Acetylation with succinimidyl acetate affects both the catalytic site and the regulation of the erythrocyte Ca^{2+} pump

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Acetylation of lysine residues of the erythrocyte Ca^{2+} pump using succinimidyl acetate (SA) led to its complete inactivation. In the absence of any of the major activators of the pump (namely calmodulin and acidic phospholipids), ATP fully protected the pump from inactivation by SA, with a $K_{0.5}$ of 13 μM . This value is very close to the K_m of the high-affinity site for ATP, thus suggesting that the residue(s) involved is(are) near or at the catalytic site of the Ca^{2+} -ATPase. Furthermore, the presence of 500 μM ATP prevented the acetylation of about two residues per molecule of enzyme. Acetylation by SA also prevented the activation of the Ca^{2+} pump by calmodulin, acidic phospholipids

or controlled trypsin proteolysis. This effect of SA treatment was not avoided by the presence of ATP in the preincubation medium, indicating a second set of modified residues. The fact that the three modes of activation were cancelled in a similar fashion by SA suggests that, although acting via different mechanisms, they share at least a common step in which SA-sensitive lysine residues may participate. Moreover, modification of the pump by SA plus ATP decreased the K_{Ca} when the activity was measured in both the absence and presence of calmodulin, suggesting that the residue(s) modified in this case is(are) involved directly in the regulation of the affinity for Ca^{2+} .

INTRODUCTION

The Ca^{2+} pump of plasma membranes is an enzyme that catalyses active transport of Ca^{2+} coupled to ATP hydrolysis. It is an enzyme of the $(\text{E}_1\text{--}\text{E}_2)\text{--P}$ -type, i.e. its reaction mechanism involves the covalent phosphorylation of the E_2 conformer of the protein by the γ -phosphate from ATP, followed by a conformational transition to the E_2 form, phosphate release and later, reconversion of the E_2 into the E_1 conformer. Ca^{2+} -ATPase activity may be increased in several ways: (1) by the presence of calmodulin, which increases both the V_{max} and the apparent affinity for Ca^{2+} , changing the Ca^{2+} activation curve from hyperbolic to sigmoidal; (2) by controlled partial proteolysis, as the removal of the C-terminal domain of the protein causes calmodulin-like activation; (3) by acidic phospholipids which increase the V_{max} and the apparent affinity for Ca^{2+} without generating co-operativity.

We have recently studied the effect of modifying Ca^{2+} -ATPase lysine residues by reaction with methyl acetimidate, which does not modify their net charge, and found that such treatment caused partial inhibition of enzyme activity [1]. The present work describes the effect of modifying lysine residues in Ca^{2+} -ATPase by treatment with *N*-hydroxysuccinimidyl acetate (SA), which acetylates primary amino groups thus changing the net charge of the protein. The effect of acetylation with SA on Ca^{2+} -ATPase activity appeared to be stronger than the effect of methyl acetimidate, and involves both catalytic properties and regulation of the enzyme.

MATERIALS AND METHODS

Fresh blood from haematologically normal adults, collected in acid/citrate/dextrose solutions, was utilized. Erythrocyte membranes were prepared as described by Gietzen et al. [2] as follows: 1 vol. of erythrocytes (washed three times with 150 mM NaCl) was lysed in 8 vol. of lysing solution (1 mM EGTA, 15 mM Tris/HCl, pH 7.7) at 4 °C. Membranes were sedimented by

spinning at 17000 *g* for 20 min, and washed twice with the lysing solution. They were then suspended in 8 vol. of lysing solution, incubated at 37 °C for 15 min in this solution and centrifuged again at 17000 *g* for 20 min. This step was repeated once. Membranes were then washed with 8 vol. of 15 mM Tris/HCl (pH 7.4), resuspended in 1 vol. of the same solution, and stored at –20 °C. This procedure yields membranes devoid of endogenous calmodulin. Calmodulin was purified from bovine brain as described by Kakiuchi et al. [3].

SA and succinimidyl [2- ^{14}C]acetate were synthesized in our laboratory. EGTA, *trans*-1,2-diaminocyclohexane-*NNN'*-tetra-acetic acid (CDTA), ATP (grade I), Tris, SDS, trypsin, soya-bean trypsin inhibitor and *p*-nitrophenyl phosphate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Salts and reagents were of analytical grade.

For treatment with trypsin, membranes were washed and suspended in a medium containing 120 mM KCl, 30 mM Tris/HCl (pH 7.20 at 4 °C), 0.5 mM EGTA and 10 $\mu\text{g}/\text{ml}$ trypsin (60 units/mg). The mixture was incubated at 4 °C and, after 8 min, trypsin action was terminated by the addition of soya-bean trypsin inhibitor (final concentration 200 $\mu\text{g}/\text{ml}$). Then membranes were sedimented and resuspended in 50 mM Bicine (potassium salt; pH 8.3 at 25 °C). Control experiments indicated that, after this treatment, Ca^{2+} -ATPase activity was approximately two to three times the initial value and was insensitive to calmodulin.

Ca^{2+} -ATPase activity was measured at 37 °C in medium containing 120 mM KCl, 3.75 mM MgCl_2 , 30 mM Tris/HCl (pH 7.40 at 37 °C), 1.0 mM EGTA, 0 or 120 nM calmodulin, 2 mM ATP, 60–80 $\mu\text{g}/\text{ml}$ membrane protein and the concentration of CaCl_2 necessary to obtain the specified free Ca^{2+} concentration. The release of P_i from the nucleotide was estimated by the procedure of Fiske and Subbarow [4] or by the measurement of ^{32}P released from [γ - ^{32}P]ATP as described by Richards et al. [5]. Ca^{2+} -dependent activity was determined by subtracting the phosphate release into a medium similar to those described

Abbreviation used: SA, *N*-hydroxysuccinimidyl acetate.

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above but without CaCl_2 . The reaction was started by transferring the tubes to a 37 °C bath and stopped by returning them to an ice-water bath.

Activation of Ca^{2+} -ATPase by acidic phospholipids was carried out by addition of liposomes obtained by sonication of phospholipids from a Folch extract (fraction I) of bovine brain (from Sigma) to the incubation media. This mixture is composed of 50–60% phosphatidylserine and 10–20% phosphatidylinositides, as well as several other brain lipids.

The free Ca^{2+} concentration in the incubation medium was measured with an IS-561 Ca^{2+} -electrode [6]. Protein content was determined by the method of Bradford [7].

Incubation of the calmodulin-free membranes with SA was performed at 25 °C in 50 mM Bicine (potassium salt; 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 total protein concentration of 0.5–0.8 mg/ml.

Ca^{2+} -ATPase was isolated from calmodulin-depleted membranes by the calmodulin-affinity-chromatography procedure as described by Roufogalis and Villalobo [8], but with pure phosphatidylcholine as the phospholipid and polyoxyethylene 10 lauryl ether as the detergent. Fractions were selected according to their specific ATPase activity. Purified enzyme was stored in liquid nitrogen until use.

The acetate incorporated into the enzyme was estimated using *N*-hydroxysuccinimidyl [$2\text{-}^{14}\text{C}$]acetate, hereinafter referred to as ^{14}C -SA. A portion of ^{14}C -SA in chloroform was dried and redissolved in Bicine buffer (pH 8.4 at 25 °C) containing unlabelled SA. This solution was immediately added to purified enzyme that had been preincubated for 3 min with 0 or 500 μM ATP. After 8 min, the incorporation was stopped by the addition of a 20-fold excess of glycine. The enzyme was precipitated with 6% trichloroacetic acid, and the suspension obtained was filtered through fibre-glass filters (Whatman; pore size 1.2 μm). They were washed three times with 10 ml of 6% trichloroacetic acid and three times with 10 ml of chloroform/methanol (3:1, v/v) in order to eliminate phospholipids. Radioactivity retained in the filters was estimated in a liquid-scintillation counter.

The values presented in the Results section are representatives of two to four experiments. Each of the measurements was performed in triplicate and the individual values did not differ by more than 5% from the mean. Equations were adjusted to the experimental results by least-squares non-linear regression, by using the algorithm of Gauss-Newton with optional damping [9]. Concentration variables were assumed to have negligible error and the velocity variable to be homoscedastic. The equations used to fit the experimental points were chosen on a best-fit basis as judged by the minimum standard deviation of the regression. The squared standard deviation of the regression results from the addition of the squared errors divided by the number of degrees of freedom. Calculations were performed with a microcomputer, using a program that allows any function with up to two independent variables and up to 15 adjustable parameters to be fitted [10].

RESULTS

Acetylation with SA inactivates Ca^{2+} -ATPase activity in the presence and absence of calmodulin

Figure 1 shows the results of an experiment in which the Ca^{2+} -ATPase activity of erythrocyte membranes was measured as a function of the preincubation time with 2.5 mM SA. In both the presence and absence of calmodulin the activity was suppressed

Figure 1 Time course of inhibition of basal Ca^{2+} -ATPase by SA

A suspension of erythrocyte membranes was incubated with 2.5 mM SA and, at the times stated, samples were diluted into an equal volume of 250 mM Tris/HCl (pH 7.4 at 37 °C). Ca^{2+} -ATPase activity was measured in the medium as described in the Materials and methods section, in the absence (●) or presence (△) of 120 nM calmodulin.

Figure 2 Time course of inhibition of Ca^{2+} -ATPase by SA in the presence of ATP

Membranes were treated as described in Figure 1, except that the reaction with SA was carried out in the presence of 0 (●), 50 (○) or 200 (△) μM ATP. Ca^{2+} -ATPase activity was measured in the absence of calmodulin; (a) and (b) are the direct and semilog representations of the same data.

on incubation with the reagent, following a simple exponential curve. However, the effect of SA was faster on the calmodulin-dependent than on the calmodulin-independent activity, i.e. after

Figure 3 Effect of ATP concentration on the rate of inhibition of Ca^{2+} -ATPase by SA

Experiments like those described in Figure 2 were performed using different concentrations of ATP. Each pseudo-first-order rate constant was plotted against the corresponding ATP concentration. The continuous curve represents the best-fitting hyperbola obtained by regression, which has a $K_{0.5}$ of $13 \pm 4 \mu\text{M}$.

Table 1 Effect of the presence of ligands of the catalytic site on inhibition of Ca^{2+} -ATPase by SA

Membranes were treated with 2.5 mM SA in the presence of the ligands indicated, and Ca^{2+} -ATPase activity was measured without calmodulin. A value of 100% represents the activity of control membranes treated under the same conditions except that SA was omitted. The values are shown as means $\pm$ S.D.

Ligand	Concentration (mM)	Ca^{2+} -ATPase activity remaining (%)
None	—	33 ± 2
AMP	1	38 ± 2
AMP	2	43 ± 1
AMP	4	69 ± 2
<i>p</i> -Nitrophenyl phosphate	30	105 ± 3

10 min of incubation with SA, the activity in the absence of calmodulin was about 30% of the control whereas the stimulation by calmodulin had already been eliminated. Furthermore, the value of the pseudo-first-order constant for the inactivation by SA was $0.16 \pm 0.02 \text{ min}^{-1}$ when the activity was measured in the absence of calmodulin and $0.33 \pm 0.01 \text{ min}^{-1}$ in the presence of 120 nM calmodulin.

SA acetylates a residue in Ca^{2+} -ATPase related to the catalytic site

Calmodulin-independent Ca^{2+} -ATPase activity can be protected from the deleterious effect of SA by the presence of ATP (Figure 2). The presence of $50 \mu\text{M}$ ATP lowered the value of the SA inactivation rate constant from $0.19 \pm 0.01 \text{ min}^{-1}$ to $0.09 \pm 0.01 \text{ min}^{-1}$. An increase in ATP concentration to $200 \mu\text{M}$ counteracted the inactivating effect of SA completely.

The substrate curve of the Ca^{2+} pump shows two kinetically

Table 2 Incorporation of [^{14}C]acetate from ^{14}C -SA into the Ca^{2+} pump

Purified Ca^{2+} pump was acetylated by the addition of ^{14}C -SA (specific radioactivity $2 \times 10^7 \text{ d.p.m./}\mu\text{mol}$), and acetate incorporation was determined as described in the Materials and methods section. The number of modified residues per molecule is shown as mean $\pm$ S.E.M. for the number of experiments shown in parentheses. $P < 0.05$ compared with control (Student's *t* test).

[ATP] (μM)	Number of residues acetylated
0	66.60 ± 0.10 (3)
500	64.75 ± 0.84 (6)

Figure 4 Time course of inhibition by SA of Ca^{2+} -ATPase activated by calmodulin

A suspension of membranes was incubated with 2.5 mM SA plus 5 mM ATP and, at the times indicated, samples were diluted into an equal volume of 250 mM Tris/HCl (pH 7.4 at 37°C). The resultant Ca^{2+} -ATPase activity was measured in the presence of calmodulin. The dotted line shows the basal value of Ca^{2+} -ATPase.

distinct components [11]: one with high apparent affinity ($K_m = 5\text{--}10 \mu\text{M}$) and low maximal velocity, and the other with lower apparent affinity ($K_m = 100\text{--}500 \mu\text{M}$) and high maximal velocity. In an attempt to relate the protection by ATP against SA inactivation to either of those components, a series of experiments relating basal Ca^{2+} -ATPase activity to reaction time were performed at different ATP concentrations. Values for the pseudo-first-order rate constant of inactivation by SA were obtained and plotted against the ATP concentration in the preincubation medium (Figure 3). A simple model was examined, in which it is assumed that the enzyme is in equilibrium with ATP, and that enzyme with bound ATP is unable to react with SA at the site whose modification suppresses basal activity. This can be represented by the following scheme:

Figure 5 Time course of inhibition by SA of Ca^{2+} -ATPase activated by proteolysis

○, Membranes were submitted to controlled partial proteolysis with trypsin under the conditions detailed in the Materials and methods section, and the time course of inhibition of Ca^{2+} -ATPase by SA was determined. Ca^{2+} -ATPase activity was measured in the absence of calmodulin. ●, Membranes were treated under the conditions described above, but without trypsin. Ca^{2+} -ATPase activity was measured in the presence of calmodulin.

Figure 6 Effect of SA treatment on the activation of Ca^{2+} -ATPase by acidic phospholipids

Membranes were incubated with 0 (●) or 2.5 (○) mM SA plus 5 mM ATP for 20 min, and Ca^{2+} -ATPase was then determined in terms of the acidic phospholipids incorporated.

where E-NH_2 is the native enzyme, $\text{E-NH}_2 \cdot \text{ATP}$ the enzyme-substrate complex, K_{ATP} the equilibrium constant of the binding of ATP to the pump, and E-NH-COCH_3 the enzyme that has had a lysine or group of lysines related to the catalytic site acetylated, thus being rendered inactive.

Making the inactivation rate of basal Ca^{2+} -ATPase equal to the acetylation rate, the former can be expressed as a pseudo-first-order equation:

$$-\frac{d(\text{activity})}{dt} = \frac{d[\text{E-NH-COCH}_3]}{dt} = k'[\text{E-NH}_2]$$

Figure 7 Effect of SA treatment on the activation of Ca^{2+} -ATPase by Ca^{2+}

(a) Membranes were incubated with 0 (●) or 2.5 (○) mM SA plus 5 mM ATP for 20 min, and Ca^{2+} -ATPase was then determined as a function of Ca^{2+} concentration, in the absence of calmodulin. (b) Membranes were incubated with 0 (●) or 2.5 (○) mM SA plus 5 mM ATP for 20 min, and Ca^{2+} -ATPase was then determined against Ca^{2+} concentration, in the presence of calmodulin. The abscissa is a logarithmic scale.

On the other hand, as the enzyme is in equilibrium with ATP:

$$[\text{E-NH}_2] = \frac{[\text{E}_{\text{total}}]}{\left(1 + \frac{[\text{ATP}]}{K_{\text{ATP}}}\right)}$$

where $[\text{E}_{\text{total}}]$ represents the total enzyme concentration; the following hyperbolic relation is obtained for the dependence of the apparent pseudo-first-order rate constant (k'_{app}) and the ATP concentration:

$$k'_{\text{app}} = \frac{k'_{i([\text{ATP}]=0)}}{\left(1 + \frac{[\text{ATP}]}{K_{\text{ATP}}}\right)} \quad (1)$$

By adjusting the parameters in eqn. (1) to the data shown in Figure 3, a K_{ATP} of $13 \pm 4 \mu\text{M}$ was obtained. This value is very close to the K_m of the high-affinity component of the ATP curve, thus suggesting that interaction of ATP with the catalytic site of the pump protects the calmodulin-independent Ca^{2+} -ATPase activity from inhibition by SA. Moreover, this result indicates that one of the target residues could be related to the active site of the enzyme.

In order to obtain further support for this idea, the effect of different ligands with affinity for the catalytic site on inactivation by SA was tested. Table 1 shows the effects of AMP, an analogue of ATP, and *p*-nitrophenyl phosphate, the substrate of the

Table 3 Kinetic parameters of Ca²⁺-ATPase activation by Ca²⁺ in membranes treated with SA plus ATP

Best-fit values $\pm$ S.E.M. were obtained by adjusting the following equations to the data in Figure 7:

(a) Activity without calmodulin (Figure 7a):

$$v = \frac{V_{\max} [S]}{K_{Ca} + [S]}$$

(b) Activity in the presence of calmodulin (Figure 7b):

$$v = \frac{V_{\max} [S]^h}{(K_{Ca}^h + [S]^h)}$$

where $V_{\max}$ is the activity at non-limiting concentrations of Ca²⁺, K_{Ca} is the concentration of Ca²⁺ for half-maximal activation and h is the Hill coefficient.

[SA] (mM)	Without calmodulin		With calmodulin		
	$V_{\max}$ ($\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$)	K_{Ca} (μM)	$V_{\max}$ ($\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$)	K_{Ca} (μM)	h
0	1.06 ± 0.05	13.3 ± 0.5	2.70 ± 0.07	6.1 ± 0.2	2.16 ± 0.07
2.5	0.56 ± 0.05	3.5 ± 0.9	1.0 ± 0.1	2.4 ± 0.5	2.1 ± 0.7

phosphatase activity of the pump [12]. It can be seen that 4 mM AMP was able to protect the enzyme, increasing its activity from $33 \pm 2\%$ to $69 \pm 2\%$ with respect of that of the control. On the other hand, 30 mM *p*-nitrophenyl phosphate was able to protect the ATPase fully from inactivation by SA.

To characterize the number of sites that ATP protects against modification, acetylation of purified enzyme by SA was determined using this reagent labelled with ¹⁴C in the acetate. Table 2 shows that, in the presence of an ATP concentration that saturates the catalytic site, acetate incorporation was lower than in its absence. The difference corresponds to the protection of about two lysine residues.

Effect of acetylation by SA on calmodulin-dependent Ca²⁺-ATPase activity

In contrast, calmodulin-dependent Ca²⁺-ATPase activity could not be protected by ATP from inactivation by SA treatment. Figure 4 shows the effect of incubation of the membranes with 2 mM SA and 5 mM ATP for different time periods on Ca²⁺-ATPase activity measured in the presence of 120 nM calmodulin. The calmodulin-dependent activity decreases on treatment with SA to a value that does not differ significantly from the activity in the absence of calmodulin. Therefore SA treatment suppressed the activation induced by calmodulin and this effect could not be prevented by ATP, thus suggesting that the residue(s) involved in calmodulin activation, which is(are) affected by SA, is(are) not related to the catalytic site.

SA treatment of Ca²⁺-ATPase suppresses calmodulin-like activation by modifying one or more residues lying outside the calmodulin-binding site

As the calmodulin-binding site contains lysine residues [13], it might be inferred that the loss of response to calmodulin on SA treatment could be a consequence of the modification of such residues by SA. An alternative method of stimulating the Ca²⁺ pump is by cleaving off the calmodulin-binding domain by proteolysis. In order to verify whether the modification of lysine residues within the calmodulin-binding domain was responsible for the loss of calmodulin activation, we compared the rate of inactivation induced by SA treatment of the enzyme previously subjected to tryptic proteolysis with the inactivation rate of the

native enzyme, the latter activity being measured in the presence of calmodulin. Figure 5 shows that SA inactivated the Ca²⁺-ATPase of both membrane preparations along superimposable curves. The rate constant of inactivation was $0.42 \pm 0.06 \text{ min}^{-1}$ for the proteolysed pump and $0.40 \pm 0.05 \text{ min}^{-1}$ for the intact pump. The fact that SA inactivated the two enzymes at the same rate strongly suggests that the residue, the modification of which by SA produced the loss of calmodulin response, lies outside the calmodulin-binding domain.

SA treatment blocks the activation of the Ca²⁺ pump by acidic phospholipids

Ca²⁺-ATPase activity was measured as a function of added phospholipids in control membranes and in membranes that had been preincubated with 2.5 mM SA and 5 mM ATP so as to discover whether activation by acidic phospholipids was also affected by acetylation. Figure 6 shows that, in the absence of acidic phospholipids, treatment with SA did not impair the activity. However, whereas the untreated pump was activated more than fourfold by acidic phospholipids, the pump pretreated with SA plus ATP was not affected by the action of acidic phospholipids.

The putative lipid-binding region of the Ca²⁺ pump is rich in lysine residues, which are supposedly susceptible to SA modification. In order to investigate whether the suppression of the stimulation by the acidic lipids was caused by acetylation of those residues, the Ca²⁺-ATPase was allowed to react with 2.5 mM SA in the absence and presence of 2.0 mg of added phospholipids per ml. On SA treatment, Ca²⁺-ATPase activity, measured in the presence of acidic phospholipids, fell from 0.7 to approximately 0.2 $\mu\text{mol/h}$ per mg in both cases, thus indicating that acidic phospholipids were unable to protect the Ca²⁺ pump from inactivation by SA. This result suggests that modification of the putative lipid-binding domain may not be responsible for the lack of response of the modified enzyme to acidic phospholipids.

Acetylation by SA increases the apparent Ca²⁺ affinity of the Ca²⁺-ATPase

As ATP can prevent the effect of SA on the catalytic site, but not on the regulatory properties of the pump, we examined whether

modification by SA in the presence of ATP affects a highly regulated parameter of this pump, namely the apparent affinity for Ca^{2+} . Erythrocyte membranes were incubated with 2.5 mM SA for 20 min in the presence of 5 mM ATP and, after washing as described in the Materials and methods section, Ca^{2+} -ATPase activity was measured as a function of Ca^{2+} concentration, in both the absence and presence of calmodulin. Figure 7 shows that, regardless of the presence of calmodulin, treatment with SA led to activation at very low Ca^{2+} concentrations and inhibition at higher Ca^{2+} concentrations. These facts reflect the parameter fitting the experimental data (Table 3): acetylation by SA in the presence of ATP resulted in an increase in the apparent affinity for Ca^{2+} , and a decrease in $V_{\max}$, without any change in cooperativity.

It is worth noting that the effect of SA treatment on the $V_{\max}$ was greater when the activity was measured in the presence of calmodulin, whereas the opposite was true for the effect on K_{Ca} .

DISCUSSION

The present work shows that SA, a reagent that specifically acetylates lysine residues in proteins [14] thus modifying their net charge, completely inactivates the Ca^{2+} pump with $t_{0.5}$ under 2 min. This sharply contrasts with the partial inhibition obtained by modification with methyl acetimidate [1].

So far it is not possible to pinpoint whether the difference is due to a different pattern of reaction or is a consequence of the preservation of the net charge as a requirement for those residues to play their role in the reaction cycle.

The presence of ATP during the incubation with SA allowed us to differentiate two effects of this reagent: (1) complete inactivation of the enzyme, prevented by ATP (at Ca^{2+} concentrations around 20 μM), thought to be related to modification of a lysine residue within the catalytic site of the enzyme, and (b) suppression of the regulation of the activity stimulated by calmodulin, acidic phospholipids or proteolysis, none of them being prevented by ATP.

Effect of SA on the catalytic site of Ca^{2+} -ATPase

Basal Ca^{2+} -ATPase is suppressed by treatment with SA. Evidence indicating that the residue responsible for this effect lies within the catalytic site arises mainly from the fact that ATP protects the pump against SA inactivation with a $K_{0.5}$ of about 13 μM , which is close to the K_m of the high-affinity site for ATP [15,16] and the K_m for the formation of the phosphoenzyme [12]. Furthermore, the extent of acetylation was less when the enzyme was treated in the presence of a saturating ATP concentration, corresponding to the protection of two residues against modification. When the activity was measured in the absence of calmodulin, full protection was obtained with concentrations at which ATP binds to the high-affinity site, and no low-affinity effects of ATP were detected. This differs from the pattern obtained with fluorescein isothiocyanate [17,18], and also from our previous results with methyl acetimidate [1], thus suggesting that the target for the modification by SA is none of those affected by the above. Stefanova et al. [19], using a fluorescent derivative of SA, found that Lys-492 of sarcoplasmic-reticulum Ca^{2+} -ATPase was the residue most affected, and also that this modification was prevented by ATP. Although labelling of this residue with the fluorescent derivative was not deleterious to activity, as a lysine residue in a comparable position is conserved in the plasma-membrane Ca^{2+} pump [20], it might be considered a potential target for modification by SA. In addition, Lys-684, which has also been proposed to play an important role in the

transfer of the phosphate group in the sarcoplasmic-reticulum Ca^{2+} pump, is conserved in the plasma-membrane Ca^{2+} pump [21].

The ATP protection experiments were carried out in the presence of CDTA, a chelator of Ca^{2+} and Mg^{2+} ions. Therefore neither Ca^{2+} nor Mg^{2+} was required for the protection of basal Ca^{2+} -ATPase. Furthermore, the presence of micromolar concentrations of Ca^{2+} and Mg^{2+} diminishes the effectiveness of ATP. The lack of effect of bivalent cations in the protection provided by ATP suggests that SA modification affects the ATP-binding site rather than the site of phosphorylation.

It is worth pointing out that AMP and *p*-nitrophenyl phosphate (for which there is evidence indicating that the phosphatase substrate binds to the catalytic site of the Ca^{2+} pump [16]) were able to mimic the protective effect of ATP.

Effect of SA on the regulation of Ca^{2+} -ATPase

Even when SA treatment was carried out in the presence of 5 mM ATP, some residues were still modified by this reagent, resulting in the Ca^{2+} -ATPase being rendered unable to undergo activation by calmodulin, acidic phospholipids or proteolysis. It is noteworthy that, for a wide range of Ca^{2+} concentration, the basal activity of the Ca^{2+} pump was negligibly affected when treatment with SA was performed in the presence of ATP.

Kinetics of the inhibition of Ca^{2+} -ATPase by SA

Inactivation of basal Ca^{2+} -ATPase follows monoexponential kinetics, suggesting that it is due to modification of a single site. Inactivation of the calmodulin- or proteolysis-activated Ca^{2+} -ATPase follows a monoexponential curve also. Consequently, it might be inferred that both effects of SA, the impairment of the catalytic-site function and lack of physiological-modulator effectiveness, are due to modification of just one site. However, it does not agree with the fact that ligands of the catalytic site only partially prevent inhibition, thus suggesting that there are two distinct SA targets. Nevertheless, it should be noted that Ca^{2+} -ATPase activated by calmodulin or proteolysis is inhibited with a pseudo-first-order constant that is twice that obtained for basal activity. These results can be explained by assuming two sites of similar reactivity to SA, one of which can be protected by combination of the substrate with the catalytic site (see the Appendix).

Location of the modified residue responsible for the changes in Ca^{2+} -ATPase activity

The data currently available do not allow us to pinpoint the location of the modified residues that cause the changes in kinetic behaviour. However, it is possible to exclude relevant modifications in some domains of the pump. After stimulation by proteolysis, ATPase was inactivated by SA at the same rate as the enzyme activated by calmodulin. As the proteolysed enzyme lacks its original calmodulin-binding domain, the residues involved in the SA effect must be located outside the calmodulin-binding site.

It has been demonstrated that acidic phospholipids bind to both a putative phospholipid-binding site and the calmodulin-binding site, and that calmodulin is also able to interact with these two sites [22,23]. Although the putative phospholipid-binding domain has a high content of lysine residues, it is not likely that relevant residues undergo modification in such a domain, as acidic phospholipids failed to prevent inactivation by SA.

Therefore an hypothesis can be proposed assuming that this

residue(s) is(are) located in a region other than that mentioned above, but involved in activation of the ATPase by calmodulin, acidic phospholipids and proteolysis. This suggestion implies that, although activation by acidic phospholipids and calmodulin have different mechanisms [24,25], they share at least one step. In this connection, SA treatment in the presence of ATP increased the apparent affinity for Ca²⁺ when the activity was measured in both the absence and presence of calmodulin, thus suggesting that the residue(s) modified under such conditions might be involved in regulation of the affinity for Ca²⁺. Figure 6 shows that the Ca²⁺-ATPase activity of membranes preincubated with SA and ATP was slightly higher than that of untreated membranes, as expected for activities measured at Ca²⁺ concentrations below 20 μ M (see Figure 7a). However, the addition of acidic phospholipids eliminates such behaviour. This fact further suggests that modification of the enzyme by SA affects a site relating the interaction with modulators and Ca²⁺ binding.

In conclusion, our present efforts were aimed at identifying the domains of the Ca²⁺ pump that are modified by SA, especially those related to the modulation of activity.

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APPENDIX

Inactivation of Ca²⁺-ATPase measured in the presence of calmodulin can be described by a single exponential curve. However, the fact that ligands for the catalytic site only partially prevent inhibition by SA suggests a more complex model involving two lysine residues, one related to the catalytic site and the other to regulation. These facts are depicted in the following scheme:

where E represents the native enzyme, E* is the enzyme that has been modified thereby losing its ability to be stimulated by calmodulin, and inactive E is the enzyme with a modified catalytic site, i.e. it has no activity. According to this, the remaining Ca²⁺-ATPase activity should be:

$$\text{Activity} \propto [E] + \frac{[E^*]}{n}$$

where n is the calmodulin activation factor. The variation of $[E]$ and $[E^*]$ with time is described by the following differential equations:

$$\frac{d[E]}{dt} = -(k + k_1)[SA][E]$$

$$\frac{d[E^*]}{dt} = k_1[SA][E] - k[SA][E^*]$$

Integration of this expression leads to:

$$\text{Activity} \propto \left(\frac{n-1}{n}\right)e^{-(k+k_1)[SA]t} + \left(\frac{1}{n}\right)e^{-k[SA]t}$$

If one assumes that $k \cong k_1$, the following is obtained:

$$\text{Activity} \propto \left(\frac{n-1}{n}\right)e^{-2k[SA]t} + \left(\frac{1}{n}\right)e^{-k[SA]t} \quad (\text{A1})$$

It can be demonstrated that, for $n \cong 4$, eqn. (A1) produces a curve that is practically superimposed on that yielded by:

$$\text{Activity} \propto e^{-2k[SA]t} \quad (\text{A2})$$

The results of performing regression of these two equations to

one set of data from a Ca^{2+} -ATPase-inactivation experiment in the presence of calmodulin are:

Eqn. (A1):

$$A_0 = 3.166 \pm 0.08$$

$$k' = 0.198 \pm 0.035$$

Eqn. (A2):

$$A_0 = 3.104 \pm 0.069$$

$$k' = 0.145 \pm 0.015$$

$$n = 7.3 \pm 3.5$$

$$\text{S.D.} = 0.1610$$

$$\text{S.D.} = 0.1566$$

Therefore, although inhibition is caused by modification of two sites, it is not possible to distinguish the curve obtained from a monoexponential one.