+}-ATPase are a dimer and a monomer-calmodulin complex. J. Biol. Chem. **271**, 9987–9991

11 Bredeston, L. M. and Rega, A. F. (1999) Phosphatidylcholine makes specific activity of the purified Ca^{2+}-ATPase from plasma membranes independent of enzyme concentration. Biochim. Biophys. Acta **1420**, 57–62

12 Caride, A. J., Rega, A. F. and Garrahan, P. J. (1986) The reaction of Mg^{2+} with the Ca^{2+}-ATPase from human red cell membranes and its modification by Ca^{2+}. Biochim. Biophys. Acta **863**, 165–177

13 Gietzen, K., Tejcka, M. and Wolf, H. U. (1980) Calmodulin affinity chromatography yields a functional purified erythrocyte (Ca^{2+}, Mg^{2+})-dependent adenosine triphosphatase. Biochem. J. **189**, 81–88

14 Castello, P. R., Caride, A. J., Gonzalez Flecha, F. L., Fernandez, H. N., Rossi, J. P. F. C. and Delfino, J. M. (1994) Identification of tansmembrane domains of the red cell calcium pump with a new photoactivable phospholipidic probe. Biochem. Biophys. Res. Commun. **201**, 194–200

15 Kosk-Kosicka, D. and Inesi, G. (1985) Cooperative calcium binding and calmodulin regulation in the calcium-dependent adenosine triphosphatase purified from the erythrocyte membrane. FEBS Lett. **189**, 67–71

16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275

17 Peterson, G. L. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. **83**, 346–356

18 Mardh, S. and Zetterqvist, O. (1974) Phosphorylation and dephosphorylation reactions of bovine brain ($Na^+ - K^+$)-stimulated ATP phosphohydrolase studied by a rapid mixing technique. Biochim. Biophys. Acta **350**, 473–483

19 Glynn, I. M. and Chappell, J. B. (1964) A simple method for the preparation of ^{32}P-labelled adenosine triphosphate of high specific activity. Biochem. J. **90**, 147–149

20 Mahaney, J. E., Froehlich, J. P. and Thomas, D. D. (1995) Conformational transitions of the sarcoplasmic reticulum Ca-ATPase studied by time-resolved EPR and quenched-flow kinetics. Biochemistry **34**, 4864–4879

21 Lehotsky, J., Raeymaekers, L., Missiaen, L., Wuytack, F., De Smedt, H. and Casteels, R. (1992) Stimulation of the catalytic cycle of the Ca^{2+} pump of porcine plasma-membranes by negatively charged phospholipids. Biochim. Biophys. Acta **1105**, 118–124

22 Takisawa, H. and Tonomura, Y. (1978) Factors affecting the transient phase of the Ca^{2+}, Mg^{2+}-dependent ATPase reaction of sarcoplasmic reticulum from skeletal muscle. J. Biochem. (Tokyo) **83**, 1275–1284

23 Froehlich, J. P. and Taylor, E. W. (1975) Transient state kinetic studies of sarcoplasmic reticulum adenosine triphosphatase. J. Biol. Chem. **250**, 2013–2021

24 Sumida, M., Wang, T., Swartz, A., Younkin, C. and Froehlich, J. P. (1980) The Ca^{2+}-ATPase partial reactions in cardiac and skeletal sarcoplasmic reticulum. A comparison of transient state kinetic data. J. Biol. Chem. **255**, 1497–1503

25 Filoteo, A. G., Engedi, A. and Penniston, J. T. (1992) The lipid-binding peptide from the plasma membrane Ca^{2+} pump binds calmodulin, and the primary calmodulin-binding domain interacts with lipid. J. Biol. Chem. **267**, 11800–11805

26 Brodin, P., Falchetto, R., Vorherr, T. and Carafoli, E. (1992) Identification of two domains which mediate the binding of activating phospholipids to the plasma-membrane Ca^{2+} pump. Eur. J. Biochem. **204**, 939–946

27 Jencks, W. P. (1989) How does a calcium pump pump calcium? J. Biol. Chem. **255**, 1497–1503

28 Ikemoto, N. and Nelson, R. (1984) Oligomeric regulation of the later reaction steps of the sarcoplasmic reticulum calcium ATPase. J. Biol. Chem. **259**, 11790–11797

29 Froehlich, J. P., Taniguchi, K., Fendler, K., Mahaney, J. E., Thomas, D. D. and Albers, R. W. (1997) Complex kinetic behavior in the Na,K- and Ca-ATPases. Evidence for subunit-subunit interactions and energy conservation during catalysis. Ann. N.Y. Acad. Sci. **834**, 280–296

30 Froehlich, J. P., Bamberg, E., Kane, D. J., Mahaney, J. E. and Albers, R. W. (2000) Contribution of quaternary protein interaction to the mechanism of energy transduction in Na^+/K^+-ATPase. In Na/K-ATPase and Related ATPases (Taniguchi, K. and Kaya, S., eds.), pp. 349–356, Elsevier Science, Amsterdam

31 Kosk-Kosicka, D. and Bzdega, T. (1990) Effects of calmodulin on erythrocyte Ca^{2+}-ATPase activation and oligomerization. Biochemistry **29**, 3772–3777

32 Vorherr, T., Kessler, T., Hofmann, F. and Carafoli, E. (1991) The calmodulin-binding domain mediates the self-association of the plasma membrane Ca^{2+} pump. J. Biol. Chem. **266**, 22–27

33 Levi, V., Rossi, J. P. F. C., Castello, P. R. and Gonzalez Flecha, F. L. (2000) Oligomerization of the plasma membrane calcium pump involves two regions with different thermal stability. FEBS Lett. **48**, 99–103

34 Strehler, E. E., James, P., Fischer, R., Heim, R., Vorherr, T., Filoteo, A. G., Penniston, J. T. and Carafoli, E. (1990) Peptide sequence analysis and molecular cloning reveal two calcium pump isoforms in the human erythrocyte membrane. J. Biol. Chem. **265**, 2835–2842

Received 4 April 2001/1 October 2001; accepted 5 November 2001