Calcium Occlusion in Plasma Membrane Ca\(^{2+}\)-ATPase*

Received for publication, May 31, 2011, and in revised form, July 1, 2011 Published, JBC Papers in Press, July 27, 2011, DOI 10.1074/jbc.M111.266650

Mariela S. Ferreira-Gomes‡,* Rodolfo M. González-Lebrero‡, María C. de la Fuente‡, Emanuel E. Strehler‡, Rolando C. Rossi‡,†,‡ and Juan Pablo F. C. Rossi‡,†

In this work, we set out to identify and characterize the calcium occluded intermediate(s) of the plasma membrane Ca\(^{2+}\)-ATPase (PMCA) to study the mechanism of calcium transport. To this end, we developed a procedure for measuring the occlusion of Ca\(^{2+}\) in microsomes containing PMCA. This involves a system for overexpression of the PMCA and the use of a rapid mixing device combined with a filtration chamber, allowing the isolation of the enzyme and quantification of retained calcium. Measurements of retained calcium as a function of the Ca\(^{2+}\) concentration in steady state showed a hyperbolic dependence with an apparent dissociation constant of 12 ± 2.2 \(\mu\)M, which agrees with the value found through measurements of PMCA activity in the absence of calmodulin. When enzyme phosphorylation and the retained calcium were studied as a function of time in the presence of La\(^{3+}\) (inducing accumulation of phosphorylation and the retained calcium), we obtained apparent rate constants not significantly different from each other. Quantification of the retained calcium in steady state and obtaining stoichiometry of one mole of occluded calcium per mole of phosphoenzyme. These results demonstrate for the first time that one calcium ion becomes occluded in the \(E_1P\)-phosphorylated intermediate of the PMCA.

The plasma membrane calcium ATPase (PMCA)\(^3\) is a calmodulin-modulated P-type ATPase responsible for the maintenance of low intracellular concentrations of Ca\(^{2+}\) in most eukaryotic cells. It couples the transport of Ca\(^{2+}\) out of cells with the hydrolysis of ATP into ADP and inorganic phosphate. PMCA\(^s\) consists of a single polypeptide chain of 127,000 to 137,000 Da. Mammalian PMCA\(^s\) are encoded by four separate genes (PMCA1–4), and additional isoforms are generated via alternative RNA splicing, which augments the number of variants to >20 (1).

The current kinetic model for PMCA function proposes that the enzyme exists in two main conformations, \(E_1\) and \(E_2\). \(E_1\) has a high affinity for Ca\(^{2+}\) and is readily phosphorylated by ATP, whereas \(E_2\) has a low affinity for Ca\(^{2+}\) and can be phosphorylated by P\(_i\). After binding of intracellular Ca\(^{2+}\) to high affinity sites, \(E_1\) can be phosphorylated by ATP with formation of the intermediate \(E_1P\). After a conformational transition to \(E_1P\), Ca\(^{2+}\) would be released to the extracellular medium from low affinity sites, followed by the hydrolysis of the phosphoenzyme to \(E_2\) and a new conformational transition to \(E_1\) (Fig. 1) (2). During some stages of the reaction cycle, Ca\(^{2+}\) becomes occluded, i.e. trapped in the enzyme machinery while it is transported from one side to the other side of the membrane. The principal aim of this study is to identify and kinetically characterize the intermediate(s) of the PMCA containing occluded calcium. Evidence for occlusion in Na,K-ATPase and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) has been well established (3), and a good deal of information exists about the occlusion and deocclusion steps of the transport cations in these pumps. Na\(^+\) and K\(^+\) are occluded in the \(E_1P\) and \(E_2\) intermediates of the Na\(^+\)/K\(^+\)-ATPase (4), respectively, and Ca\(^{2+}\) becomes occluded in the \(E_1P\) intermediate of the SERCA (5).

By analogy with SERCA, one would expect (5) that Ca\(^{2+}\) occlusion occurs in the \(E_1P\) state of the PMCA. However, it has not been possible until now to obtain definitive experimental evidence of such a phenomenon, mainly because PMCA in microsomal preparations obtained from natural sources is not sufficiently abundant and pure. To overcome this difficulty, we have used a procedure to overexpress PMCA in Sf9 insect cells using a baculovirus system and also to isolate the microsomal membranes. Binding of Ca\(^{2+}\) to these membranes was measured as a function of time using a method (6) that combines a quench-flow apparatus with a rapid filtration device, with a time resolution of 3.5 ms. The procedure included inhibition of endogenous SERCA and the permeabilization of microsomal vesicles using the pore-forming peptide alamethicin, which prevents \(45\)Ca\(^{2+}\) accumulation (7). Our results suggest that a single calcium ion is occluded in \(E_1P\) of the PMCA and that the formation of this phosphoenzyme and the occlusion of calcium are simultaneous events.

**EXPERIMENTAL PROCEDURES**

**Expression of PMCA in Sf9 Cells**—The Sf9 cells (derived from pupal ovarian tissue of the fall armyworm Spodoptera frugiperda) were grown in suspension at 27 °C in Grace Medium

---

*This work was supported, in whole or in part, by National Institutes of Health, Fogarty International Center Grant R03TW006837. This work was also supported by Agencia Nacional de Promoción Científica y Tecnológica, Consejo Nacional de Investigaciones Científicas y Técnicas, and Universidad de Buenos Aires, Ciencia y Técnica from Argentina.

‡The online version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

†To whom correspondence may be addressed: Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, and Universidad de Buenos Aires, Ciencia y Técnica from Argentina.

‡‡The abbreviations used are: PMCA, plasma membrane calcium pump; \([\text{I}^{25}\text{S}]\text{TID-PC/16}, 1\text{-O-hexadecanoyl-2-0-[9\]([2\text{-}\text{I}^{125}\text{S}]\text{iodo-4-(trifluoromethyl)-3H- diazirin-3-y1]benzoyl]oxy]carbonyl]nonanoyl\}); sn-glycero-3-phosphocholine.
Calcium Occlusion in PMCA

supplemented with 10% fetal bovine serum, 1% Pluronic, and 1× antibiotic-antimycotic (Invitrogen, catalog no. 15240-062). The expression for protein production was carried out by infecting Sf9 cells in suspension in complete Grace Medium with the recombinant virus at a multiplicity of infection of 1–2. After 48 h of incubation at 27 °C in the dark, the cells were harvested. A 250-ml culture gave 250×10⁶ cells. The cells were washed with phosphate-buffered saline buffer containing 1 mM EDTA and protease inhibitors, quickly frozen, and then kept at −80 °C until microsome processing.

Recombinant Baculovirus—The viral stocks for expression of human PMCA4b have been described (8) and were kindly provided by Ariel J. Caride and Adelaida G. Filoteo (Mayo Clinic, Rochester, MN).

Amplification of Recombinant Baculovirus—The viral stocks were amplified using a multiplicity of infection of 0.1–0.2 following standard procedures, and the titer of the amplified stock was determined. The viral stock was kept at 4 °C in the dark.

Measurements of Free Ca²⁺ Concentrations—The Ca²⁺ concentration in the incubation medium was measured using a selective Ca²⁺ electrode (93–20, Orion Research, Inc.), as described by Kraljevic et al. (10).

Thapsigargin Treatment—Thapsigargin (octanoyl acid derivative of azulene[4,5-b]furan) was obtained from Sigma (catalog no. T9033). Thapsigargin was dissolved in dimethyl sulfoxide to a concentration of 153.6 μM. Dilutions of this solution were added to a suspension containing the protein. The final concentration of dimethyl sulfoxide never exceeded 0.1% in volume. Controls of Ca²⁺-ATPase activity with and without dimethyl sulfoxide showed no significant differences. In agreement with Sagara et al. (11), we found that inhibition of SERCA activity was achieved after a 15-min incubation of the microsomal preparation with 200 nM thapsigargin at 25 °C. This inhibition lasted for at least 3 min after addition of Ca²⁺. Therefore, all experiments were performed within this time frame.

Measurements of Calcium Retained—Calibration and standards were obtained using the method of Rossi et al. (6) where the rapid mixing apparatus is connected to a quenching-and-washing chamber (supplemental Fig. S1), through a suitable polyethylene tubing. In a typical experiment, one volume of a microsomal preparation suspended in a solution with 30 mM MOPS (pH 7.4 at 25 °C), 120 mM KCl, and 400 mM thapsigargin was mixed with the same volume of a solution containing the same concentrations of MOPS and KCl, plus 6 mM MgCl₂ and enough ATP and (⁴⁵Ca²⁺)CaCl₂ to obtain the concentrations of the nucleotide and of free Ca²⁺ indicated in the figures. For some experiments, 100 μM La³⁺ was also included in the latter solution. Measurements were carried out at 25 °C. Reactions were quenched after the appropriate time by injecting the reaction mixture into the quenching-and-washing chamber at a flow rate of 1–5 ml/s. During the injection process, the fluid was mixed with an ice-cold washing solution flowing at a rate of 30–40 ml/s and then filtered through a Millipore filter (AA, 0.8-μm pore size) placed in the quenching-and-washing chamber to retain the microsomal suspension that includes the enzyme. From control experiments using a microsomal preparation covalently labeled with [¹²⁵I]TID-PC ([¹²⁵I]TID (3-(trifluoromethyl)-3-[(m-[¹²⁵I]iodophenyl)diazirin) (13), and measuring the radioactivity on the Millipore filters of 0.22–0.80-μm pore size after a quenching and washing run, at least 99% of the MgCl₂, 200 nM thapsigargin, and enough CaCl₂ to obtain the concentration of free Ca²⁺ indicated in the figures. The concentrations of other components varied according to the experiments and are indicated in the figure legends. For measuring time courses of phosphoenzyme and Ca²⁺ retained in the millisecond-second timescale, we used a rapid mixing apparatus SFM4 from Bio-Logic.
labeled enzyme was recovered irrespective of the filter pore size.

To ensure that the initial temperature in the quenching-and-
washing chamber was 1–2 °C and that the flow was constant,
~50 ml of washing solution was allowed to run through the
filter prior to the injection of the reaction mixture, and 240 ml
of washing solution was applied to the filter from that moment.
The composition of the washing solution was 10 mM Tris, 10
mM EDTA, pH 7.4, at 2 °C. Control experiments show that the
washing procedure effectively removes the unbound 45Ca2+.
After the washing solution was drained, the filter was removed,
dried under a lamp, and counted for 45Ca2+ radioactivity in a
scintillation counter. This was converted into nanomoles of
Ca2+ using the specific activity value of the 45Ca2+ in the
reaction mixture. Retained Ca2+ was considered equal to the
45Ca2+ radioactivity retained by the enzyme after subtracting
the blank values. These were estimated from the amount of
45Ca2+ retained by the filters in the presence of enzyme that
was heat-inactivated for 2 h at 50 °C (see supplemental Figs. S2
and S3).

Determination of Phosphorylated Intermediates—The phos-
phorylated intermediates (EP), were measured as the amount of
acid-stable 32P incorporated in the enzyme from [γ-32P]ATP
after stopping the reaction with an ice-cold solution containing
10% trichloroacetic acid. The isolation of the intermediate was
performed according to two methodologies.

In the first methodology, the suspension was transferred to
Millipore filters (Type GS, 0.22 μm pore size) where the phos-
phoenzyme was washed with 20 ml of a solution of 10% trichlo-
roacetic acid and 50 mM H3PO4. For the blanks, similar exper-
iments were done with heat-inactivated enzyme.

In the second methodology, when the microsomes were
phosphorylated with high concentration of ATP, we used the
method described by Echarte et al. (14). The phosphorylation
reaction was stopped, and the tubes were spun down at 7000
rpm for 3.5 min at 4 °C. The samples were then washed once
with 7% TCA, 150 mM H3PO4, and once with double-distilled
and stored for SDS-PAGE. For this purpose, the pellets
were dissolved in a medium containing 150 mM Tris-HCl
(pH 6.5 at 14 °C), 5% SDS, 5% DTT, 10% glycerol, and bromphe-
nol blue (sample buffer). Electrophoresis was performed at pH
6.3 (14 °C) in a 7.5% polyacrylamide gel. The reservoir buffer
was 175 mM MOPS, pH 6.5, with 0.1% SDS. Migration of the
sample components took place at 14 °C, with a current of 60
mA until the tracking dye reached a distance of ~10 cm from
the top of the gel. Gels were stained, dried, and exposed to a
Storage Phosphoscreen of Molecular Dynamics (Amersham
Biosciences). Unsaturated autoradiograms and stained gels
were scanned with an HP Scanjet G2410 scanner. Analysis of
the images was performed with GelPro Analyzer. EP quantifi-
cation was achieved as described in Echarte et al. (14).

Alamethicin Treatment—Alamethicin was obtained from
Sigma (catalog no. A4665). This peptide was dissolved in 60%
(v/v) ethanol to a concentration of 20 mg/ml (7). This solution
was added to a suspension containing the protein. Final con-
centrations of alamethicin added to the microsomes are
expressed on a weight basis relative to microsomal protein. The
final ethanol concentration of the microsomes after adding
alamethicin never exceeded 0.1%, and for all of the experi-
ments, an equivalent amount of ethanol was added to control
membranes not treated with alamethicin.

Data Analysis—Theoretical equations were fitted to the
results by nonlinear regression based on the Gauss-Newton
algorithm using commercial programs (Excel and Sigma-Plot
for Windows, the latter being able to provide not only the best
fitting values of the parameters but also their S.E.). The good-
ness of fit of a given equation to the experimental results was
evaluated by the corrected AIC criterion defined in Equation 1,

$$AIC_c = N \ln(SS/N) + 2PN/(N - P - 1)$$ \hspace{1cm} (Eq. 1)

where N is the number of data, P is the number of parameters
plus one, and SS is the sum of weighted square residual errors
(15). Unitary weights were considered in all cases, and the best
equation was chosen as that giving the lower value of AICc. The
AIC criterion is based on information theory and selects an
equation among several possible equations on the basis of its
capacity to explain the results using a minimal number of
parameters.

RESULTS

Calcium Retained by Microsomal Vesicles Expressing PMCA—
According to earlier findings on SERCA (16), one would expect
to observe occlusion of Ca2+ in PMCA as a fast phase early in
the time course of 45Ca2+ uptake by microsomal vesicles.

Fig. 2 shows the amount of 45Ca2+ retained by microsomal
vesicles (Ca_{ret}) measured as a function of time in media with 25
μM or 2000 μM ATP, 3 mM MgCl2, and 60 μM [45Ca] Ca2+ at
25 °C. For both concentrations of ATP, the time course can be
described by the sum of two increasing exponential functions of
time,

$$Ca_{ret} = A_1(1 - e^{-k_1t}) + A_2(1 - e^{-k_2t})$$ \hspace{1cm} (Eq. 2)

where A1 and A2 are maximal amounts of Ca_{ret} and k1 and k2
are rate coefficients. The best fitting values of the parameters

FIGURE 2. Time course of retained calcium by microsomal preparations of
PMCA at 25 μM or 2 mm ATP. Measurements of retained calcium by hPMCA4b were carried out at 25 °C in a reaction medium containing 60
μg/ml total protein, 3 mM MgCl2, 0.2 μM thapsigargin (Δ), 25 μM or 2000 μM
ATP (○), and enough (45Ca)CaCl2 to give concentrations of 100 μM free Ca2+.

Calcium Occlusion in PMCA

FIGURE 2.
are shown in Table 1, where it appears that $A_1$ and $k_1$, as well as $k_2$ increase with the concentration of ATP. If one assumes that the fast component is due to the occlusion of Ca$^{2+}$ whereas the slow one reflects the accumulation of Ca$^{2+}$ into vesicles of the microsomal preparation, $A_1$ should be on the order of the amount of Ca$^{2+}$ ATPase, while $A_2$ (but not necessarily $A_1$) should decrease upon addition of a permeabilizing agent. Results of experiments to test these predictions are described in the following paragraphs.

Enzyme Concentration—La$^{III}$ is known to prevent the Mg$^{2+}$-dependent transition $E_1P\rightarrow E_2P$, acting noncompetitively with respect to Ca$^{2+}$ and ATP (17, 18). Thus, the amount of PMCA can be evaluated by measuring the concentration of enzyme phosphorylated from [$\gamma$-32P]ATP in the presence of this inhibitor, producing accumulation of $E_1P$ with the inhibition of the ATPase activity (17, 18).

Fig. 3A shows the amount of phosphorylated intermediates, $EP (= [E_1P] + [E_2P])$, as a function of [La$^{III}$]. $EP$ increased with the concentration of La$^{III}$ along the following hyperbolic function,

$$EP = EP_0 + \frac{(EP_{\text{max}} - EP_0) \times [\text{La}^{III}]}{K_{0.5} + [\text{La}^{III}]} \quad \text{(Eq. 3)}$$

where $EP_0$ and $EP_{\text{max}}$ are the amounts of PMCA phosphorylated in the absence of lanthanum and in the presence of non-limiting concentrations of the inhibitor, respectively, and $K_{0.5}$ is the concentration of La$^{III}$ at which $EP = (EP_{\text{max}} + EP_0)/2$. The best fitting values for $EP_0$, $EP_{\text{max}}$, and $K_{0.5}$ were 0.030 ± 0.002 nmol $EP$ mg$^{-1}$, 0.0765 ± 0.0024 nmol $EP$ mg$^{-1}$, and 5.25 ± 1.33 $\mu$M, respectively. In the inset of Fig. 3A, we plotted Ca$^{2+}$-ATPase activity as a function of the concentration of La$^{III}$. The best fit to the experimental data were a hyperbolic decreasing function (see legend to Fig. 3), where the $K_i$ for La$^{III}$ was 3.5 ± 0.8 $\mu$M (cf. with the value of $K_{i,\text{La}}$ for La$^{III}$ for EP formation). The time course of Ca$^{2+}$ retained in the presence of 50 $\mu$M La$^{III}$ was measured under similar conditions, although using a different preparation (Fig. 3B). Best fitting values using equation 2 were $A_1 = 0.0774 ± 0.0072$ nmol mg$^{-1}$, $A_2 = 0.314 ± 0.014$ nmol mg$^{-1}$, $k_1 = 2.17 ± 0.38$ s$^{-1}$, and $k_2 = 0.059 ± 0.007$ s$^{-1}$, respectively.

Note that the value of $A_1$ is of the same order of magnitude as that of $EP_{\text{max}}$ above, which can be taken as evidence that the first fast phase of the time course of calcium retained is due to the occlusion of Ca$^{2+}$ in the PMCA. Moreover, although La$^{III}$ almost completely inhibits the ATPase activity, it does not significantly decrease the fraction of the slow phase in the time course of Ca$^{2+}$ retained.

Effect of Alamethicin on Ca$^{2+}$ Accumulation by Microsomes—If the slow phase of the time course in Figs. 2 and 3B is due to the uptake of Ca$^{2+}$ into vesicles, addition of permeabilizing agents should facilitate the washing out of Ca$^{2+}$ in the quenching and washing chamber and accordingly remove a possible confounding factor for the detection of Ca$^{2+}$-occluded states in the PMCA. We tested this by treating the microsomal membranes with alamethicin, a peptide that forms large pores allowing the passage of organic molecules of considerable size such as ATP. Alamethicin has been used previously in transport studies of the SERCA (7) and determinations of occlusion of Rb$^+$ in the H$^+$/K$^+$-ATPase (19).

Following incubation of microsomal membranes with different concentrations of alamethicin for 30 min at 25°C, retained calcium was measured as a function of time from 1.5 to 8.5 s (Fig. 4A, inset). In Fig. 4A, we plotted both the Ca$^{2+}$-ATPase activity and the slopes of the curves of retained calcium versus time as a function of the alamethicin concentration. The data show that, as [alamethicin] increases, the slope decreases, whereas the PMCA activity remains nearly constant, at least up
The amount of bound calcium was measured at 25 °C in medium containing microsomal preparations of hPMCA4b treated with or without alamethicin. concentrations of 60 mM MgCl$_2$, 2000 μM ATP, and enough $^{45}$CaCl$_2$ to give concentrations of 60 μM free Ca$^{2+}$. The inset shows the calcium retained over time as a function of different ratios of alamethicin:total protein. Each slope line represents the velocity of calcium retention at a given alamethicin:protein ratio. Measurements of Ca$^{2+}$-ATPase activity were carried out at 25 °C in medium containing 60 μg/ml total protein, 3 mM MgCl$_2$, 2000 μM ATP, and enough CaCl$_2$ to give concentrations of 60 μM free Ca$^{2+}$. B, time course of calcium retained by microsomal preparations of hPMCA4b treated with or without alamethicin. The amount of bound calcium was measured at 25 °C in medium containing 60 μg/ml total protein, 3 mM MgCl$_2$, 25 μM ATP without (△) or with (○) 12 μg/ml alamethicin and enough $^{45}$CaCl$_2$ to give concentrations of 60 μM free Ca$^{2+}$.

to 0.3 mg of alamethicin per mg of total protein. In a similar experiment (data not shown), we found that it was safe to use this peptide in a concentration of 0.4 mg per mg of total protein without affecting the enzyme activity. Based on these results, we performed subsequent experiments adding alamethicin at a concentration of 0.2–0.4 mg per mg of total protein. This substantially reduced (but did not totally eliminate) the interference due to the microsomal uptake and accumulation of Ca$^{2+}$ in our measurements of Ca$_{ret}$. As a further refinement of these measurements we also took into account a slow residual linear component of bound Ca$^{2+}$ that was observed as well for heat inactivated enzyme and was therefore subtracted as background (supplemental Fig. S2).

Fig. 4B compares the time course of calcium retained measured either in the absence of alamethicin or in the presence of 0.2 mg alamethicin per mg of total protein. Notice that La$^{3+}$ was not present in this experiment. Although the time course with no added alamethicin shows a behavior similar to that in Fig. 2 and can be described by the same function of time, results obtained in the presence of alamethicin are well fitted by a single increasing exponential function of time plus a slow linear component. The faster exponential component of both curves has similar values (0.0269 ± 0.0041 nmol Ca$^{2+}$·mg$^{-1}$ in the absence and 0.0224 ± 0.0012 nmol Ca$^{2+}$·mg$^{-1}$ in the presence of alamethicin) and rate coefficient (2.963 ± 0.764 s$^{-1}$ in the absence and 2.336 ± 0.439 s$^{-1}$ in the presence of alamethicin). This indicates that alamethicin permits efficient washing of the permeabilized vesicles without affecting the capacity of PMCA to bind calcium.

**Steady-state Level of Calcium Occluded and Ca$^{2+}$-ATPase Activity as a Function of [Ca$^{2+}$]—**If the retained calcium that accumulates in the presence of alamethicin corresponded to a reaction intermediate of the PMCA, its steady-state amount should vary with the concentration of a substrate with the same affinity as that of the ATPase activity. To test this, we measured the amount of retained calcium in the steady state (Fig. 5, A and B) as well as the Ca$^{2+}$-ATPase activity (Fig. 5C) as a function of [Ca$^{2+}$]. Fig. 5A shows the time courses of retained calcium, after subtracting the small linear component due to nonspecific binding (see supplemental Fig. S3), measured at different [Ca$^{2+}$]. All the curves can be described by a single increasing exponential function of time,

$$Ca_{osc} = A(1 - e^{-kt}) \quad \text{(Eq. 4)}$$

where $A$ is the steady-state amount of the “specific” retained Ca$^{2+}$, and $k$ is an apparent rate coefficient. The best fitting values of $A$ from Fig. 5A were plotted as a function of [Ca$^{2+}$] as shown in Fig. 5B.

Both the value of $A$ from Equation 4 and the Ca$^{2+}$-ATPase activity can be described by a rectangular hyperbola as a function of [Ca$^{2+}$],

$$Y = \frac{Y_{max}[Ca^{2+}]}{K_{0.5} + [Ca^{2+}]} \quad \text{(Eq. 5)}$$

where $Y_{max}$ is the Ca$^{2+}$-ATPase activity or the value of $A$ when the calcium concentration tends to infinity, and $K_{0.5}$ represents the [Ca$^{2+}$] at which the half-maximum effect is achieved. The best fitting values of $K_{0.5}$ were 12.5 ± 1.3 μM and 12.7 ± 2.2 μM for activity and occluded calcium, respectively. The fact that these values are not significantly different from each other indicates that the retained calcium measured in media with alamethicin is due to an intermediate of the reaction cycle, probably that containing occluded calcium.

**Stoichiometry of Occluded Calcium—**As shown in Fig. 3, the amount of PMCA, evaluated as the maximal concentration of EP that accumulates as $E$P in the presence of La$^{3+}$, is of the same order of magnitude as the size of the fast component of the time course of Ca$_{ret}$. Because the yield of PMCA expressed in S9 cells varies between different preparations, a strictly quantitative comparison between Ca$_{ret}$ and EP requires performing experiments using the same preparation under the same conditions. Results of parallel experiments in media with
La\textsuperscript{III} measuring steady-state levels of both \( E \)\textsubscript{P} and \( Ca^{2+} \) occluded, and the ratio \( Ca_{\text{ooc}}/E \)\textsubscript{P} are shown in Table 2 for two different microsomal preparations. Although the values of both experimentally determined levels vary between the preparations, the ratio \( Ca_{\text{ooc}}/E \)\textsubscript{P} is nearly equal to 1, which is consistent with the stoichiometry of one \( Ca^{2+} \) transported per molecule of ATP hydrolyzed (20–22) and with the hypothesis that, in the presence of ATP, calcium is occluded in the \( E \)\textsubscript{P} conformation of the PMCA.

**Time Course of \( E \)\textsubscript{P} and Occluded Calcium**—To determine whether calcium occlusion in the PMCA is concomitant with the formation of the \( E \)\textsubscript{P} phosphorylated enzyme intermediate, we measured the time course of \( E \)\textsubscript{P} and calcium occlusion under the same experimental conditions. Fig. 6 shows that both phosphorylation and calcium occlusion increase simultaneously and can be described by a single exponential as a function of time adjusted to Equation 4, with best fitting values of \( k = 2.10 \pm 0.30 \text{ s}^{-1} \) and \( 2.09 \pm 0.31 \text{ s}^{-1} \), for \( E \)\textsubscript{P} and occluded calcium, respectively. Furthermore, the data reveal a stoichiometry of 1:1 for calcium occlusion and PMCA phosphorylation over time, showing that both reactions occur concomitantly.

**FIGURE 5.** Steady-state level of retained calcium and \( Ca^{2+} \)-ATPase activity as a function of \( [Ca^{2+}] \). A, the retained calcium was measured at 25 °C in reaction medium containing 60 \( \mu \text{g} \)/ml total protein, 3 \( \text{mM MgCl}_2 \), 25 \( \mu \text{M ATP} \), 12 \( \mu \text{g/ml} \) alamethicin, and enough \( \text{(45Ca)}\text{CaCl}_2 \) to give 2 \( \mu \text{M} \) free \( Ca^{2+} \) \( (\bigcirc) \); 10 \( \mu \text{M} \) free \( Ca^{2+} \) \( (\cdot) \); 25 \( \mu \text{M} \) free \( Ca^{2+} \) \( (\bigtriangleup) \); and 60 \( \mu \text{M} \) free \( Ca^{2+} \) \( (\square) \). B, plot of the values of maximal retained \( Ca^{2+} \) as a function of the free \( Ca^{2+} \) concentration as obtained from an analysis of the data in A. C, \( Ca^{2+} \)-ATPase activity measurements were carried out at 25 °C in reaction medium containing 60 \( \mu \text{g/ml} \) total protein, 3 \( \text{mM MgCl}_2 \), 25 \( \mu \text{M} \) ATP, and 12 \( \mu \text{g/ml} \) alamethicin enough \( Ca^{2+} \) to give different concentrations of free \( Ca^{2+} \).

**FIGURE 6.** Time course of phosphorylated intermediate formation and occluded calcium in the presence of lanthanum. The phosphorylation of \( h \text{PMCA}4b \) and the amount of occluded calcium were measured at 25 °C in reaction medium containing 60 \( \mu \text{g/ml} \) total protein, 3 \( \text{mM MgCl}_2 \), 25 \( \mu \text{M} \) ATP, 50 \( \mu \text{M La}^{\text{III}} \), and enough \( Ca^{2+} \) to give concentrations of 60 \( \mu \text{M} \) free \( Ca^{2+} \).
Calcium Occlusion in PMCA

DISCUSSION

This work provides the first conclusive evidence that Ca\textsuperscript{2+} becomes occluded in the plasma membrane Ca\textsuperscript{2+}-ATPase, with a stoichiometry of one Ca\textsuperscript{2+} transported per ATP hydrolyzed. The evidence is based on the following findings. First, during ATPase activity, the uptake of Ca\textsuperscript{2+} by microsomes enriched in PMCA shows a time course with a fast component whose amplitude is compatible with the amount of enzyme, and a slow component, which is related to accumulation of Ca\textsuperscript{2+} into vesicles. In support of this interpretation, addition of the pore-forming peptide alamethicin tends to eliminate the slow component without affecting the fast one. Second, measurements in the presence of alamethicin and La\textsuperscript{III} show that the time course of the fast component of Ca\textsuperscript{2+} uptake is concomitant with that of enzyme phosphorylation, with a stoichiometry of one Ca\textsuperscript{2+} retained per phosphorylation site. Third, steady-state measurements of calcium retained and ATPase activity in the presence of alamethicin exhibit the same $K_0$ for Ca\textsuperscript{2+}. These results indicate that Ca\textsuperscript{2+} becomes occluded in the $E_1P$ intermediate of the PMCA, with a stoichiometry of one Ca\textsuperscript{2+} per phosphorylation site, but they do not rule out the possibility that other intermediates of the reaction cycle can occlude Ca\textsuperscript{2+} as well.

Steps toward Detailed Kinetic Model for PMCA—Although there is a large body of information on the kinetics of the PMCA, most of this information has not been incorporated into a detailed unified model because of the heterogeneity of the data. The main reason for this has been the lack of a reliable standard preparation of the pump. Unlike for other P-type ATPases, there are no known tissue sources rich enough in a single isoform of PMCA. Even the purified erythrocyte preparation is a mixture of PMCA4b and PMCA1b (23). Additionally, methodological challenges have made it very difficult to obtain reliable measurements of phosphorylated species at physiological (millimolar) concentrations of ATP. One of the main differences between purified enzyme preparations from erythrocytes and the insect cell-derived recombinant enzyme preparation used in this work is that in the former case the enzyme is solubilized in detergents and included in phospholipid micelles, whereas in the latter it remains embedded in the lipid bilayer of the plasma membrane. Therefore, besides the fact that the PMCA is kept in its original environment, an important advantage of the S99 insect cell-expressed PMCA preparation is the possibility of isolating the enzyme containing occluded ions by filtration on Millipore-type membranes. This, plus the use of alamethicin as a permeabilizing agent for efficient washing out of unbound Ca\textsuperscript{2+}, provides an excellent system to measure occlusion of this cation in the PMCA.

By analogy with Ca\textsuperscript{2+} occlusion in SERCA and Na\textsuperscript{+} occlusion in Na,K-ATPase (4, 5), Ca\textsuperscript{2+} occlusion is thought to occur in the $E_1P$ state of the PMCA. To detect Ca\textsuperscript{2+}-occluded states the strategy therefore must be to work under conditions where the expected amount of $E_1P$ is maximal, and its rate of breakdown is minimal. These conditions can be deduced from kinetic studies determining the ADP-sensitive phosphoenzyme (24), but an alternative approach is to perform the reactions in the presence of La\textsuperscript{III} (17, 18), which blocks the Mg\textsuperscript{2+}-depen-

4 M. S. Ferreira-Gomes, R. M. Gonzalez-Lebrero, M. C. de La Fuente, E. E. Strehler, R. C. Rossi, and J. P. F. C. Rossi, unpublished observations.
detailed reaction model of the plasma membrane Ca\textsuperscript{2+}-ATPase. Future experiments using the methodology employed in this paper will also include a comparative kinetic study of Ca\textsuperscript{2+} occlusion in different PMCA isoforms. Of particular interest will be studies on PMCA2, which differs significantly from PMCA4 in activation kinetics and basal activity (28). These studies may then also be extended to PMCA mutants with changes in amino acids thought to be involved in the state transitions linked to Ca\textsuperscript{2+} occlusion.

REFERENCES

1. Strehler, E. E., and Zacharias, D. A. (2001) *Physiol. Rev.* **81**, 21–50
2. Rega, A. F., and Garrahan, P. J. (1986) *The Ca\textsuperscript{2+} Pump of Plasma Membranes*, CRC Press Inc., Boca Raton, FL
3. Glynn, I. M., and Karlsh, S. J. (1990) *Annu. Rev. Biochem.* **59**, 171–205
4. Glynn, I. M., and Richards, D. E. (1982) *J. Physiol.* **330**, 17–43
5. Takisawa, H., and Makinose, M. (1983) *J. Biol. Chem.* **258**, 2986–2992
6. Rossi, R. C., Kaufman, S. B., González-Lebrero, R. M., Norby, J. G., and Garrahan, P. J. (1999) *Anal. Biochem.* **270**, 276–285
7. Ritov, V. B., Murzakhmetova, M. K., Tverdislova, I. L., Menshikova, E. V., Butylin, A. A., Avakian, T. Yu, and Yakovenko, L. V. (1993) *Biochim. Biophys. Acta* **1148**, 257–262
8. Caride, A. J., Penheiter, A. R., Filoteo, A. G., Bajzer, Z., Enyedi, A., and Penniston, J. T. (2001) *J. Biol. Chem.* **276**, 39797–39804
9. Verma, A. K., Enyedi, A., Filoteo, A. G., Strehler, E. E., and Penniston, J. T. (1996) *J. Biol. Chem.* **271**, 3714–3718
10. Kratje, R. B., Garrahan, P. J., and Rega, A. F. (1983) *Biochim. Biophys. Acta* **731**, 40–46
11. Sagara, Y., Fernandez-Belda, F., de Meis, L., and Inesi, G. (1992) *J. Biol. Chem.* **267**, 12606–12613
12. Schwarzbaum, P. J., Kaufman, S. B., Rossi, R. C., and Garrahan, P. J. (1995) *Biochim. Biophys. Acta* **1233**, 33–40
13. Weber, T., and Brunner, J. (1995) *J. Am. Chem. Soc.* **117**, 3084–3095
14. Echarte, M. M., Levi, V., Villamil, A. M., Rossi, R. C., and Rossi, J. P. (2001) *Anal. Biochem.* **289**, 267–273
15. Akaike, H. (1974) *IEEE Transactions of Automatic Control* **19**, 716–723
16. Sumida, M., and Tonomura, Y. (1974) *J. Biochem.* **75**, 283–297
17. Lutterbacher, S., and Schatzmann, H. J. (1983) *Experientia* **39**, 311–312
18. Herscher, C. J., and Rega, A. F. (1996) *Biochemistry* **35**, 14917–14922
19. Montes, M. R., Spiaggi, A. J., Monti, J. L., Cornelius, F., Olesen, C., Garrahan, P. J., and Rossi, R. C. (2011) *Biochim. Biophys. Acta* **1808**, 316–322
20. Schatzmann, H. J. (1973) *J. Physiol.* **235**, 551–569
21. Larsen, F. L., Hinds, T. R., and Vincenzi, F. F. (1978) *J. Membr. Biol.* **41**, 361–376
22. Clark, A., and Carafoli, E. (1983) *Cell Calcium* **4**, 83–88
23. Strehler, E. E., James, P., Fischer, R., Heim, R., Vorherr, T., Filoteo, A. G., Penniston, J. T., and Carafoli, E. (1990) *J. Biol. Chem.* **265**, 2835–2842
24. Andersen, J. P., Jørgensen, P. L., and Møller, J. V. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4573–4577
25. Garrahan, P. J., and Rega, A. F. (1978) *Biochim. Biophys. Acta* **513**, 59–65
26. Moreira, O. C., Rios, P. F., and Brrabin, H. (2005) *Biochim. Biophys. Acta* **1708**, 411–419
27. Kaufman, S. B., González-Lebrero, R. M., Schwarzbaum, P. J., Norby, J. G., Garrahan, P. J., and Rossi, R. C. (1999) *J. Biol. Chem.* **274**, 20779–20789
28. Caride, A. J., Filoteo, A. G., Penheiter, A. R., Pászty, K., Enyedi, A., and Penniston, J. T. (2001) *Cell Calcium* **30**, 49–57