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Chemical Modification Reveals Involvement of Different Sites for Nucleotide Analogues in the Phosphatase Activity of the Red Cell Calcium Pump

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Abstract. The calcium pump of plasma membranes catalyzes the hydrolysis of ATP and phosphoric esters like p-nitrophenyl phosphate (pNPP). The latter activity requires the presence of ATP and/or calmodulin, and Ca^{2+} [22, 25]. We have studied the effects of nucleotide-analogues and chemical modifications of nucleotide binding sites on Ca^{2+}-pNPPase activity. Treatment with fluorescein isothiocyanate (FITC), abolished Ca^{2+}-ATPase and ATP-dependent pNPPase, but affected only 45% of the calmodulin-dependent pNPPase activity. The nucleotide analogue eosin-Y had an inhibitory effect on calmodulin-dependent pNPPase ($Ki_{\text{eosin-Y}}$ = 2 μM). FITC treatment increased $Ki_{\text{eosin-Y}}$ 15 times. Acetylation of lysine residues with N-hydroxysuccinimidyl acetate inactivates Ca^{2+}-ATPase by modifying the catalytic site, and impairs stimulation by modulators by modifying residues outside this site [9]. Acetylation suppressed the ATP-dependent pNPPase with biphasic kinetics. ATP or pNPP during acetylation cancels the fast component of inactivation. Acetylation inhibited only partially the calmodulin-dependent pNPPase, but neither ATP nor pNPP prevented this inactivation. From these results we conclude: (i) ATP-dependent pNPPase depends on binding of ATP to the catalytic site; (ii) the catalytic site plays no role in calmodulin-dependent pNPPase. The decreased affinity for eosin-Y of the FITC-modified enzyme, suggests that the sites for these two molecules are closely related but not overlapped. Acetimidation of the pump inhibited totally the calmodulin-dependent pNPPase, but only partially the ATP-pNPPase. Since calmodulin binds to E_1, the E_1 conformation or the $E_2 \Leftrightarrow E_1$ transition would be involved during calmodulin-dependent pNPPase activity.

Key words: PMCA — Phosphatase activity — Nucleotide analogues — Lys residues — Chemical modification — Partial reactions

Introduction

The plasma membrane calcium pump (PMCA) shares with other P-type ATPases several features regarding the mechanism of hydrolysis of ATP, namely: (i) the involvement of a phosphorylated intermediate; (ii) the biphasic dependence of the hydrolytic activity with ATP concentration, which can be described as a sum of two hyperbolae, one of low K_m (1–5 μM) and low V_{max}, and another of higher K_m (100–500 μM) and V_{max} 3- to 5-fold higher than the low K_m component; (iii) the fact that small acyl-phosphates such as p-nitrophenylphosphate (pNPP) are also hydrolyzed by this pump [20]; (iv) the ability of derivatives of fluorescein to inhibit either reversibly or irreversibly the activity of PMCA.

Despite these similarities, there are some characteristics that differentiate this pump from the most studied members of the family of P-type ATPases, i.e., the (endo) sarcoplasmic reticulum Ca^{2+} pump, and the Na,K pump. The differences more relevant to this paper are: (i) the low affinity component of the ATP curve is modulated by calmodulin or by proteolytic removal of the calmodulin binding domain [19, 22, 6]; (ii) different from the sarcoplasmic reticulum Ca^{2+} pump [14] or the Na,K pump [2], the hydrolysis of alternative substrates like pNPP or acetylphosphate is not coupled to Ca^{2+} transport [5]; (iii) the pNPPase activity requires either

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calmodulin plus calcium [25], proteolytic removal of the calmodulin binding domain [21, 7] or ATP plus Ca^{2+} [4] to be elicited.

To further characterize the peculiarities of the mechanism of substrate hydrolysis by the PMCA, we analyzed the effects of fluorescein derivatives and chemical modifications on the pNPPase activity of this pump.

Materials and Methods

Materials

All the chemicals used in this work were of analytical grade and purchased principally from Sigma Chemical (St. Louis, MO). Recently drawn human blood for the isolation of Ca^{2+}-ATPase was obtained from the Hematology Section of the Hospital de Clínicas General San Martín (Argentina).

Preparation of Human Erythrocyte Membranes

Calmodulin-depleted erythrocyte membranes were prepared using a hypotonic lysis procedure according to Gietzen et al. [12].

Measurement of Enzymic Activities

The p-nitrophenyl phosphatase (pNPPase) activity was measured by estimating the release of p-nitrophenol (pNP). The incubation medium contained (in mM): 120 KCl, 6.25 $MgCl_2$, 30 Tris/HCl (pH 7.4 at 37°C), 10 p-nitrophenyl phosphate (pNPP), 1.0 EGTA, 1 ouabain, 60–80 μg of membrane protein/ml and the concentration of $CaCl_2$ necessary to obtain 1.0 μM free Ca^{2+}. The ATP-dependent pNPPase activity was measured in the medium above described in the presence of 0.5 mM ATP, while the calmodulin-dependent pNPPase activity was measured in the presence of 120 nM calmodulin. Ca^{2+}-ATPase activity was measured at 37°C in a medium similar to that used for the estimation of the p-nitrophenyl phosphatase activity, but without the addition of p-nitrophenyl phosphate and with a final concentration of 2 mM ATP. The concentration of $MgCl_2$ in such medium was 3.75 mM.

Treatment with Methyl Acetimidate (MA)

Incubation of the calmodulin-free membranes with MA was performed at 25°C (pH 7.4 at 37°C), in 50 mM bicine-K (pH 8.3 a 25°C). The incubation time was 30 min. After this treatment, membranes were washed twice with (in mM): 15 Tris-HCl (pH 7.4 at 37°C), 40 KCl, 1.8 $MgCl_2$ and resuspended in the same buffer at a total protein concentration of 0.5–0.8 mg/ml.

Treatment with N-Hydroxysuccinimidyl Acetate (SA)

SA was synthesized in our laboratory [9]. Incubation of calmodulin-free membranes with SA was performed at 25°C in 50 mM bicine potassium, (pH 8.3 at 25°C). Membranes were then spun down and washed twice with 15 mM Tris-HCl (pH 7.4 at 37°C) containing 40 mM KCl and 1.8 mM $MgCl_2$ and finally resuspended in the same buffer, at a protein concentration of 0.5–0.8 mg/ml.

Treatment with Fluorescein Isothiocyanate (FITC)

The treatment with FITC was performed as described in Kosk-Kosicka and Bzdega [15]. Protein concentration was determined according to the method of Bradford [3].

Curve Fitting

Curves were fitted to the experimental results by least-squares nonlinear regression by using the algorithm of Gauss Newton with optional dumping. The concentration variables were assumed to have negligible error and the velocity variation to be homoscedastic. The equations used to fit the experimental points were chosen in the basis of this best fitting, as judged by the minimum SD of the regression. The SD of the regression is the sum of the square errors divided by the number of parameters. The program used allows us to fit any function with up to two independent variables and up to 15 adjustable parameters and their SD values.

Abbreviations

FITC	fluorescein isothiocyanate
MA	methyl acetimidate
SA	N-hydroxysuccinimidyl acetate
PMCA	plasma membrane calcium ATPase
SERCA	sarcoplasmic reticulum calcium ATPase
pNPPase	p-nitrophenyl phosphatase

Results

Effect of Treatment with FITC on pNPPase Activity

Erythrocyte membranes were chemically modified by treatment with FITC, and Ca^{2+}-ATPase and Ca^{2+}-pNPPase activities dependent of ATP or calmodulin were measured. As can be seen in Fig. 1*a*, such treatment abolished both, Ca^{2+}-ATPase and the ATP-dependent pNPPase, but the enzyme retained about 55% of calmodulin-dependent pNPPase activity. When the latter activity was measured as a function of the pNPP concentration the FITC-treated enzyme showed both simulation at lower and inhibition at higher concentrations of the substrate (Fig. 1*b*). Analysis by nonlinear regression of data in Fig. 1*b* (*see* legend) reveals that FITC causes a decrease of the V_{max} with no effect in the apparent affinity for pNPP as activator. The inhibition by pNPP does not seem to occur with a significant change of affinity either.

Effect of Eosin-Y on Ca^{2+}-pNPPase Activity

It has been described that eosin-Y binds to ATP binding sites in several P-type ATPases: K^+-H^+-ATPase [13], Na^+K^+-ATPase [24] and Ca^{2+}-ATPase from muscle sarcoplasmic reticulum [17]. To further characterize which nucleotide binding sites are involved in the pNPPase

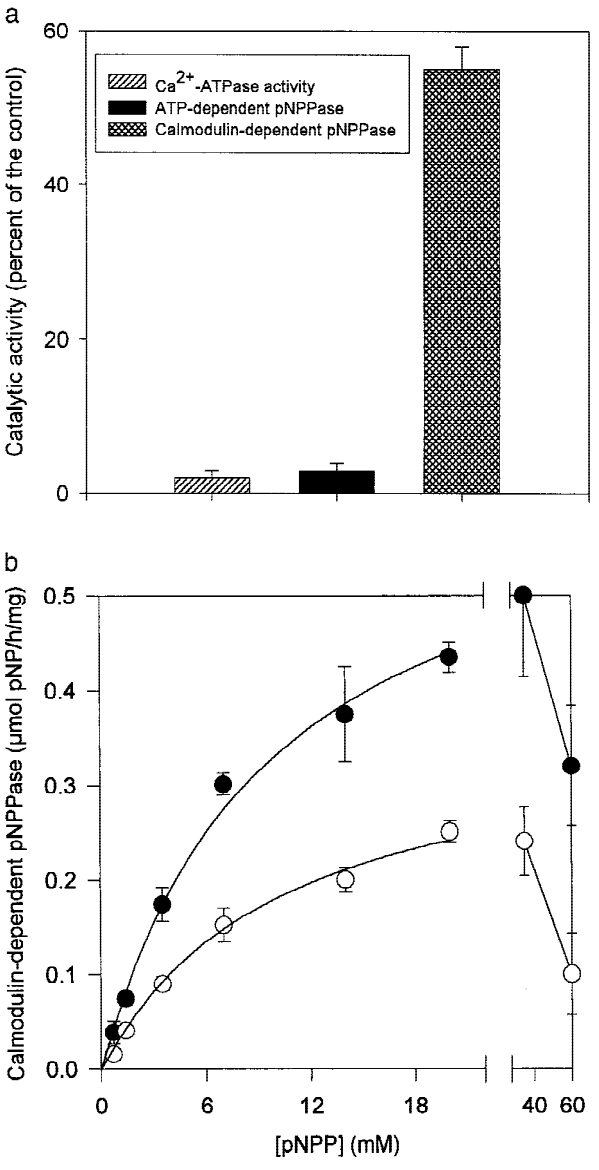

Fig. 1. Effect of FITC. Comparison among different catalytic activities of the Ca^{2+} pump. Membranes were incubated with 0 or 18 μM of FITC, and different catalytic activities were determined, namely Ca^{2+}-ATPase activity, ATP dependent pNPPase activity, and calmodulin-dependent pNPPase activity. Vertical bars are standard errors. Effect of pNPP on calmodulin pNPPase activity. Membranes were treated with 0 μM (●) or 18 μM (○) FITC and the calmodulin pNPPase activity was measured as a function of pNPP. The continuous lines between 0 and 20 mM pNPP were obtained adjusting the following equation to the experimental points (*see* Rossi et Caride [23] for justification):

$$v = V_m/(1 + K_m/[\text{pNPP}])^2 \quad (1)$$

with $V_m = 0.547 \pm 0.023$ μmol pNP/mg protein/hr, and $K_m = 2.55 \pm 0.25$ mM for the control and $V_m = 0.316 \pm 0.021$ μmol pNP/mg protein/hr, and $K_m = 3.05 \pm 0.45$ mM for the FITC-treated enzyme. Vertical bars represent standard errors and where not shown fall within the size of the symbols.

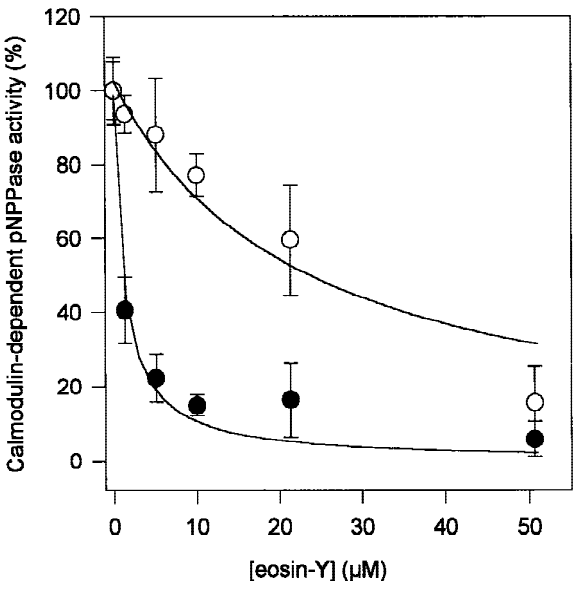

Fig. 2. Effect of eosin-Y on calmodulin-pNPPase activity of membranes treated with FITC. Calmodulin-pNPPase activity was measured as a function of increasing concentrations of eosin-Y for membranes treated (○) or untreated (●) with FITC. The continuous lines were obtained adjusting the following equation to the experimental points:

$$V = V_m/(1 + [\text{eosin-}Y]/K_i) \quad (2)$$

With $V_m = 100.8 \pm 4.4\%$ and $K_i = 1.2 \pm 0.3$ μM for the control and $V_m = 102 \pm 3.6\%$ and $K_i = 22.5 \pm 6.7$ μM for the enzyme treated with FITC. Vertical bars represent standard errors.

activity of PMCA, we planned to use eosin-Y as a tool. In the experiment (results shown in Fig. 2), the calmodulin-dependent pNPPase activity was measured as a function of eosin-Y concentration, both in native and in FITC-treated membranes. Different from the effects on the sarcoplasmic reticulum Ca^{2+}-ATPase [16], eosin-Y had no stimulating effect either in control or in FITC-treated PMCA. A pure inhibitory effect can be seen with a K_i of 1.2 μM on control enzyme. The effect persisted in the enzyme modified by FITC, but the K_i for eosin-Y was increased 15 times. These results suggest that eosin-Y binds to a site related but not identical to the FITC site.

Effect of Treatment with MA on pNPPase Activity

Modification of lysine residues in erythrocyte membranes with the reagent MA was shown to partially inhibit Ca^{2+}-ATPase activity. As we have demonstrated before, this modification seems to be different from that by FITC, since high (mM) concentrations of ATP, in the absence of divalent cations, failed to prevent this inhibition [8]. MA treatment also produced a total inactivation

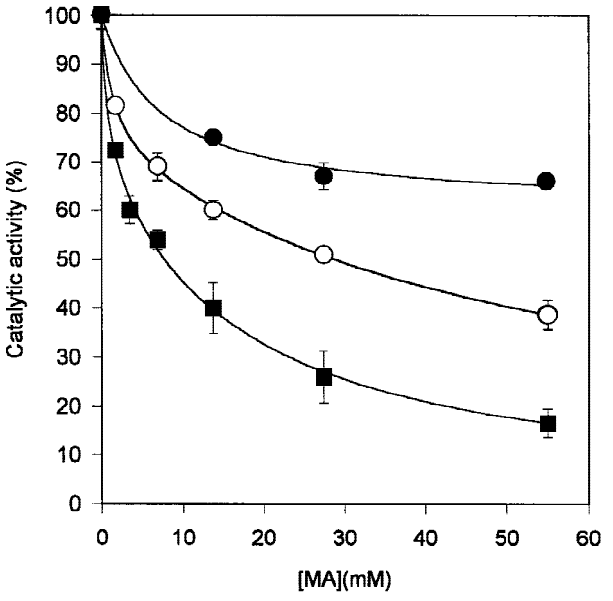

Fig. 3. Effect of MA on catalytic activities of the Ca^{2+}-pump. Membranes were treated with different concentration of MA as described in Materials and Methods and the following activities of the pump were measured: Ca^{2+}-ATPase (●), ATP-dependent pNPPase (○) and calmodulin-dependent pNPPase (■). Vertical bars represent standard errors and where not shown fall within the size on the symbols. The continuous lines were obtained adjusting the following equation to the experimental points:

$$V = Vm_1/(1 + [MA]/Ki_1) + Vm_2/(1 + [MA]/Ki_2) \quad (3)$$

The kinetic parameters are shown in Table 1.

of calmodulin-dependent pNPPase [8]. Figure 3 shows an experiment in which membranes have been pretreated with different concentrations of MA, and then Ca^{2+}-ATPase and both pNPPase activities were measured. It can be seen that inhibition of ATP-dependent pNPPase by MA was larger than inhibition of Ca^{2+}-ATPase, but lesser than the inactivation of calmodulin-dependent activity, which was almost complete. These results are op-

Table 1. Kinetic parameters describing the concentration-dependence of MA treatment

Parameters	Ca^{2+}-ATPase	ATP-dependent pNPPase	Calmodulin-dependent pNPPase
Vm_1 (%)	39.6 ± 4.4	29.6 ± 3.9	30.6 ± 1.8
Ki_1 (mM)	7.3 ± 3.3	1.35 ± 0.43	0.65 ± 1.10
Vm_2 (%)	60.4 ± 3.9	70.4 ± 3.8	69.4 ± 17.5
Ki_2 (mM)	NA	64.0 ± 12.1	16.8 ± 6.9

Best fit-parameters obtaining by adjusting Eq. 3 to the data in Fig. 3. NA = nonapplicable. A value of 100% represents the activity of control membranes treated under the same conditions except that MA was omitted. The values are shown as mean ± SD.

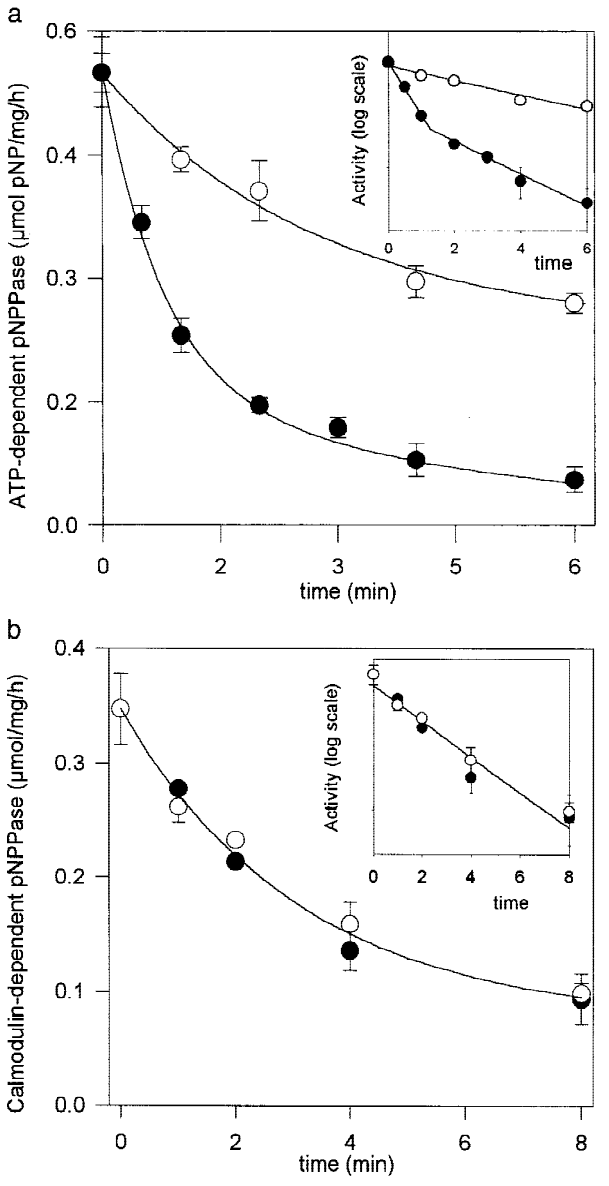

Fig. 4. Effect of acetylation with SA on pNPPase activities. Erythrocyte membranes were treated with 2.5 mM SA in the presence (○) or the absence (●) of 3 mM ATP during 0 to 6 or 8 min. ATP-dependent (*a*) or calmodulin-dependent (*b*) phosphatase activities were determined. 100% represents the activity of control membranes treated under the same conditions except that SA was omitted. The values are shown as mean ± SD. The insets show the same data plotted in logarithmic scale. The continuous lines were obtained adjusting the following equation to the experimental points for the experiments of ATP-dependent pNPPase in the absence of ATP:

$$a = a_1 \cdot \exp(-k_1 t) + a_2 \cdot \exp(-k_2 t) \quad (4)$$

where a_1 is the activity of the first component inhibitable by SA and a_2 the activity for the second component inhibitable by SA. For the curve of ATP-dependent pNPPase treated with SA in the presence of ATP and for calmodulin-dependent pNPPase the equation was reduced to:

$$a = a_1 \cdot \exp(-k_1 t) + a_\infty \quad (5)$$

where a_∞ is the noninhibitable activity by SA treatment. Vertical bars represent standard errors and where not shown fall within the size on the symbols. The kinetic parameters are shown in Table 2.

Table 2. Kinetic parameters of the time course of inhibition of Ca^{2+}-pNPPase by SA

(a) ATP-dependent pNPPase

	[ATP] = 0 mM	[ATP] = 3 mM
Equation	$a = a_1 \cdot \exp(-k_1 t) + a_2 \cdot \exp(-k_2 t)$	$a = a_1 \cdot \exp(-k_1 t) + a_\infty$
a_1 (μmol/mg/hr)	0.381 ± 0.110	0.377 ± 0.032
k_1 (min^{-1})	1.322 ± 0.421	0.370 ± 0.009
a_2 (μmol/mg/hr)	0.172 ± 0.113	
k_2 (min^{-1})	0.202 ± 0.154	
a_∞ (μmol/mg/hr)		0.173 ± 0.047

(b) Calmodulin dependent pNPPase

	[ATP] = 0 mM	[ATP] = 3 mM
Equation	$a = a_1 \cdot \exp(-k_1 t) + a_\infty$	
a_1 (μmol/mg/hr)	0.281 ± 0.014	0.268 ± 0.021
k_1 (min^{-1})	0.343 ± 0.043	0.291 ± 0.058
a_∞ (μmol/mg/hr)	0.073 ± 0.014	0.074 ± 0.022

posite to those obtained by labeling the enzyme with FITC, thus further supporting that MA modifies a different site, which is necessary for pNPPase activity.

Effect of Treatment with SA on pNPPase Activity

Acetylation of lysine residues with SA has been reported to inactivate Ca^{2+}-ATPase by: (i) modifying the catalytic site and (ii) impairing the stimulation of this activity through the modification of other/s residue/s lying outside the catalytic site [9]. Modification of one or two lysine residues related to the catalytic site could be prevented by the presence of saturating concentration of ATP [9].

Erythrocyte membranes were treated with SA or SA plus ATP, and pNPPase activities were measured, in the presence of ATP or calmodulin. Figure 4*a* shows the time course of inactivation of the ATP-dependent pNPPase by SA or SA plus ATP. These curves were analyzed by nonlinear regression, and the results of this procedure are presented in Table 2. Incubation of the enzyme with SA fully canceled out ATP-dependent pNPPase, following a complex kinetic in which two components of different velocity of inhibition can be distinguished. ATP during SA treatment partially protected against this inhibitory effect, by neutralizing the rapid first component. The behavior of calmodulin-dependent pNPPase was different, as can be observed in Fig. 4*b*. In this case, SA inhibited only partially, following monoexponential kinetics. The constant for the inactivation is similar to that of the slow component of the inactivation of the ATP-dependent pNPPase. The presence of ATP did not alter the time course of inactivation.

To further study the effect of acetylation by SA on the substrate sites, we performed an experiment in which membranes were treated with SA during one minute. The resultant ATP-dependent pNPPase was measured as a function of ATP (Fig. 5*a*) and pNPP (Fig. 5*b*) concentrations. The concentrations of substrates used allowed us to analyze both the activating as well as the inhibitory regions of the curves [4]. It can be seen that the rapid inhibitory effect occurs with no significant change in the apparent affinities (either as activators or inhibitors) for both ATP and pNPP.

We assayed also the effect of pNPP during the treatment of membranes with SA, in order to know whether it could protect the ATP-dependent and the calmodulin-dependent pNPPase against inhibition. Results are shown in Table 3. pNPP prevented partially ATP-dependent pNPPase from inhibition by SA. pNPP also prevents the inhibition of Ca^{2+}-ATPase by SA [9] but had no effect on inhibition of calmodulin-dependent pNPPase activity.

Discussion

We have modified the PMCA with different probes of the nucleotide binding region, and tested their effect on the pNPPase activity, in order to get information about the relationship between the nucleotide binding site(s) and the site for the substrate of the phosphatase activity. FITC is the most widely known modifier of the nucleotide-binding site of this pump [19, 10, 15]. Modification by FITC irreversibly inhibited 100% of the ATPase activity and the ATP-dependent pNPPase activity of the pump. However, the calmodulin-dependent pNPPase activity was inhibited by only 45% (Fig. 1*a*). Related to this, acetylation by SA, another reagent that modifies Lys residues but not as bulky as FITC, also fully can-

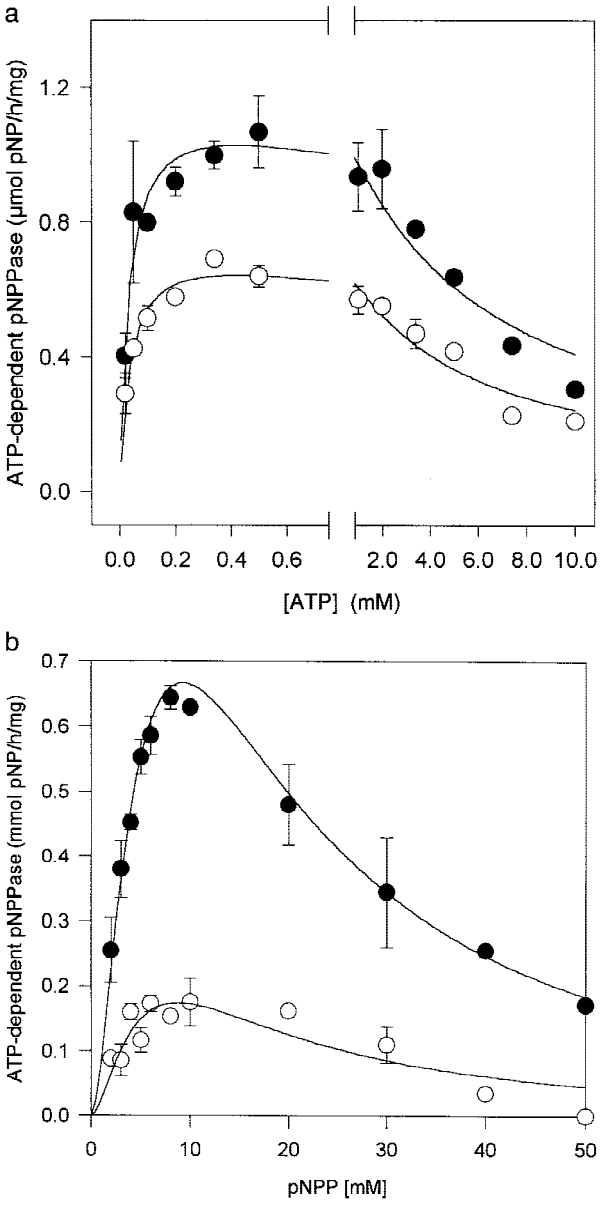

Fig. 5. Effect of acetylation on the fast component of inactivation by SA on ATP-dependent pNPPase activity by ATP and pNPP. Membranes were preincubated during one minute with 0 (●) or 2.5 (○) mM SA as described in Materials and Methods and the ATP-dependent pNPPase activity was assayed as function of concentration of ATP (*a*) or pNPP (*b*). (*a*) Effect of ATP. The continuous lines that fit the experiments represent the equation:

$$v = Vm/(1 + Ka/[\mathrm{ATP}] + [\mathrm{ATP}]/Ki)^2 \quad (6)$$

with $Vm = 2.30 \pm 0.13$ µmol pNP/mg protein/hr, $Ka = 22.7 \pm 4.9$ µM and $Ki = 2.58 \pm 0.60$ mM for the control and $Vm = 1.44 \pm 0.08$ µmol pNP/mg protein/hr, $Ka = 24.3 \pm 4.9$ µM and $Ki = 2.60 \pm 0.50$ mM for the enzyme treated with SA. (*b*) Effect of pNPP. The continuous lines that fit the experiments is the solution of Eq. (6) except that pNPP replaces ATP as variable with: $Vm = 2.13 \pm 0.15$ µmol pNP/mg protein/hr, $Km = 3.74 \pm 0.27$ mM and $Ki = 21.8 \pm 1.4$ mM for the control and $Vm = 0.66 \pm 0.47$ µmol pNP/mg protein/hr, $Km = 4.1 \pm 2.9$ mM and $Ki = 18 \pm 12$ mM for the enzyme treated with SA.

Table 3. Effect of pNPP during treatment with SA

[pNPP] (mM)	ATP-dependent	Calmodulin-dependent
	pNPPase (%)	pNPPase (%)
0	3 ± 2	28 ± 5
30	14 ± 2	26 ± 7

celled Ca^{2+}-ATPase [9] and ATP-dependent pNPPase (Fig. 4*a*) activities. However, the fast component of the curve was absent when the incubation with SA was performed in the presence of ATP (Fig. 4*a*). On the other hand, calmodulin-dependent pNPPase activity was inhibited by SA along a monoexponential curve, with a rate of inactivation comparable to that of the slow component of the ATPase activity. ATP (Fig. 4*b*) did not prevent the inactivation of the calmodulin-dependent pNPPase activity. Taken together, these results suggest that modification of Lys residues inhibited Ca^{2+}-ATPase and ATP-dependent pNPPase by preventing ATP binding to the high affinity site in the pump. This finding is in agreement with previous results that suggest the requirement of ATP binding to its high affinity site in order to activate the pNPPase [4]. The modification of the Lys residues involved in high affinity ATP binding does not seem to affect by itself the hydrolysis of pNPP stimulated by calmodulin, since its rate of inactivation by SA is the same regardless the presence of ATP during the treatment.

The partial inactivation that is produced by FITC is probably related to the steric effect produced by the bulky fluorescein moiety, or by modification of other Lys residues by FITC. It is worth mentioning that Adamo et al. [1] reported that a version of the human isoform 4b of the PMCA in which Lys-591 was mutated to Arg, still was irreversibly inactivated by FITC.

T test whether the fast and slow components of the inhibition of ATP-dependent pNPPase by SA correspond to the activating and inhibiting sites for pNPP, or for ATP, the enzyme was submitted to a brief treatment with SA. During the selected time, the first rapid component of the inhibition of ATP-dependent activity is expected to be almost completed while the second slow component is barely manifest. However, the enzyme treated in this way showed both inhibitory and activating effects for ATP and pNPP, with nonsignificant change in the apparent affinities (Fig. 5*a* and *b*). Hence, the fast component of the inhibition of the pNPPase activity by SA cannot be related to a deleterious modification of the sites for ATP or pNPP. Moreover, the presence of pNPP during acetylation with SA did not prevent inhibition of the calmodulin-dependent phosphatase, and only partially protected against the inhibition of the ATP-dependent activity (Table 3), which is also against the possibility that SA modifies a site for pNPP.

Eosin-Y has been proved to be a potent inhibitor of the calcium pump of plasma membranes (PMCA) [11]. In our hands, eosin-Y inhibited the calmodulin-dependent pNPPase activity also, both in native and in FITC-modified enzyme (Fig. 2). However, modification by FITC decreased the affinity for eosin-Y about 15-fold. This result suggests that the site for eosin-Y is related to the FITC-site, which is not surprising since both molecules are derivatives of fluorescein. However, the fact that covalent modification by FITC did not inactivate completely the calmodulin-dependent pNPPase, and noncovalent binding of eosin-Y did, suggests that the site for eosin-Y is close but not the same as the FITC site. These results are in agreement with those of Gatto and Milanick [11], who suggested that FITC and eosin-Y reacted at different but not completely independent sites.

In the Ca^{2+} pump from sarcoplasmic reticulum (SERCA), it has been found [16] that eosin-Y has a biphasic effect on the Ca-ATPase activity of this pump. It activates at low concentrations, and higher concentrations inhibit this activity. When the SERCA was labeled with FITC the activating phase was absent, and only the inhibitory effect of eosin-Y was observed. As eosin-Y is considered to be a nucleotide analogue, these observations were interpreted as evidence of two separated nucleotide-binding sites. The results in this paper showed that, in the PMCA, eosin-Y had no activating effect on the pNPPase activity. Besides, Gatto and Milanick [11] have shown that in the PMCA, eosin-Y does not compete with ATP. All these evidences point out that the structure of the substrate binding site(s) of PMCA and SERCA are different. Taken together, these results suggest that extreme caution should be exerted when extrapolating results of other P-type ATPases to interpret results in the PMCA. Opposite to what has been described for FITC and SA, MA inhibited completely the calmodulin-dependent pNPPase, in conditions in which the effect on the Ca^{2+}-ATPase and the ATP-dependent pNPPase are only partial (Fig. 3). These results are interesting because MA modifies Lys residues in a conformation dependent way [8]. Inactivation of the Ca^{2+}-ATPase activity is maximal in the presence of Ca^{2+} and Mg^{2+} (E_1), while it is prevented by vanadate, which stabilizes E_2 [8]. The fact that the calmodulin-dependent pNPPase is so sensitive to inactivation by MA suggests that the E_1 conformation or at least the E_2–E_1 transition is involved during the calmodulin-dependent pNPPase activity. In case of the ATP-dependent pNPPase, transition towards E_1 may occur through the cycle of ATP hydrolysis and Ca^{2+} transport. On the other hand, in the presence of Ca^{2+} and calmodulin, E_1 can be regenerated through a futile cycle that hydrolyzes pNPP but does not elicit Ca^{2+} transport.

It can be concluded from the experimental evidence presented in this paper that calmodulin-dependent pNPPase activity depends on a site which is not the catalytic one, while the ATP-dependent pNPPase depends on at least two, one of them being the catalytic site.

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