The activation of purified and phospholipid-depleted plasma membrane Ca$^{2+}$-ATPase by phospholipids and ATP was studied. Enzyme activity increased with [ATP] along biphasic curves representing the sum of two Michaelis-Menten equations. Acidic phospholipids (phosphatidylinositol (PI) and phosphatidylserine (PS)) increased $V_{\text{max}}$ without affecting apparent affinities of the ATP sites. In the presence of 20 $\mu$M ATP, phosphorylation of the enzyme preincubated with Ca$^{2+}$ (CaE$_1$) was very fast ($k_{\text{app}} = 400$ s$^{-1}$), $v_{\text{app}}$ of phosphorylation of CaE$_1$ increased with [ATP] along a Michaelis-Menten curve ($K_{M}$ of 15 $\mu$M) and was phospholipid-independent. Without Ca$^{2+}$ preincubation ($E_i + E_a$), $v_{\text{app}}$ of phosphorylation was also phospholipid-independent, but was slower and increased with [ATP] along biphasic curves. This high affinity component reflected rapid phosphorylation was also phospholipid-independent, but was slower and increased with [ATP] along biphasic curves. This high affinity component reflected rapid phosphorylation without Ca$^{2+}$ preincubation ($E_i + E_a$, $v_{\text{app}}$ for $E_a$), further increased by ATP. Steady-state $v_{\text{app}}$ for $E_a$ shifted, which accelerated to a rate higher than that of the ATPase activity when ATP was bound to the regulatory site. Dephosphorylation of EP did not occur without ATP. Dephosphorylation increased along a biphasic curve with increasing [ATP], showing that ATP accelerated dephosphorylation independently of phospholipid. PI, but not phosphatidylethanolamine (PE), accelerated dephosphorylation even in the absence of ATP. $k_{\text{app}}$ for dephosphorylation was 57 s$^{-1}$ at 0 $\mu$M ATP; that rate was further increased by ATP. Steady-state $[\text{EP}] \times k_{\text{app}}$ for dephosphorylation varied with [ATP], and matched the Ca$^{2+}$-ATPase activity measured under the same conditions. ApoE$_0$, the catalytic cycle is rate-limited by dephosphorylation. Acidic phospholipids stimulate Ca$^{2+}$-ATPase activity by accelerating dephosphorylation, while ATP accelerates both dephosphorylation and the conformational change from E$_2$ to E$_1$, further stimulating the ATPase activity.

PMCA, which couples ATP hydrolysis to the extrusion of Ca$^{2+}$ from the cytosol across the plasma membrane of most cells, is strongly modulated by natural PLs and ATP. Acidic PLs like PS and PI increase the enzyme’s apparent affinity for Ca$^{2+}$ and its turnover, whereas neutral PLs like PC and PE are without effect (1, 2). Acidic PLs may exert their effect by simultaneously interacting with the C-terminal regulatory region of PMCA and with a region between putative transmembrane domains 2 and 3 (3, 4). PS has also been reported to increase the phosphorylation, and therefore the activity, of Na/K-ATPase (5), but more recent reports suggest that the key determinant of Na/K-ATPase activity resides in the length of the fatty acyl chains rather than in the polar heads of the PLs (6, 7). Acidic PLs have no significant effect on the affinity of Ca$^{2+}$ for SERCA, but do reduce the level of ATP binding, thereby inhibiting the enzyme activity (8). There are reports of activation of SERCA by PI, but that involves a complex mechanism involving phosphorylation of PI by ATP in the absence of Ca$^{2+}$ (9, 10). It seems, therefore, that activation by acidic PLs is unique to PMCA. On the other hand, the binding of ATP at a regulatory site with lower affinity than the catalytic site increases the rate of ATP hydrolysis (11) and Ca$^{2+}$ transport (12) by PMCA, as well as the activities of Na/K-ATPase (13, 14, 15) and SERCA (16, 17, 18).

We previously reported that PS lowers the apparent dissociation constant for ATP at the low affinity site on PMCA from 9700 to 640 $\mu$M in red cell membranes (19), an observation that others confirmed and extended to PI with PMCA purified from porcine erythrocytes (20). The fact that the concentration of ATP in the cytoplasm of most cells is near 1 mM suggests activation of PMCA by ATP is a physiologically significant event, and that it would only exert its effect in the presence of acidic PL. Likewise, PL would only have an effect on PMCA activity in the presence of ATP. Little specific information about the relationship between PL, ATP, and PMCA is currently available, however.

One way to approach this question is to measure the effects of PL and ATP on the partial reactions of the PMCA catalytic cycle in Scheme I (21). We recently measured the phosphorylation and dephosphorylation (Scheme I, Reactions 3 and 6) of purified PL-depleted PMCA under pre-steady-state conditions and found that aselectin, a mixture of acidic PLs, accelerated dephosphorylation of EP (21). This enzyme preparation should be suitable for studying the effects of ligands on partial reactions because: (i) it is virtually free of contaminant protein, (ii) the radioactivity incorporated from [$\gamma$-32P]ATP is largely EP, (iii) it is free of CaM, which usually contaminates membrane preparations, and (iv) the nature of accompanying lipids can be controlled with pure PLs. In this work we present the results of rapid mixing experiments designed to test the effects of pure acidic and neutral PLs and ATP on the kinetics of the partial reactions that take place during hydrolysis of ATP by PMCA.
Activation of Ca\(^{2+}\)-ATPase by ATP and Acidic Phospholipids

**EXPERIMENTAL PROCEDURES**

**Reagents**—\(\gamma\)-\[^{32}\text{P}\]\text{ATP} was prepared by the method of Glynn and Chappell (22), except that no unlabelled orthophosphate was added to the incubation medium. Carrier-free \(\[^{32}\text{P}\]\text{H}_3\text{PO}_4\) was provided by the Comisión Nacional de Energía Atómica (Buenos Aires, Argentina). 1-α-phosphatidylinositol, 1-α-phosphatidyl-l-serine, 1-α-phosphatidyl-ethanolamine Type V, l-α-phosphatidylcholine, C\(_{12}\)E\(_{10}\), CaM-agarose, and the enzymes and cofactors for the synthesis of \(\gamma\)-\[^{32}\text{P}\]\text{ATP} were all obtained from Sigma Chemical Co. Salts and reagents were of analytical reagent grade.

**Isolation of PMCA**—Cell membranes depleted of CaM were prepared from pig red blood cells using the method of Gietzen et al. (23) with some modification. PL-depleted PMCA was isolated by affinity chromatography on a CaM-agarose column as described by Penniston et al. (24), with C\(_{12}\)E\(_{10}\), K-MOPS and 20% (w/v) glycerol replacing Triton X-100, TES, and PC, respectively. No PLs were added to the medium. The CaM-agarose column with the PMCA bound was washed with 30-column volumes of washing buffer, after which the eluate was eluted with buffer containing 20 mM K-MOPS (pH 7.40 at 4 °C), 100 mM KCl, 0.5 mM MgCl\(_2\), 0.5 mg/ml C\(_{12}\)E\(_{10}\), 2 mM dithiothreitol, 20% (w/v) glycerol, and 1 mM EGTA. No measurements were made of the amount of PL that may have remained with the enzyme. However, assuming that the activity of the soluble enzyme was fully dependent on PL, comparison of the ATPase activity in the absence and presence of added PL indicated the level of delipidation to be close to 90%. Protein concentrations were measured by the method of Lowry (25) after the protein had been precipitated using deoxycholate and trichloroacetic acid to avoid interference (26). Bovine serum albumin was used as the standard.

**Estimation of ATPase Activity**—ATPase activity was estimated from the release of \[^{32}\text{P}\]\text{IP} from \(\gamma\)-\[^{32}\text{P}\]\text{ATP} at 25 °C. Samples (1–2 μg) of PMCA were preincubated for at least 10 min in 0.15 ml of buffer containing 0.5 mM EGTA, 100 mM KCl, 0.5 mM MgCl\(_2\), 50 mM Tris-HCl (pH 7.40 at 25 °C), and 150 μM free Ca\(^{2+}\), 140 μg/ml C\(_{12}\)E\(_{10}\), 20% (w/v) glycerol, and 0 or 66 μg/ml PL. The reaction was started by addition of 0.15 ml of the same buffer containing the indicated concentrations of \(\gamma\)-\[^{32}\text{P}\]\text{ATP} plus an equimolar concentration of MgCl\(_2\).

**Phosphorylation and Dephosphorylation of PMCA**—Phosphorylation of PMCA using \(\gamma\)-\[^{32}\text{P}\]\text{ATP} was carried out at 25 °C in a rapid mixing apparatus adapted for chemical quenching (Intermekron AB, Uppsala, Sweden) based on the design of Mardh and Zetterqvist (27). In a typical experiment, one syringe contained 10–15 μg of purified PMCA, 140 μg of C\(_{12}\)E\(_{10}\), 20% (w/v) glycerol, and 0 or 66 μg of pure PL in 1 ml of buffer containing 0.5 mM EGTA, 100 mM KCl, 0.5 mM MgCl\(_2\), 50 mM Tris-HCl (pH 7.40 at 25 °C), and 0 or 150 μM free Ca\(^{2+}\). A second syringe contained \(\gamma\)-\[^{32}\text{P}\]\text{ATP}, which had been passed through a Millipore filter (type HAWP, 0.45-μm pore size) before use, plus an equimolar concentration of MgCl\(_2\) in 1 ml of the same buffer. The enzyme in the buffer was preincubated with or without Ca\(^{2+}\) at 25 °C for at least 10 min before phosphorylation. The reaction was started by mixing the contents of the two syringes and was ended by collecting the mixture in 9 ml of denaturing solution containing 8.5% (w/v) trichloroacetic acid, 10 mM ATP, and 50 mM H\(_2\)PO\(_4\) at 0 °C. The EP in the denaturing solution was collected by vacuum filtration by carefully adding the mixture, drop by drop, onto the center 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 and 50 mM H\(_2\)PO\(_4\), after which the filters were dried, and the radioactivity was measured by counting in 3 ml of Optiphase scintillation liquid (Fisher Chemical Co.). A blank was prepared by measuring the radioactivity incorporated by the enzyme in medium containing no CaCl\(_2\). The value of the blank was dependent on the amount of \(\gamma\)-\[^{32}\text{P}\]\text{ATP} added, but it did not vary with 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 (21). EP was formed in the lines between the first and second mixing chambers and was chased by the lines between the first and second mixing chambers and was ended by collecting the mixture in 9 ml of a denaturing solution containing 8.5% (w/v) trichloroacetic acid, 10 mM ATP, and 50 mM H\(_2\)PO\(_4\) at 0 °C. The EP in the denaturing solution was collected by vacuum filtration by carefully adding the mixture, drop by drop, onto the center 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 and 50 mM H\(_2\)PO\(_4\), after which the filters were dried, and the radioactivity was measured by counting in 3 ml of Optiphase scintillation liquid (Fisher Chemical Co.). A blank was prepared by measuring the radioactivity incorporated by the enzyme in medium containing no CaCl\(_2\). The value of the blank was dependent on the amount of \(\gamma\)-\[^{32}\text{P}\]\text{ATP} added, but it did not vary with the reaction time and was subtracted from the EP measured in the presence of Ca\(^{2+}\).
activity was measured in the same medium following addition of appropriate amounts of ATP and Ca\(^{2+}\). We found that incubation without Ca\(^{2+}\) and PL inactivated PMCA with a \(t_{1/2} = 30\) min. The enzyme was fully protected by 33 \(\mu\)g/ml PE or 100 \(\mu\)M Ca\(^{2+}\) reduced the amount of inactivation after 60 min incubation to only 20% of that seen in their absence. In the presence of 100 \(\mu\)M Ca\(^{2+}\), either PI, PE, or PC stabilized the activity of detergent-purified PMCA such that it remained unchanged even after 60 min of incubation. Given these observations, one would expect no more than 10% inactivation of the enzyme during the 10-min preincubation in medium containing glycerol, Ca\(^{2+}\), and no PL. Consequently, we are confident that the results shown in Fig. 1 do not reflect protection of the enzyme against inactivation. The information provided by the inactivation experiments was also useful for determining the conditions employed in the experiments described below.

**Effect of PL on the Phosphorylation Kinetics**—Fig. 2 shows the kinetics of EP formation when PMCA was preincubated in medium containing Ca\(^{2+}\) and Mg\(^{2+}\), with and without the indicated PLs, and then phosphorylated in the same medium containing 20 \(\mu\)M \([\gamma\text{-}^{32}\text{P}]\text{ATP}\) at 25 \(^\circ\)C. In the absence of added PL, the time course of phosphorylation was biphasic, with a rapid component \((k_{\text{app}} = 367 \text{ s}^{-1})\) that reached a maximum of 843 pmol/mg of protein, followed by a slow component \((k_{\text{app}} = 20 \text{ s}^{-1})\) that reached a maximum of 270 pmol/mg of protein. The steady-state concentration of EP was increased somewhat by PE and reduced somewhat by PC without changing the shape of the curve. By contrast, in the presence of the acidic PI or PS, the concentration of EP increased rapidly up to a maximum close to 600 pmol/mg of protein in 4 ms with \(k_{\text{app}}\) values of 325 s\(^{-1}\) and 427 s\(^{-1}\) for PI and PS, respectively, and then declined more slowly to a steady-state level of near 470 pmol/mg of protein, a value about half that reached in the absence of acidic PL. These results confirm and extend to pure PC and PS our earlier findings on the effects of asolectin and pure PE and PI on the time course of phosphorylation of PL-depleted PMCA (21), and lend further support to the conclusion that the initial rate of phosphorylation (Scheme I, Reaction 3) is PL-independent.

Our earlier findings (21) suggest that the data shown in Fig. 2 might be the result of a transient fast phosphorylation of Ca\(E_1\) occurring during preincubation and leading to a maximum EP concentration at about 4 ms, followed by a slower phosphorylation limited by the rate of Ca\(E_1\)P formation from Ca\(E_1\)P across Reactions 4, 5, 6, 7, and 7 in Scheme I. Moreover, under conditions that stimulate PMCA overall activity, acidic PLs reduced by 50% the steady-state concentration of EP. This suggests that acidic PLs accelerate at least one of the reactions following Reaction 3.

**Effect of the Timing of the Addition of Ca\(^{2+}\) on the Kinetics of Phosphorylation**—During the experiments summarized in Fig. 2, the enzyme was preincubated with Ca\(^{2+}\) and then phosphorylated by the addition of ATP. Fig. 3 shows the kinetics of the phosphorylation when Ca\(^{2+}\) and ATP were added simultaneously to PMCA preincubated with PI in the absence of Ca\(^{2+}\).

![Figure 2](http://www.jbc.org/)

**Table 1**

| PL | \(K_{n1}\) \(\mu\)M | \(K_{n2}\) \(\mu\)M | \(V_{n1}\) \(\mu\)mol/mg of protein/min | \(V_{n2}\) \(\mu\)mol/mg of protein/min |
|----|----------------|----------------|--------------------------------|--------------------------------|
| PE | 2.6 ± 0.5      | 190 ± 54       | 1.2 ± 0.1                      | 1.2 ± 0.1                      |
| PS | 3.4 ± 1        | 133 ± 52       | 3.3 ± 0.5                      | 6.3 ± 0.4                      |
| PI | 2.8 ± 0.4      | 111 ± 15       | 3.8 ± 0.2                      | 5.2 ± 0.2                      |

*The values ± S.D. correspond to the curves in Fig. 1.*

![Figure 3](http://www.jbc.org/)
observation that preincubating PMCA in medium with limiting concentrations of Ca\(^{2+}\) lowered the rate of phosphorylation (21). Comparison of results in Figs. 2 and 3 also shows that: (i) the steady-state [E] was the same regardless of the presence of Ca\(^{2+}\) during preincubation, and (ii) steady-state was reached at 20 ms in the presence while at 120 ms in the absence of Ca\(^{2+}\) during phosphorylation. This is consistent with the idea that the activation of the PMCA by Ca\(^{2+}\) during phosphorylation depends on a slower reaction prior to phosphorylation, as will be shown below.

**Effect of PI and ATP on the Steady-state Concentration of Phosphoenzyme**—It has been shown previously that steady-state [E] increases along a hyperbolic curve as a function of the ATP concentration up to 100 \(\mu\)M in human red cell membranes \((K_{m,}\, 6.5 \,\mu\text{M at } 0\, ^\circ\text{C}) (30)\) as well as in preparations of purified enzyme \((K_{m,}\, 8.2 \,\mu\text{M at } 25\, ^\circ\text{C}) (31)\). Furthermore, using a wider range of ATP concentrations, including some high enough for ATP to interact with the low affinity site, [E] was shown to increase along a biphasic curve with a high affinity component \((K_{m,}\, 3.2\text{--}12.7 \,\mu\text{M})\) and a low affinity component \((K_{m,}\, 515\text{--}665 \,\mu\text{M})\) (32). As a result, the [E] at 2 m\(\text{M}\) ATP is 3--5 times higher than at 0.1 m\(\text{M}\) ATP, which is indicative of the large effect exerted by the nucleotide binding to the low affinity site.

Because the results in Fig. 2 were obtained at a single ATP concentration, they do not provide information about the effects of ATP on steady-state [E] or on the initial rate of phosphorylation. To measure those parameters simultaneously is troublesome, so we looked first at the effects of ATP (2--1000 \(\mu\text{M}) on steady-state [E] in the presence and absence of PI. The results in Fig. 4 show that \(K_{m,}\) and \(K_{m,}\) were 2 and 155 \(\mu\text{M}\), which is consistent with the values in Table I for the activity of the enzyme in the absence of PI. The low affinity component in Fig. 4, [E] in Table I, cannot be correlated with \(V_{\text{max,}}\) in Table I, except with respect to its relative contribution to the total [E], which is calculated as the ratio \([\text{[E]}]/[\text{[E]}]_1 + [\text{[E]}]_2\) and gave a value of 17\%. Its equivalent for the ATPase activity calculated using the appropriate values in Table I \((V_{\text{max,}}/V_{\text{max,}} + V_{\text{max,}})\) was 24\%.

Fig. 4 also shows the effects of ATP on [E] in the presence of PI. As expected from the results in Fig. 2, PI reduced [E]. Here again the values of \(K_{m,}\) and \(K_{m,}\) were consistent with the corresponding values in Table I. Under these conditions \([\text{[E]}]/[\text{[E]}]_1 + [\text{[E]}]_2\) = 38\%, while \(V_{\text{max,}}/V_{\text{max,}} + V_{\text{max,}}\) = 58\%. Thus, an increase in [E] at high ATP concentrations in the presence of PI does not account for the increase in ATPase activity observed in Fig. 1 under similar conditions.

**Effects of PL, ATP, and the Timing of the Addition of Ca\(^{2+}\) on the Initial Rate of Phosphorylation**—The effect of ATP on \(v_{\text{max}}\), the rate of phosphorylation was tested on PMCA preincubated with or without Ca\(^{2+}\) and then phosphorylated using increasing concentrations of \([\gamma^3\text{P}][\text{ATP}]\) for up to 3 ms. Fig. 5 shows that when preincubated with Ca\(^{2+}\) plus either the acidic PI or the neutral PE, the initial rate of phosphorylation increased with the ATP concentration along a Michaelis-Menten-like curve \((K_{m,}\, 15.0 \,\mu\text{M})\) to a maximum of 570 pmol/mg of protein/ms and then remained constant up to 800 \(\mu\text{M}\) ATP. Since the \(K_{m,}\) value was close to those in the literature \((30,\, 31)\), it was concluded that, at any ATP concentration, phosphorylation of the enzyme preincubated with Ca\(^{2+}\) followed the binding of ATP to the high affinity catalytic site and was PL-independent.

Fig. 5 also shows the initial rate of phosphorylation of PMCA preincubated with either PI or PE in the absence of Ca\(^{2+}\) prior to simultaneous addition of \([\gamma^3\text{P}][\text{ATP}]\) and Ca\(^{2+}\). Confirming the results in Fig. 3, phosphorylation was significantly slower...
than when PMCA was preincubated with Ca\textsuperscript{2+}. It could be argued that this was a consequence of the conditions used in this particular experiment, which could have retarded the association of ATP with the enzyme, thereby reducing the number of reacting units. That possibility was dismissed, however, after a control experiment showed that addition of 0, 10, or 100 μM ATP to the Ca\textsuperscript{2+}-free preincubation medium had no effect on the phosphorylation rate. In the absence of Ca\textsuperscript{2+}, the rate of phosphorylation of preincubated PMCA increased along curves having two components whose parameters are given in the legend to Fig. 5. The rates obtained with PE were lower than with PI, a difference attributed to partial inactivation of the enzyme in the absence of Ca\textsuperscript{2+}, as mentioned above. Regardless of the PL present, the values of Km\textsubscript{2} were close to the Km of the enzyme preincubated with Ca\textsuperscript{2+}, and were ascribed to the catalytic site; those of K\textsubscript{m2} were comparable to the low affinity regulatory site.

Interpreting the results summarized in Fig. 5 on the basis of the consecutive catalytic cycle (Scheme I), we recognized that since the affinity of E\textsubscript{1} for Ca\textsuperscript{2+} is more than 10\textsuperscript{3} times higher than that of E\textsubscript{2}, after preincubation with excess Ca\textsuperscript{2+} most of the enzyme will be in the CaE\textsubscript{1} form (33). Upon addition of ATP, Reactions 2 and 3 take place, leading to formation of phosphoenzyme, and according to our results these reactions are insensitive to high concentrations of ATP and to acidic PLs. The maximum rate was 570 pmol/mg of protein/s, which is more than 3 times higher than the maximum ATPase activity (Fig. 1), again showing that phosphorylation is not rate-limiting during the PMCA catalytic cycle (34).

During preincubation in the absence of Ca\textsuperscript{2+}, PMCA remains distributed between conformers E\textsubscript{1} and E\textsubscript{2}, the latter of which is unfavorable for phosphorylation and must shift to E\textsubscript{1}. Consequently, following addition of Ca\textsuperscript{2+} plus [γ\textsuperscript{32P}]ATP two events likely take place: (i) the existing E\textsubscript{1} rapidly forms the CaE\textsubscript{A}ATP complex and is phosphorylated to CaE\textsubscript{A}P, and (ii) E\textsubscript{2} shifts to E\textsubscript{1} to form the CaE\textsubscript{A}ATP complex and phosphorylated to CaE\textsubscript{AP}. It seems reasonable to assume that the first event gives rise to most of the high affinity component of the biphasic curve in Fig. 5, since reaction 7 is slower than phosphorylation (33). v\textsubscript{a} increased even at ATP concentrations above those needed for occupation of the catalytic site, which is in keeping with the idea that ATP at the regulatory site increases the concentration of E\textsubscript{1} by accelerating reaction 7. Based on our interpretation, v\textsubscript{a} should be directly related to the concentration of CaE\textsubscript{A}, so that the maximum rate of 570 pmol/mg of protein/ms reached by PMCA preincubated with Ca\textsuperscript{2+} can be ascribed to the condition in which 100% of the enzyme is in the CaE\textsubscript{1} form. Similarly, in the presence of PI, the value of v\textsubscript{o max1} (127 pmol/mg of protein/s) for the enzyme at rest without Ca\textsuperscript{2+} can be ascribed to E\textsubscript{1} and represents 22% of the total enzyme, which is more than the 7% we reported previously for the PMCA in red cell membranes (33). Finally, v\textsubscript{o max2} (206 pmol/mg of protein/s) represented 36% of the total and should reflect the extra E\textsubscript{1} that accumulated due to occupation of the low affinity regulatory site during the phosphorylation period. If our interpretation that v\textsubscript{o max2} corresponds to the v\textsubscript{a} of react-
tion 7 is correct, comparison of its value (12.4 \mu mol/mg of protein/min) with those of the Ca\textsuperscript{2+}-ATPase activity (Fig. 1) should enable us to conclude that the conformational change \( E_2 \rightarrow E_1 \) is not rate-limiting during the PMCA catalytic cycle. Although ATP-induced acceleration of the \( E_2 \rightarrow E_1 \) shift is well documented for Na/K-ATPase (35) and SERCA (36), the present results represent the first experimental observation of such an effect in PMCA. Moreover, since the curves remained biphasic, regardless of the presence PI or PE, it can be concluded that acceleration of the conformational change by ATP is PL-independent.

**Effect of PL on the Kinetics of Dephosphorylation**—Fig. 6 shows the kinetics of the first 50 ms of the dephosphorylation of EP in the presence of various PLs. Without added PL, decomposition of EP followed simple exponential kinetics (\( k_{app} = 7 \) s\(^{-1} \)) and except for the increase in \( k_{app} \) to 16 and 25 s\(^{-1} \), respectively, PE and PC did not change the kinetics of the reaction. By contrast, EP prepared with acidic PL dephosphorylated with biphasic kinetics: \( k_{app} \) for the fast component was 100 and 124 s\(^{-1} \) for PI and PS, respectively, while that for the slow component was 5 and 9 s\(^{-1} \). This extends to pure PLs our earlier finding with asolectin (21). Although neutral PLs accelerated dephosphorylation, these results clearly demonstrate that the nearly 16-fold increase in \( k_{app} \) depended primarily on the presence of acidic PL. The fast component reflected at least 70% of [EP] at the start of the reaction with either PI or PS.

The inset to Fig. 6 shows a semi-log plot of the time course of EP dephosphorylation up to 150 ms in the presence of increasing concentrations of PI. The portion of [EP] that decomposed rapidly at the start of the reaction increased from 66% with 15 \mu g of PI/ml, to 80% with 33 \mu g of PI/ml, and to 86% with 66 \mu g of PI/ml; the portion that decomposed slowly decreased in the same proportion. The rate of the slow decomposition of EP was independent of the PI concentration and equal to that of the delipidated PMCA. This suggests that 10–20% of the PMCA did not react with, or was insensitive to, PI, causing an apparently biphasic time course of the dephosphorylation of PMCA in the presence of acidic PL.

We have no definitive explanation for the biphasic kinetics of the dephosphorylation of EP. We previously suggested (21) that one way of interpreting it is to consider PMCA to be a molecular dimer, as has been proposed for SERCA (37) and Na/K-ATPase (38). In a dimeric enzyme, full dephosphorylation implies sequential liberation of P\(_i\) from the two enzyme subunits. Acceleration of one of the subunits by PL could explain the biphasic decomposition of EP, though it does not explain why part of the enzyme continued to behave as if it were delipidated.

**The Effects of ATP on the \( k_{app} \) for Dephosphorylation**—To determine whether phosphorylation at less than 20 \mu M ATP changed the behavior of PMCA during dephosphorylation, control experiments were run in which EP was prepared using 1.5, 3.0, 7.5, or 20 \mu M \([\gamma\textsuperscript{32P}]\text{ATP} \) and dephosphorylated in the presence of 13.3 \mu M ATP. No significant differences in the rates of dephosphorylation were observed (results not shown).

During the experiments summarized in Fig. 6, the dephosphorylation medium contained 13.3 \mu M ATP. Fig. 7 shows the effect of increasing the concentration of ATP in the dephosphorylation medium on the \( k_{app} \) for the reaction. By extrapolating the curves back to the ordinate, the dephosphorylation rate in medium without ATP could be estimated. The rate of decomposition of the delipidated EP in the absence of ATP was zero. In the presence of ATP, \( k_{app} \) increased as a function of the ATP concentration along a biphasic curve from 0 to 4 s\(^{-1} \) at low ATP concentrations, and to 33 s\(^{-1} \) at higher ATP concentrations; \( K_m \) values for the two components were consistent with those for the catalytic and the regulatory sites. Addition of PE had little effect on the dephosphorylation response to ATP: \( k_{app} \) increased from 0 to 2 s\(^{-1} \) and to 37 s\(^{-1} \) within the low and high ATP concentration ranges, respectively. On the other hand, the effect of ATP on the \( k_{app} \) for dephosphorylation was strongly affected by the presence of PI. By extrapolating back to the ordinate, the \( k_{app} \) at 0 \mu M ATP was estimated to be 57 s\(^{-1} \). This is the first demonstration that PI increased the rate of dephosphorylation via a mechanism independent of other PMCA ligands. Furthermore, comparison of the effects of PI with those of PE (Fig. 7) enabled us to conclude that activation of dephosphorylation in the absence of ATP was a specific effect of acidic PLs. In the presence of PI, increasing ATP caused \( k_{app} \) to increase along a biphasic curve up to 119 s\(^{-1} \) at low ATP concentrations (\( K_{app} \), of 13 \mu M) and then up to 192 s\(^{-1} \) at higher ATP concentrations (\( K_{app} \) of 132 \mu M). Clearly, ATP accelerated the dephosphorylation of PMCA. The same effect has been observed with SERCA (39), but not with Na/K-ATPase, where the rate of dephosphorylation was unaffected by ATP, either in the presence or absence of K\(^+\) (40).

Activation of delipidated PMCA in the presence of PE was stimulated mainly via the low affinity regulatory site. In the presence of PI, by contrast, 46% of the maximum activation by ATP was apparent at concentrations too low to be mediated via the regulatory site. Without discarding alternative possibilities, occupation of the catalytic site by ATP would seem a simple way to account for this partial activation. Moreover, since Ca\textsuperscript{2+} was absent during dephosphorylation, the effect must be independent of phosphorylation. It is therefore notable that P-nitrophenylphosphatase activity, which is thought to reflect the dephosphorylation reaction catalyzed by the \( E_0 \) conformer, is activated by either high affinity ATP binding (41) or CaM (42), and is further activated by ATP in the presence of CaM (43). The \( K_m \) for ATP as an activator of P-nitrophenylphosphatase is equal to the \( K_m \) of the Ca\textsuperscript{2+}-ATPase (41). However, we have shown that the ATP-dependent activity of P-nitrophenylphosphatase from red cell membranes persists after most of the Ca\textsuperscript{2+}-ATPase activity has stopped, and the dephosphorylation reaction has been abolished by phospholipase C (44). It is tempting to suggest that the high affinity activation of dephosphorylation with PI and phosphatase activity by ATP...
could be related phenomena. The suggestion that in SERCA the high and low affinity sites are a single site that changes its affinity for ATP during the reaction cycle may support this view (18).

We have shown (45) that in the presence of a non-limiting concentration of Mg$^{2+}$, the transition CaE$_2$P → CaE$_2$P during phosphorylation is rapid, suggesting that in the experiments summarized in Figs. 6 and 7 most of the phosphoenzyme was in the CaE$_2$P form at zero time. This means the effect of acidic PL or ATP should be exerted on a reaction downstream of that transition. However, our results do not provide enough information about whether activation of dephosphorylation by acidic PLs and ATP was the result of accelerated EP hydrolysis (Scheme I, Reaction 6) or the release of Ca from CaE$_2$P (Reaction 5) to enable us to draw a conclusion.

Comparison of the ATPase activity with the Estimated Rate of P$_i$ Production in Presence of PI

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Comparison of the ATPase activity with the Estimated Rate of P$_i$ Production in Presence of PI—Multiplication of the steady-state [EP] × the $k_{\text{app}}$ for dephosphorylation gives the rate of production of P$_i$ from EP. If the dephosphorylation that we measured represents a reaction that participates in the PMCA catalytic cycle, the rate of P$_i$ production should be close to the rate of ATP hydrolysis. Such comparison requires that [EP], $k_{\text{app}}$ and ATPase activity are measured under identical experimental conditions. This was the case when these quantities were measured as a function of the ATP concentration in the presence of PI (Figs. 1, 4, and 7). The results in Fig. 8 show good agreement between the experimental findings and the calculated activity, giving strong support to the idea that the properties of dephosphorylation shown here belong to a reaction that participates in the PMCA catalytic cycle, that activation by ATP and PI are key determinants of the activity, and that dephosphorylation of CaE$_2$P is the rate-limiting step in the catalytic cycle. Furthermore, since the concentration of ATP in the cytoplasm of most animal cells is close to 1 mM, results in this study assign to acidic phospholipids a key role in the physiological regulation of PMCA.

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