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Thermal analysis of the plasma membrane Ca^{2+}-ATPase

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Abstract

The plasma membrane Ca^{2+}-ATPase is a well known enzyme in eucaryotes able to extrude calcium to the extracellular space in order to restore intracellular calcium to very low levels. This ATPase needs plasma membrane lipids such as acidic phospholipids in order to maintain its activity. In this study, we investigated the role that calcium and cholesterol play on the thermal stability of the Ca^{2+}-ATPase isolated from cardiac sarcolemma and erythrocyte membranes. Calcium showed a stabilizing and protective effect when the enzyme was exposed to high temperatures. This stabilizing effect showed by calcium was potentiated in the presence of cholesterol. These protection effects were reflected on several thermodynamic parameters such as T_{50}, ΔH_{vh} and apparent ΔG, indicating that calcium might induce a conformational change stabilized in the presence of cholesterol that confers enzyme thermostability. The effect shown by cholesterol on ΔH_{vh} and apparent $\Delta H^{\ddagger}$ open the possibility that this lipid decreases cooperativity during the induced transition. Despite that a binding site for cholesterol has not been identified in the plasma membrane Ca^{2+}-ATPase, our results supports the proposal that this lipid interacts with the enzyme in a direct fashion. (Mol Cell Biochem **209:** 105–112, 2000)

Key Words: Ca2+-ATPase, thermal analysis, cholesterol, calcium

Introduction

The plasma membrane Ca^{2+}-ATPase [PM-(Ca^{2+})-ATPase] is a ubiquitous enzyme of eukaryotic cells [1]. This enzyme couples the hydrolysis of ATP to the transport of calcium from the cytoplasm to the extracellular space, and has been shown to be regulated by several factors including calmodulin, phospholipids, phosphorylation, etc. [1–3]. The lipid environment has been shown to be very important for the activity and stability of several membranes bound proteins, i.e., enzymes, receptors, channels, etc. For some of these proteins there is a strict lipid requirement in order to maintain a biological active conformation. Changes in the lipids surrounding these proteins can alter their activity, structure, and stability. For the PM-(Ca^{2+})-ATPase, acidic phospholipids, such as phosphatydilserine and phosphatidic acid stimulate enzyme activity [4]. In our laboratory we have shown that cholesterol also regulates the pump activity, in such a way that high membrane cholesterol concentrations correlate with low ATPase activities, and *vice versa,* a low cholesterol content induces a stimulation of the ATPase [5, 6]. We have also found that increased concentrations of cholesterol in cardiac sarcolemma, stabilize the enzyme against thermal inactivation [7, 8]. Moreover, this lipid has been found to stabilize the Ca^{2+}-ATPase from sarcoplasmic reticulum [9], as well as the acetylcholine receptor [10], and the UDP-glucoronyl transpherase [11]. For the acetylcholine receptor, the presence of cholesterol seems to be necessary to maintain a proper receptor function and also to stabilize its structure [12]. In a similar way, studies performed with cytochrome oxidase have shown that activity and stability are optima when the enzyme is reconstituted into liposomes with phospholipids containing fatty acids with acyl chains ranging between 18 and 20 carbons [13, 14], conclusion reached using gel thermal analysis and differential scanning calorimetry (DSC) [13, 14].

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On the other hand, the binding of different ligands and metal ions to different proteins has been reported to induce conformational changes that stabilize their structures. These conformational changes can be followed by different spectroscopic techniques, like fluorescence, circular dichroism, nuclear magnetic resonance, and electron paramagnetic resonance. Thermal analysis offers the unique opportunity to investigate structural changes, based on the differential sensitivity of proteins to temperature, when binding of ligands or metal ions has been occurred. This approach has been successfully applied to study the effect of calcium on thermal inactivation and denaturation of sarcoplasmic reticulum Ca^{2+}-ATPase [15], alkaline phosphodiesterase I [16], α-lactoglobulin [17], and β-glucanase [18]. In all these proteins, calcium binding induces an increment on thermally induced unfolding transitions. Other studies where modification of the calcium binding sites take place, have proven that calcium stabilizes proteins such as subtilisin [19], and lysozyme [20, 21]. Pronounced protein stabilization mediated by metal ions has been reported for the Copper-Zinc superoxide dismutase [22] and the zinc metalloenzyme alkaline phosphatase [23]. Enzyme stabilization by cations has also been observed for the membrane bound pyrophosphatase from the photosynthetic bacteria *R. rubrum*, where binding of Zn^{2+}, Mg^{2+}, and Ca^{2+} protects the enzyme against thermal inactivation [23].

This study employs thermal analysis to investigate the role of calcium and cholesterol on the thermal stability of the PM-(Ca^{2+})-ATPase isolated from two different cell systems. Although an increase in the number of potential ion-pair contacts [25], mechanical and dynamical properties [26] as well as the improvement of hydrophilic packing of proteins [27] have been characteristics associated to thermal stability, our study for the first time provides an insight into the thermodynamic properties of the PM-(Ca^{2+})-ATPase when stabilized by calcium and cholesterol against thermal inactivation.

Materials and methods

Materials

All reagents were of the highest quality available. Salts, Triton X-100, cholesterol, asolectins and sepharose-4B were obtained from Sigma Company (St. Louis, MO, USA). Human erythrocytes were kindly provided by the National Institute of Neurology and Neurosurgery (Mexico City).

Cardiac microsomes

The microsomal enriched fraction was isolated from calf ventricular muscle based in a procedure reported by van Alstyne *et al*. [28] with several modifications [29]. The procedure involves the following steps: Calf ventricular tissue is cleaned and finely minced with cold scissors, homogenized twice with a blender for 2 min and rehomogenized with an ultra turrax five cycles for 5 sec each in a buffer containing 10 mM $NaHCO_3$ (pH 7.0), and 0.5 mM sodium azide (buffer A). After filtration with four layers of gauze, the suspension is centrifuged at 8,700 g for 30 min and the supernatant separated. The pellets are blender homogenized for 2 min with 5–6 vol of buffer A, recentrifuged at 8,700 g for 30 min and the supernatants mixed and centrifuged at 22,000 g for 60 min. Pellets from this spin are homogenized with a Potter pestle in 5–6 volumes of a buffer containing 20 mM malic acid (pH 6.8), and 0.6 M KCl (buffer B). The suspension centrifuged at 33,000 g for 60 min and the pellets resuspended and homogenized with a Potter pestle in 5–6 vol of a buffer containing 10 mM HEPES-KOH (pH 7.4), 2.0 mM EDTA. The suspension is mixed for 10 min with a magnetic stirrer and centrifuged at 50,000 g for 30 min. Pellets are resuspended in 2 vol of a buffer containing 50 mM Tris-malate (pH 7.4), 0.5 mM $MgCl_2$, 50 μM $CaCl_2$, 2 mM DTT (buffer C), separated in aliquots, and stored at –70°C.

Erythrocyte ghosts

Membranes from healthy human donors were prepared as previously reported [30]. Red cells are washed by centrifugation at 1,500 g in a buffer containing 130 mM KCl and 20 mM Tris-HCl (pH 7.4) (buffer A) for 10 min. The supernatant is discarded and the pellet resuspended in a hypotonic buffer (buffer B) containing 1mM EDTA, 5 mM Tris-HCl (pH 7.4) and centrifuged at 27,500 g for 30 min. The pellet is recovered and washed several times using the same conditions in order to eliminate haemoglobin. The final pellet is resuspended in buffer C containing 10 mM Tris-HCl (pH 7.4), and then washed twice by centrifugation using buffer A at 34,800 g for 10 min. The pellet containing the erythrocyte ghosts is resuspended in buffer A and aliquots stored at –70°C until use.

Ca^{2+}-ATPase isolation from cardiac microsomes and from erythrocytes ghosts

The Ca^{2+}-ATPase from cardiac sarcolemma was isolated employing a calmodulin-sepharose-4B column as described by Niggli *et al*. [31]. Briefly, the microsomal membrane suspension is adjusted to 5 mg of protein/ml with a buffer containing 20 mM MOPS, 130 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2 mM DTT (pH 7.4). Triton X-100 is slowly added to a final concentration of 0.5%, gently stirred during 15 min and the nonsolubilized material removed by centrifugation at 100,000 × g at 4°C for 30 min. The supernatant is recovered and sonicated soybean asolectins added to a final concentration of 0.5 mg/ml. The supernatant is loaded in a calmodulin-

sepharose-4B column, previously equilibrated with the solubilization buffer plus 0.5% Triton X-100 and asolectins 0.5 mg/ml (buffer A). The column is washed with 10–15 vol of buffer A, and with 10 vol of the same buffer with the exception that Triton X-100 is decreased to 0.05% (buffer B). Finally, the enzyme is eluted with 20 mM MOPS, 130 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, 1 mM EGTA, 10% glycerol, 0.05% triton X-100 and 0.5 mg/ml asolectins (pH 7.4) (buffer C). The isolated enzyme is stored in liquid nitrogen until use. In order to obtain the enzyme in the different lipid environments employed, the ratio cholesterol/phospholipid is changed along the purification procedure.

Since the erythrocytes preparation was exposed to EDTA during the last washing procedure, calmodulin-depleted membranes were obtained. Solubilized membranes (final concentration of 0.5% Triton X-100) are applied to a calmodulin-Sepharose column. The procedure followed to isolate the erythrocyte Ca^{2+}-ATPase was as the one employed for the sarcolemmal Ca^{2+}-ATPase.

Ca^{2+}-ATPase activity measurements

The Ca^{2+}-ATPase activity is determined at 25°C in a reaction medium containing 20 mM MOPS-KOH (pH 7.4), 130 mM KCl, 5 mM $MgCl_2$, 3 mM ATP and the free Ca^{2+} concentration adjusted to 10 μM with EGTA. After incubation for ten min, the reaction is stopped with the malaquite acid reagent and released Pi determined [32]. Free Ca^{2+} concentrations are adjusted with a computer program [33]. Protein was measured following the Bensandoun and Wenstein procedure [34].

Thermal inactivation experiments

Inactivation experiments performed as a function of temperature employ 2–3 three micrograms of isolated enzyme per sample in buffer C with the indicated concentrations of free calcium and cholesterol. The samples are heated in a water bath at a heating rate of 1.2°C/min, removed from the bath at 1°C intervals from 25–55°C, and immediately placed on ice until all samples were collected. When all samples are collected, the remaining Ca^{2+}-ATPase activity is determined at 25°C.

For the inactivation experiments as a function of time where the temperature is kept constant, the purified Ca^{2+}-ATPase contained in the different lipid mixtures is heated in buffer C. This buffer either contains 1 mM EGTA (no calcium), 10 μM or 100 μM free calcium. Samples are incubated at the desired temperatures in small glass tubes, removed at the indicated times and placed on ice. The remaining Ca^{2+}-ATPase activity is measured at 25°C as indicated below. Rates of inactivation are obtained from least-square analysis calculated from the plots of log of the remaining activity vs. time. The activation energy (E_A) is calculated from the slope of Arrhenius plots.

Results

The use of a microsomal preparation permitted us to obtain large amounts of membranes employed as raw material for the affinity chromatography procedure employed in the isolation

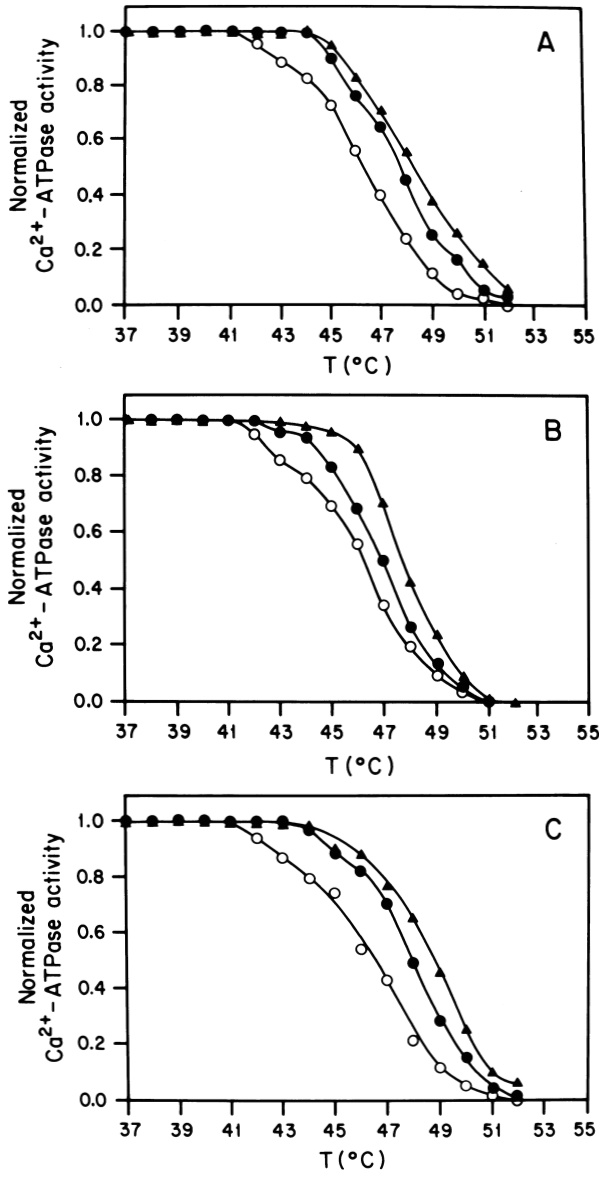

Fig. 1. Thermal inactivation profiles of the Ca^{2+}-ATPase under different phospholipid/cholesterol dispersions in the presence or the absence of calcium. A. Inactivation profiles of the enzyme in a phospholipid dispersion (0.5 mg/ml). B. Inactivation profiles of the Ca^{2+}-ATPase in a cholesterol/phospholipid (0.3/0.7) dispersion. C. Inactivation profiles of the enzyme in a cholesterol/phospholipid (0.4/0.6) dispersion. (○) 1 mM EGTA, (●) 10 μM free Ca^{2+}, and (Δ) 100 μM free Ca^{2+} employed during enzyme preincubation. Each data point on the plot is an average of 4 independent measurements and errors are estimated at ± 5% on the basis of the S.D. of the averaged values.

of the PM-(Ca^{2+})-ATPase. The use of this preparation employing a calmodulin-Sepharose column, gave excellent results since large amounts of membranes permitted to isolate good quantities of pure homogeneous Ca^{2+}-ATPase as demonstrated by SDS-PAGE and calmodulin sensitivity (data not shown).

After isolation, enzyme activity measured at 25°C without heat pretreatment was used as representative of the enzyme in the native state. The fraction of enzyme activity remaining in the native state at higher temperatures (F_n), is obtained by dividing enzyme activity values at the indicated temperatures over the activity at 25°C, as follows:

$$Fn(T) = \frac{A(T)}{A(25°C)} \quad (1)$$

where A (T) and A (25°C) represent the normalized activities at the different temperatures employed and 25°C respectively.

Inactivation profiles presented a sigmoid shape and a marked shift to higher temperatures depending on the presence of calcium ions. This effect is more evident when 100 μM free calcium is added during enzyme preincubation (Fig. 1A). These results suggest that calcium stabilizes and protects the PM-(Ca^{2+})-ATPase against thermal inactivation. When cholesterol was added to the phospholipid dispersion in a molar ratio of 0.3/0.7 or 0.4/0.6 (cholesterol/phospholipid), the inactivation profiles for the Ca^{2+}-ATPase was slightly shifted to higher temperatures, compared to the enzyme associated to phospholipids only (Fig. 1B, C).

The sigmoid-like patterns of the heat-inactivation curves were also analyzed in terms of their T_{50} values, temperatures at which half of the initial Ca^{2+}-ATPase activity remains (Table 1). Depending upon preincubation time, concentration of calcium and/or cholesterol, the T_{50} values ranged between 46.4°C in the absence of calcium to 48.9°C in the presence of 100 μM of the cation. From these data we can observe that in the absence of calcium, cholesterol seems to have no effect on enzyme stabilization, since the cholesterol effect is only appreciated when calcium is present in the preincubation media. These results suggest that calcium *per se* have an important role on enzyme stabilization against thermal inactivation. However at 10 μM free calcium, the addition of cholesterol seems to optimize the stabilizing effect of calcium. These results also suggest that calcium binding to the enzyme might expose certain areas of the protein susceptible to interact with cholesterol, since in the absence of calcium there is no change in the T_{50} and ΔH_{vh}, in contrast to the values found when calcium ions were only employed (Table 1).

Using the data shown in Fig. 1, the apparent equilibrium constants corresponding to the ratio of the population of molecules in the inactive vs. native state, were calculated:

$$K_{app}(T) = \frac{1\text{-}F_n(T)}{Fn(T)} \quad \frac{F_d(T)}{F_n(T)} \quad (2)$$

Apparent equilibrium values can be used to estimate thermodynamic parameters like the van't Hoff enthalpy parameter. van't Hoff plots for the calculated apparent equilibrium constants obtained from data shown in Fig. 1 are presented in Fig. 2. The linearity of these plots suggests that even though the thermal inactivation of the Ca^{2+}-ATPase is irreversible, calculated *kapp* obeys an exponential dependence on the inverse of the absolute temperature. The addition of calcium to the enzyme produces an increase in the slopes of the van't Hoff plots, whereas the addition of cholesterol causes a decrease. Although the presence of calcium and cholesterol seems to increase the thermal stability of the enzyme reflected in higher T_{50} values, stability values decrease when cholesterol is present in the lipid dispersion.

It has been proposed that under irreversible processes or reversible multistage transitions, the van't Hoff enthalpy change can be only considered as a measure of the sharpness of a thermally induced transition rather than to the effects associated with the process [14]. If we assume this idea, addition of calcium to the preincubation media produces sharper transitions during enzyme inactivation, suggesting that calcium might induce high cooperativity during thermal inactivation of the Ca^{2+}-ATPase.

Once we established the temperatures for the transition of the Ca^{2+}-ATPase (active-inactive), we carried out inactivation experiments at fixed temperatures as a function of time. Inactivation plots of remaining Ca^{2+}-ATPase activity at different conditions are presented in Fig. 3 and Fig. 4. It is shown that enzyme inactivation obeyed pseudo-first-order kinetics. Addition of calcium during preincubation stabilizes the Ca^{2+}-ATPase activity against thermal inactivation, reflected in a decrease of

Table 1. Influence of calcium and cholesterol on T_{50} and ΔH_{vh} of the plasma membrane Ca^{2+}-ATPase from cardiac sarcolemma

	ΔH_{vh} (kcal/mol)			T_{50} (°C)		
Free [Ca^{2+}] μM	0	10	100	0	10	100
PL	138	184	223	46.4	47.1	48.8
PL/CHOL (0.7/0.3)	139	155	135	46.4	47.8	48.4
PL/CHOL (0.6/0.4)	139	157	154	46.5	48.1	48.9

PL, in the presence of phospholipid; PL/CHOL, in the presence of phospholipid/cholesterol. Each value is the average of 4 independent measurements and errors are estimated at ± 5% on the basis of the S.D. of the averaged values.

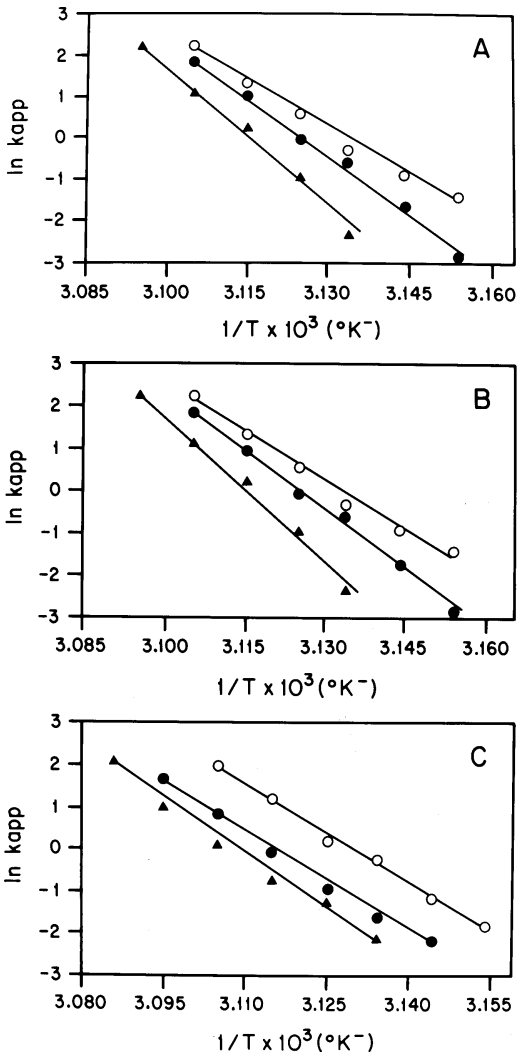

Fig. 2. van't Hoff plots of the Ca^{2+}-ATPase inactivation profiles. Data obtained from results shown in Fig. 1. Panels and symbols as in Fig. 1.

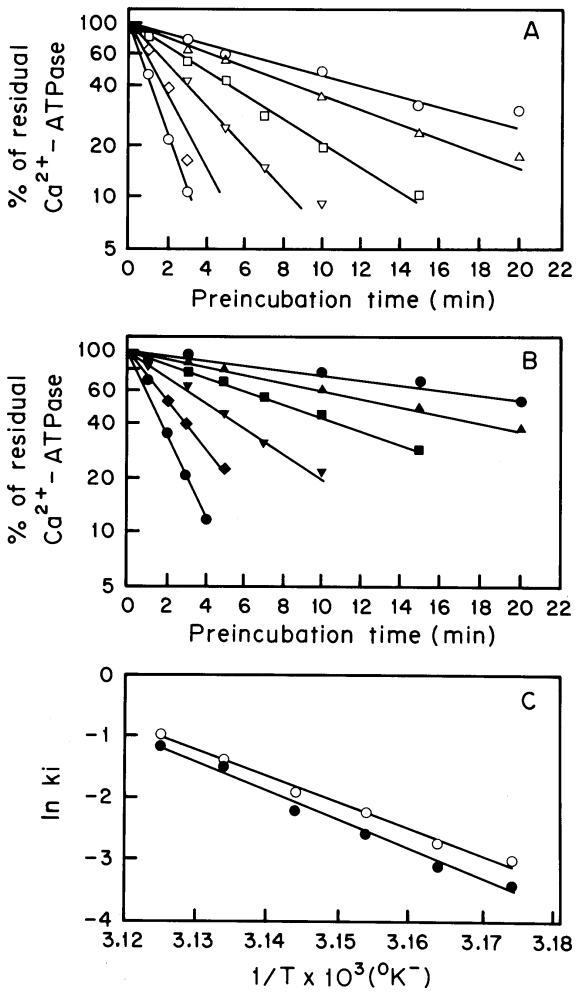

Fig. 3. Thermal inactivation profiles of the Ca^{2+}-ATPase dependent on temperature and preincubation time in the presence of a phospholipid dispersion (0.5 mg/ml). A. Inactivation plots of the enzyme preincubated in the presence of 1 mM EGTA (no calcium). B. Inactivation of the enzyme preincubated in the presence of 10 μM free calcium. C. Arrhenius plots obtained from calculated inactivation rates of data shown in panels A and B. Inactivation in the presence of 1 mM EGTA, (○) or 10 μM of free Ca^{2+} (●). Each data point on the plot is an average of 4 independent measurements and errors are estimated at ± 5% on the basis of the S.D. of the averaged values. (○, ●) 43; (Δ, ▲) 44; (□, ■) 45; (∇, ▼) 46; (◊, ♦) 47°C.

decrease of the inactivation rates at each temperature (Fig. 3). Arrhenius plots, obtained from the data presented in panels 3A and 3B show that the addition of calcium during preincubation produces an increase of about 10 kcal/mol in the activation energy of the process (Fig. 3C, Table 2). In a similar set of experiments, our results show that the inclusion of cholesterol in the lipid mixture decreases the inactivation rates, where the addition of calcium seems to further stabilize the enzyme against the heat challenge, producing a higher decrease in the inactivation rates (Fig. 4). Even though cholesterol seems to stabilize the enzyme, we observe a decrease in the activation energy (around 8 kcal/mol), compared to the enzyme present in phospholipid dispersions only (Fig. 4C, Table 2). However, when calcium and cholesterol are present during preincubation, the activation energy increases again, close to those values obtained when phospholipids and calcium are present during preincubation (Table 2).

In order to obtain more information about the activation thermodynamic parameters involved in the thermal inactivation of the PM-(Ca^{2+})-ATPase from cardiac sarcolemma, the data presented in Figs. 3, 4 were reploted as ln*(k/T)* vs (1/*T*) (Fig. 5) and analyzed with the following equation:

$$\ln(k/T) = \frac{-\Delta H^{\ddagger}}{R}\,(1/T) + \frac{\Delta S^{\ddagger}}{R} + \ln(k'/h) \qquad (3)$$

where $\Delta H^{\ddagger}$ is the activation enthalpic change, $S^{\ddagger}$ the activation entropic change, *R* the gas constant, *k* the Boltzman constant, and *h* the Plank's constant.

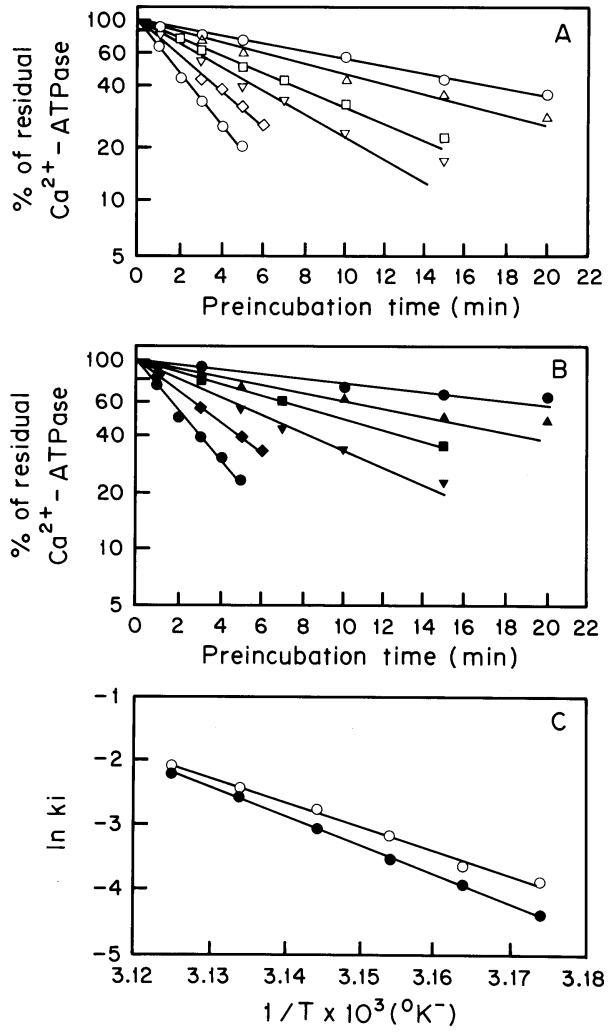

Fig. 4. Thermal inactivation profiles of the Ca^{2+}-ATPase dependent on temperature and preincubation time in the presence of a dispersion cholesterol/phospholipid (0.3/0.7) (0.5 mg/ml). A. Inactivation plots of the enzyme preincubated at the indicated temperatures in the presence of 1 mM EGTA (no calcium). B. Inactivation of the enzyme preincubated in the presence of 10 μM free Ca^{2+}. C. Arrhenius plots obtained from the calculated inactivation rates of the data shown in panels A and B. Inactivation in the presence of 1 mM EGTA (○) or 10 μM free Ca^{2+} (●). Each data point on the plot is an average of four independent measurements and errors are estimated at ± 5% on the basis of the S.D. of the averaged values. (○, ●) 43; (Δ, ▲) 44; (□, ■) 45; (∇, ▼) 46; (◊, ♦) 47°C.

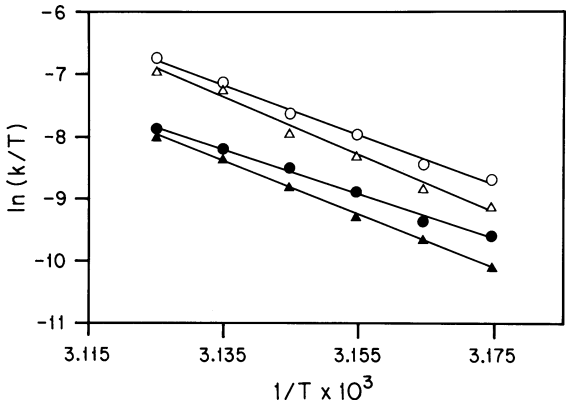

Fig. 5. Replot of the data presented in Figs. 3, 4. Inactivation of the Ca^{2+}-ATPase in a phospholipid dispersion (○, ●). Inactivation of the enzyme in cholesterol/phospholipid dispersions (0.3/0.7) (Δ, ▲). Open symbols; preincubation of the enzyme with 1 mM of EGTA (no calcium). Closed symbols; preincubation of the enzyme in the presence of 10 μM free Ca^{2+}.

From the slope of these plots, it is possible to estimate $\Delta H^{\ddagger}$, and from the intercept $\Delta S^{\ddagger}$. The data obtained from these calculations are summarized in Table 2. The results show that while calcium increases $\Delta H^{\ddagger}$ of the transition, cholesterol induces a decrease of $\Delta H^{\ddagger}$ values, even if calcium is present in the preincubation media. On the other hand, if we accept the hypothesis that high ΔS values correlate with a more disorganized system, the addition of cholesterol to the enzyme produces a decrease in $\Delta S^{\ddagger}$, suggesting that under these conditions the enzyme seems to be more organized. A different interpretation could be due to the possibility that the initial and transition states of the enzyme are different depending on the composition of the preincubation media.

$\Delta G^{\ddagger}$ calculated from $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ is ranged between 24 and 27 kcal/mol depending on lipid composition, and the presence or absence of calcium in the medium (Table 2). Since $\Delta G^{\ddagger}$ can be regarded as a parameter for the kinetic stability of protein, $\Delta G^{\ddagger}$ values obtained suggest that the enzyme is kinetically more stable when calcium is present in the preincubation medium. This stabilization effect seems to be enhanced when cholesterol is also present.

Table 2. Activation parameters for the inactivation of purified Ca^{2+}-ATPase from cardiac sarcolemma

	$\Delta H^{\ddagger}$ kcal/mol	$\Delta S^{\ddagger}$ kcal/mol	ΔG_{298K} kcal/mol	E_A kcal/mol
PL	83.6	200.2	23.9	81.3
PL + Ca^{2+} 10 μM	90.8	222.2	24.5	93.4
PL /CHOL	69.7	154.8	26.6	73.1
PL/CHOL + Ca^{2+} 10 μM	83.4	197.0	24.7	87.3

PL, in the presence of phospholipid; PL/CHOL, in the presence of phospholipid/cholesterol (0.7/0.3). Each value is the average of 4 independent measurements and errors are estimated at ± 5% on the basis of the S.D. of the averaged values.

Table 3. Activation parameters for the inactivation of purified Ca^{2+}-ATPase from human erythrocyte ghosts

	$\Delta H^{\ddagger}$ kcal/mol	$\Delta S^{\ddagger}$ kcal/mol	E_A kcal/mol
PL	78.52	117.26	78.94
PL + Ca^{2+} 10 μM	94.80	141.70	95.06
PL + Ca^{2+} 100 μM	112.50	169.38	113.60

PL, in the presence of phospholipid. Each value is the average of 4 independent measurements and errors are estimated at ± 5% on the basis of the S.D. of the averaged values.

When the Ca^{2+}-ATPase from human erythrocyte ghosts are assayed at different preincubation times employing temperatures that ranged from 42–47°C, similar inactivation profiles are observed in comparison to the values obtained for the Ca^{2+}-ATPase isolated from cardiac sarcolemma. The activation thermodynamic properties of the erythrocyte Ca^{2+}-ATPase are shown in Table 3. It can be observed that 10 μM and 100 μM calcium increase the values for E_A, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ very much in a similar fashion to the values obtained for the sarcolemmal Ca^{2+}-ATPase . Again, the presence of calcium seems to protect the enzyme against thermal inactivation.

Discussion

Our results show that calcium promote changes in different thermodynamic parameters like the T_{50} values, van't Hoff enthalpy and the apparent ΔG. Binding of calcium to the enzyme most probably induces structural changes reflected in higher stability of the protein. However a direct evidence of the location of calcium binding sites in the plasma membrane Ca^{2+}-ATPase and the conformational changes induced by this cation are still unknown. Extensive reports in the literature have shown that the plasma membrane Ca^{2+}-ATPase presents a K_m for calcium between 10–20 μM [1–3]. Our experiments performed at 10 μM of free calcium suggest that at this concentration, the cation can partially saturate the active site of the enzyme, that in turn might induce a conformational change reflected in higher T_{50} and ΔH_{vh} values. Furthermore, addition of 100 μM free calcium produces a higher increase in these values, inducing a structural change related to a fully saturated binding site and producing a more stable conformation of the ATPase.

Several years ago it was proposed that the PM-(Ca^{2+})-ATPase presents two non catalytic high affinity Ca^{2+} binding sites located in its carboxy terminus [35]. Therefore, an alternative explanation to our data could be that 10 μM of calcium might saturate these high affinity binding sites and partially saturate the active site, which in turn could promote stabilization of the protein. Although experiments using circular dichroism were performed, we found no differences in the spectra in the presence or the absence of calcium (data not shown), suggesting that structural changes are minor and/or that the change probably takes place on the tertiary structure of the enzyme. For the sarcoplasmic reticulum Ca^{2+}-ATPase it was found that calcium binding has no effect on its secondary structure [36]. Although the high affinity calcium binding sites proposed for the sarcoplasmic reticulum Ca^{2+}-ATPase are located between the putative transmembrane sequences M4, M5, M6 and M8 [37], a definitive determination of these sites on the PM- (Ca^{2+})-ATPase will require first the elucidation of its three-dimensional structure. Crystallographic and thermal studies performed with proteins such as Δ-glucanase, subtilisin, and lysozyme have demonstrated that metal binding induces structural changes that in turn promotes more stable conformations of these proteins [18–21].

Inactivation of the PM-Ca^{2+}-ATPase is an irreversible transition and can be explained in terms of ΔH_{vh} reflecting the sharpness of the transition but not the energy involved in the process. This assumption has been validated for irreversible transitions or multistage reversible transitions [14]. In comparison to measurements carried out in the absence of calcium, the higher ΔH_{vh} values obtained in the presence of this cation, suggest that thermal transitions of the Ca^{2+}-ATPase might be considered more cooperative under this condition.

Cholesterol presents a less prominent effect than calcium since there were no changes observed on the T_{50} and ΔH_{vh} values in the absence of calcium (Table 1), suggesting that cholesterol stabilizes the enzyme only in the presence of calcium. This possibility suggests that calcium binding to the enzyme might expose certain areas of the protein that might interact with cholesterol. The decrease in apparent $\Delta H^{\ddagger}$ induced by cholesterol, might also suggest that cholesterol decreases cooperativity during the thermally induced transition.

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