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# Phosphatidylcholine makes specific activity of the purified $Ca^{2+}$-ATPase from plasma membranes independent of enzyme concentration

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**Abstract**

$Ca^{2+}$-ATPase of plasma membranes (PMCA) was isolated from either human or pig red cells by calmodulin-affinity chromatography and supplemented with phosphatidylcholine (PC). The specific activity of the purified PMCA diluted in media with detergent ($C_{12}E_{10}$) was very low, and increased with the concentration of the enzyme along a curve that reached the maximum at 8 μg/ml with $K_{0.5}$ = 1.2–2.5 μg/ml. Such behavior has been described and attributed to self-association of the enzyme (D. Kosk-Kosicka and T. Bzdega, J. Biol. Chem. 263 (1988) 18184–18189). After heat-inactivation, the PMCA was as effective an activator as the intact enzyme, increasing, to the maximum, the specific activity of diluted enzyme with $K_{0.5}$ = 2.2 μg/ml. The inactivated PMCA failed to increase the activity of concentrated enzyme, suggesting that activation did not depend on interaction of intact with denatured enzyme molecules. When enough PC was added to the reaction medium to make its final concentration 16–33 μg/ml, the specific activity of the PMCA was maximum and independent of enzyme concentration. Under these conditions, activation by calmodulin lowered to 10%. As a function of the concentration of pure PC, maximum specific activity was reached along a curve with $K_{0.5}$ = 4 μg/ml. This curve was identical to that of activation at increasing enzyme concentration, suggesting that, in the latter case, activation could have depended on PC contributed to the assay medium by the enzyme. The results show that PC made the purified PMCA solubilized in detergent reach maximum activity at any concentration of the enzyme. 

*Keywords:* $Ca^{2+}$-ATPase; $Ca^{2+}$ pump; $Ca^{2+}$ transport

## 1. Introduction

Kosk-Kosicka and coworkers have first shown that the catalytic activity of the purified hPMCA varies with the concentration of the enzyme [1,2]. The specific activity and the apparent affinity for $Ca^{2+}$ of the diluted hPMCA during ATP hydrolysis are low and increase with CaM, while in the concentrated enzyme are maximal and insensitive to CaM [2]. It has been reported that concentration by volume exclusion with dextran increases the specific activity of diluted hPMCA [3].

Independently of enzymic activities, measurements of physical properties agree with the idea that when diluted, the hPMCA behaves as a monomer in contrast with the concentrated enzyme that behaves as a dimer [4–7]. Kosk-Kosicka and Bzdega [2] concluded

Abbreviations: PMCA, calcium ion-dependent adenosine triphosphatase from plasma membrane; prefix 'h' is for human and 'p' for pig; HIP, heat-inactivated preparation of PMCA; CaM, calmodulin; EGTA, [Ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MOPS, (3-[*N*-morpholino]propane-sulfonic acid); PC, pure phosphatidylcholine

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that at increasing concentrations, the purified hPMCA solubilized with detergent undergoes reversible self-association from monomer to dimer, that is the condition of the enzyme endowed with maximum specific activity.

Measurements of ATPase activities reported by Kosk-Kosicka et al. were made at fixed detergent concentration and molar phospholipid/enzyme ratio [2]. We found recently [8] that under these experimental conditions, the specific activity of the purified pPMCA is low at low concentration and high at high concentration of the enzyme. After inactivation by heating, the pPMCA preparation is as effective as the intact preparation in raising to the maximum the specific activity of the pPMCA at low concentration [8], demonstrating that activation of the pPMCA is independent of the functional integrity of the pPMCA. This suggested that activation of the ATPase may not depend on the concentration of pPMCA. This point is relevant to the reaction mechanism of the PMCA, particularly after the demonstration that the hPMCA in the membrane can exist as a dimer [9]. In view of this, we thought it worth studying further the dependence of the activity with the concentration of the enzyme, PC, and CaM in media with constant amounts of detergent.

## 2. Materials and methods

pPMCA was isolated by CaM affinity column chromatography following the procedure described by Penniston et al. [10] except that $C_{12}E_{10}$ and potassium-MOPS were used for Triton X-100 and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, respectively, as in [11]. Asolectin or PC were the phospholipid in the buffers during solubilization and washing, respectively, and 20 mM potassium-MOPS (pH 7.40 at 4°C), 130 mM KCl, 1 mM $MgCl_2$, 1 mg/ml PC, 0.5 mg/ml $C_{12}E_{10}$, 2 mM dithiothreitol, and 3 mM EDTA was the composition of the elution buffer. hPMCA was isolated by the same procedure except that 20% (w/v) glycerol was added to all media. Protein concentration was determined by the method described by Peterson [12] with bovine serum albumin as the standard.

ATPase activity was estimated from the release of [$^{32}$P]Pi from [$\gamma$-$^{32}$P]ATP at 37°C [13] in 0.3 ml of reaction medium containing: 50 mM Tris (pH 7.40 at 37°C), 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 70 μg/ml $C_{12}E_{10}$, 3 mM [$\gamma$-$^{32}$P]ATP, and enough $CaCl_2$ to give 100 μM of free $Ca^{2+}$. PC and Asolectin were added solubilized in detergent. To make the reaction media 70 μg $C_{12}E_{10}$/ml, the amount of detergent carried by the enzyme and other components of the mixture were taken into account. [$\gamma$-$^{32}$P]ATP (1 mCi/ml) was prepared by the method of Glynn and Chappell [14] except that no unlabeled Pi was added to the incubation medium. [$^{32}$P]Pi was provided by Comisión Nacional de Energía Atómica (Argentina). Egg yolk phosphatidylcholine Type XVI-E (Sigma P 3556), soybean phosphatidylcholine Type IV-S (Asolectin, Sigma P 3644), $C_{12}E_{10}$, CaM from bovine brain, CaM-Agarose, and enzymes for [$\gamma$-$^{32}$P]ATP synthesis were from Sigma (USA). Salts and reagents were of analytical grade. Differences in activities among experiments were due to the differences in ATPase activity among batches of enzyme.

## 3. Results and discussion

Fig. 1 shows the specific activity of the pPMCA at increasing concentrations in media with and without CaM. At less than 1.5 μg/ml the activity was very low and difficult to measure. In the absence of CaM, the specific activity of the pPMCA increased from about 1.3 to a maximum of 5.8 μmol/mg protein/min at 8.0 μg/ml. Results in Fig. 1 also show that stimulation of the activity by CaM was 90% at 1.65 and lowered to 10% at the concentrations of pPMCA that gave maximum activation. Half-maximum activation was reached at 2.7 and 2.0 μg/ml of pPMCA in absence and presence of CaM, respectively.

We have reported [8] that incubation of purified pPMCA at 65°C during 120 min renders a preparation (HIP) containing all the components of the intact enzyme preparation, but devoid of ATPase activity. Fig. 2 shows the results of an experiment in which the specific activity of intact pPMCA at 1.25 μg/ml was measured in media containing increasing concentrations of HIP. It can be seen that specific activity rose up to saturation at a maximum of 9.8 μmol/mg protein/min. This value was the same as the maximum specific activity of the intact enzyme used in the assay. The activity was half-maximum when

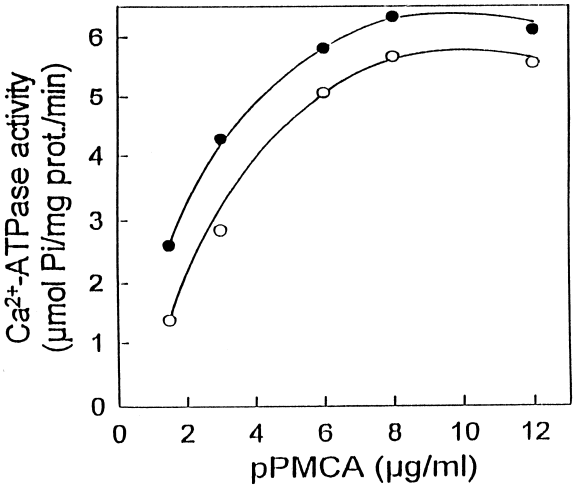


Fig. 1. Specific activity of the pPMCA at increasing concentration of the enzyme in media with (●) and without (○) CaM. The enzyme preparation used contained 2 µg of PC per µg of pPMCA. The molar concentration of CaM was 7-fold higher than that of the pPMCA. Incubation times during activity measurements lowered from 60 to 10 min as the enzyme concentration increased from 1.8 to 12 µg/ml. Under any of the conditions tested, the rate of hydrolysis of ATP was constant. Same results were obtained in four experiments with different enzyme preparations.

the total concentration of pPMCA (intact plus inactive) was 2.2 µg/ml, a value close to the concentration of intact pPMCA for the same effect during the experiment in Fig. 1.

If dimerization of the enzyme were involved, activation in the experiment in Fig. 2 should be attributed to either intact–intact or intact–inactive enzyme association. Lack of catalytic activity of HIP can reasonably be attributed to heat-denaturation of the pPMCA. Under these circumstances, it is unlikely that HIP would favor the formation of intact–intact enzyme dimers. On the other hand, the finding that synthetic peptides corresponding to the CaM-binding domain of the hPMCA stimulate the diluted enzyme [15], did not allow the possibility to be discarded that the denatured enzyme formed intact–inactive dimers fully active. This was tested by measuring the effect of HIP on concentrated enzyme. The activity of the concentrated enzyme should increase upon addition of HIP due to formation of two functional intact–inactive dimers per one intact–intact dimer in the equilibrium. The activity of a sample of pPMCA with 10.5 µg/ml was measured in absence and presence of HIP at 10.5 µg/ml. The activity in the presence of HIP was 90% of the activity in its absence. This result allowed the formation of functional intact–inactive dimers to be discarded as the cause of the activation shown in Fig. 2. This favored the idea that the activator could be among the non-enzymic components of HIP.

In experiments, such as that in Fig. 1 and in the literature [2], the enzyme concentration was varied by adding different aliquots of $C_{12}E_{10}$ and PC-containing enzyme to a constant volume of the assay medium. Under these conditions, the concentration of PC varied in parallel with the concentration of PMCA. For instance, during the experiment in Fig. 1, the concentration of PC went from 1.6 at the lower to 24 µg/ml at the higher concentration of pPMCA tested, and during the experiment in Fig. 2, the concentration of PC increased from 2 µg/ml in the absence of HIP to 16 µg/ml at the higher concentration of HIP tested. Detergent was kept constant at 70 µg/ml.

To see if the dependence of activation on increas-

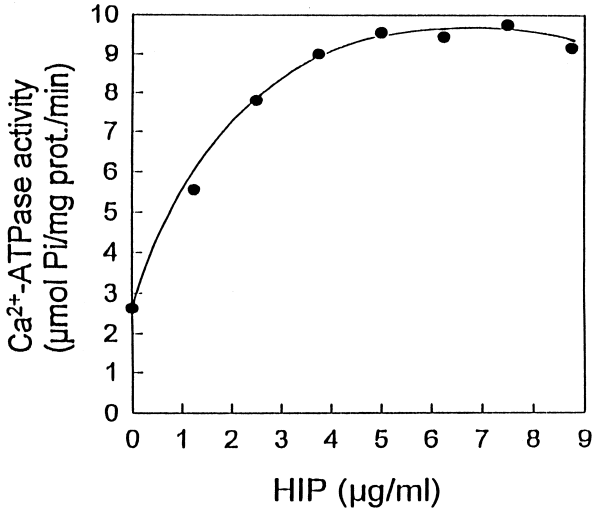


Fig. 2. The effect of HIP on the specific activity of the pPMCA at 1.25 µg/ml. The preparations of PPMCA and of HIP contained 1.6 µg of PC per µg of pPMCA. The concentration of HIP is expressed on the base of the concentration of pPMCA in the sample submitted to heating. The reaction time was 30 min. At the higher concentration of HIP, the consumption of ATP was less than 15% of the total. Under any of the conditions tested, the rate of hydrolysis of ATP was constant. Specific activity was estimated by referring the hydrolysis of ATP to the concentration of intact pPMCA. The same result was obtained in three experiments with different enzyme preparations.

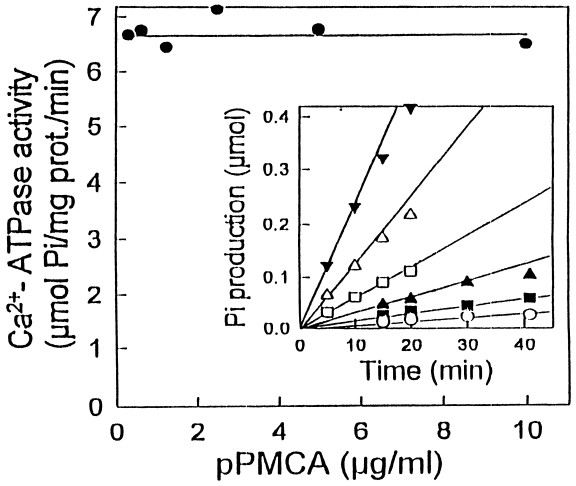


Fig. 3. Specific activity of pPMCA at increasing concentration of the enzyme in the presence of PC 16 µg/ml. The preparation used contained 1.6 µg of PC per µg of pPMCA. The reaction media received enough volume of PC 1000 µg/ml in elution buffer for a final concentration of 16 µg/ml. Specific activities were estimated from the slope of the lines in the inset. Inset: production of orthophosphate from ATP at various concentrations of pPMCA as a function of reaction time. The concentrations of pPMCA were 0.33 (○); 0.67 (■); 1.25 (▲); 2.5 (□); 5.0 (△) and 10 (▼) µg/ml. The same results were obtained in two experiments with different enzyme preparations.

ing concentrations of enzyme was still apparent in the presence of PC at high constant concentration, specific activity was measured at increasing concentrations of pPMCA in the presence of PC at 16 µg/ml. As can be seen in Fig. 3, under these experimental conditions, specific activity was high (6.7 µmol/mg protein/min) and independent of the concentration of pPMCA. All the experimental points in Fig. 3 represent maximum specific activities since they were calculated from the initial rates of ATP hydrolysis represented by the slope of the lines in the inset to Fig. 3. The effects of 300 nM CaM were tested on a sample of pPMCA at 1.25 µg/ml in the presence of 16 µg/ml of PC. The activities were 6.45 and 7.13 µmol Pi/mg protein/min in the absence and presence of CaM,respectively (11% activation). During the experiment in Fig. 1, the activity of pPMCA at 1.65 µg/ml with PC at 3.3 µg/ml increased 90% with CaM. Hence PC at 16 µg/ml lowered activation by CaM from 90 to 11% that, on the other hand, was the activation due to CaM observed at high enzyme concentration in the experiment of Fig. 1. PC at 16 µg/ml, therefore, made the specific activity of the $Ca^{2+}$-ATPase independent of the concentration of pPMCA and little sensitive to CaM.

There is general agreement on the activating effects of acidic phospholipids on the PMCA solubilized with non-ionic detergents [16,17]. In contrast with that, information on the effects of neutral phospholipids as PC is contradictory. Reports on the lack of effects [16] are counterpoised by reports on the activation of the PMCA upon exposure to small amounts of PC [17–19]. A further complication comes from the suggestion that the action of phospholipids on distinct isoforms of the PMCA may be different [20]. In view of this and that the reports on the dependence of the activation of the PMCA with the concentration of the enzyme refer to experiments made with hPMCA [2], the possibility that the results in Fig. 3 expressed a particular property of a distinct isoenzyme from the pig red cells was considered, and the effects of PC tested on hPMCA.

The specific activity of hPMCA was measured at increasing enzyme concentration with and without

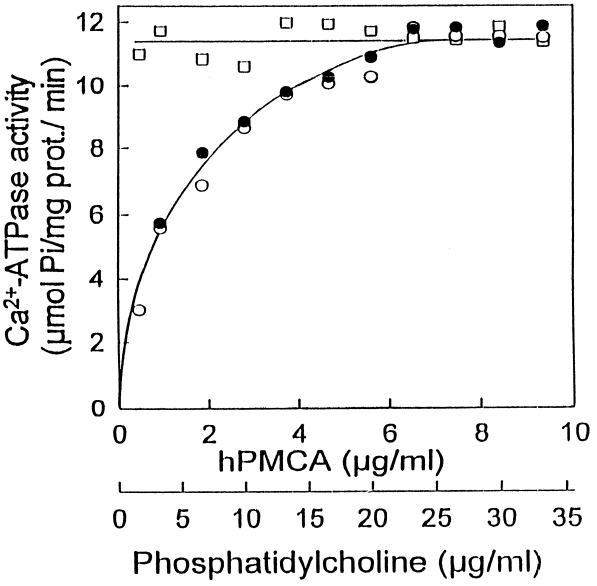


Fig. 4. Specific activity of hPMCA as a function of enzyme concentration and PC concentration. The preparation used contained 3.5 µg of PC per µg of hPMCA. Incubation times were within periods in which the rate of appearance of orthophosphate was constant. ○, activity at increasing concentrations of hPMCA and PC at a constant ratio of PC/hPMCA (w/w) of 3.5; □, activity at increasing concentration of hPMCA with PC constant at 33 µg/ml; ●, activity of hPMCA at 0.95 µg/ml at increasing concentrations of PC. The same results were obtained in two experiments with different enzyme preparations.

added PC with the results shown in Fig. 4. Without added PC the specific activity increased with $K_{0.5} = 1.2$ μg/ml reaching saturation at 11.5 μmol/mg protein/min when the concentration of hPMCA was 7.0 μg/ml. At the highest concentration of enzyme tested, the preparation raised the concentration of PC in the reaction medium to 33 μg/ml. When all reaction media were supplemented with enough PC to a final concentration of 33 μg/ml, the specific activity of the hPMCA was maximum and independent of the concentration of enzyme (Fig. 4). Comparison of these results with those in Figs. 1 and 3 allowed the conclusion to be made that the changes induced by PC on the activation of the hPMCA and the pPMCA were the same. Fig. 4 also shows the activity of a sample containing 0.95 μg/ml of hPMCA and 3.3 μg/ml of PC as a function of increasing concentrations of PC up to 33 μg/ml. Specific activity increased with $K_{0.5} = 4$ μg/ml along a curve superimposable to that drawn as a function of hPMCA concentration. Identical results were obtained when the same weight of Asolectin was used for PC. The identity of the kinetic of activation by PC and by the enzyme preparation suggest that a single mechanism was the responsible for the activation in both conditions. Furthermore, at the $K_{0.5}$ the hPMCA brought to the reaction medium 4.2 μg/ml of PC, just about the $K_{0.5}$ for activation by PC.

The results in this paper demonstrate that PC, at 16–25 μg/ml, made the specific activity of the purified PMCA constant at any enzyme concentration and almost insensitive to CaM.

The experiments were performed at a constant total $C_{12}E_{10}$ concentration of 70 μg/ml (cmc 10 μg/ml) plus various concentrations of PMCA and phospholipids. Phospholipids were added as concentrated PC or Asolectin either alone or together with the enzyme. The enzyme was either intact PMCA or inactivated PMCA in HIP. In such a heterogeneous system, protein/lipid/detergent micelles (PLDM), lipid/detergent micelles (LDM), protein/detergent micelles (PDM), detergent micelles, and monomeric detergent should be at equilibrium. After phospholipids entered the system, a new condition should have been reached with a higher lipid/detergent ratio in PLDM and LDM, and less detergent micelles and monomeric detergent. Should micellar or monomeric $C_{12}E_{10}$ have reversible deleterious effects on PMCA, lowering its concentration with PC would result in higher ATPase activity. We showed that within 50–150 μg/ml of $C_{12}E_{10}$ in media with Asolectin at 16 μg/ml the activity of PMCA at 12 μg/ml lowers only to 75% of the maximum [8], so that the above possibility was unlikely. The incorporation of PC into LDM should be without observable effects on the enzyme while in PLDM should have increased the PC/PMCA molar ratio. In view of this, we considered it reasonable to think that maximum specific activity of the $Ca^{2+}$-ATPase depended on PLDM containing enough PC to ensure optimum interaction of the phospholipid with the PMCA. This view is in keeping with the proposal that Nelson and Hanahan [17] made after finding activation of a soluble preparation of hPMCA by PC in the presence of acidic phospholipids. Furthermore, it is likely that the activation observed at high enzyme concentration was caused by the phospholipid in the enzyme preparation.

The results do not throw much light on the molecular event leading to the activation. It could be that PC either: (1) was irreplaceable for some functions of the PMCA [17]; (2) reaccommodated the components of the micelles allowing the enzyme to expose the binding sites to the ligands in the reaction medium; or (3) increased the concentration and hence the frequency of collisions between PLDM facilitating, in this way, self-association of PMCA molecules. Regardless of the molecular mechanism of activation, the results in this paper are clear in showing that PC made the specific activity of the solubilized PMCA independent of its concentration.

An alternative to activation by PC carried by the enzyme preparation as proposed above is that another activator, for instance CaM leaking from the column, accompanies the solubilized PMCA, resists the heating during preparation of HIP and causes the concentration-dependent activation. Activation by pure (activator-free) PC allows this alternative to be discarded. In addition, the results in Fig. 1 show that only partial activation should had been elicited with contaminant CaM.

At concentrations of PC producing maximum activation, the effectiveness of CaM was low, a typical behavior of the PMCA with acidic phospholipids [21]. It has been proposed that the role of PC in ATPase function, is expressed only in association

with acidic phospholipids [17]. Acidic phospholipids from the red cell membranes could have remain with the enzyme throughout the purification procedure. It has been reported that Asolectin, a commercial preparation of PC, replaces phosphatidylserine and phosphatidylinositol as hPMCA activators [17], showing that it also contains acidic phospholipids. The finding that identical results to those in Fig. 4 were obtained with Asolectin in place of PC, is in keeping with the idea that the enzyme preparations used during this study contained enough acidic phospholipids as to make it insensitive to further additions.

PC provides a simple procedure to ensure maximum activation at any concentration of the PMCA purified by the procedure of Penniston et al. [10]. This is relevant to the employ of purified PMCA for activity measurements without the risk of false interpretations of experimental results.

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