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Protein stability and the evolution of the cell membrane ☆

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Abstract

Cholesterol has been shown to regulate the activity of several membrane proteins. Although this phenomenon represents an important factor in the regulation of ion homeostasis, insights are needed to fully understand the role of this lipid in cell function in order to better comprehend the effect of bilayer components upon membrane function. Since evolution has shaped the composition of the membrane bilayer, it becomes of interest to study these changes in parallel with the many functions of membranes such as ion transport. The present study employing a plasma membrane preparation obtained from calf ventricular muscle demonstrates that cholesterol partially inhibits the Ca^{2+},Mg^{2+}-ATPase as the catalytic function of the calcium pump, when incubation reaction temperatures are below 42 °C. In contrast, when incubation reaction temperatures are above 42 °C, cholesterol apparently promotes enzyme stabilization reflected in higher activity. Although the activation energy values for the enzyme are almost the same at ranges between 15 and 40 °C, the use of elevated temperatures promote higher enzyme inactivation rates in control than in cholesterol enriched membranes. Cholesterol apparently is promoting stabilization that in turn protects the enzyme against thermal inactivation. This protective effect is reflected in a decrease of inactivation rate values and energy released during enzyme catalysis. The modification of many membrane properties throughout million of years made it possible for new evolutionary driving forces to show themselves as new characteristics in eukaryotes such as the one discussed in this study, dealing with the presence of cholesterol in the cell membrane directly associated to the promotion of protein thermostability.

Keywords: Protein stability; Cell membrane evolution; Membrane cholesterol; Ca^{2+},Mg^{2+}-ATPase activity

1. Introduction

Sterols are present in all major groups of living organisms. Nevertheless, many prokaryotes that have no cholesterol or contain small amounts of sterols may replace these molecules by pentacyclic triterpenoids such as the hopanoids (Nes and Mckean, 1977; Petterson, 1994). Although it is known that sterols have multiple roles in the physiology of a cell, in most organisms the largest amounts of sterols are employed as structural constituents of their plasma membranes.

Sterols recognized as primary components of eukaryote cell membranes, modulate the physical state of the lipid bilayer (Yeagle et al., 1990; Yeagle, 1991). Variations in cholesterol concentration correlate with changes in a broad range of cellular processes including cell proliferation (Yeagle, 1985), membrane transport (Kutryk and Pierce, 1988; Debetto et al., 1990), enzyme activity (Xie et al., 1986; Yoda and Yoda, 1987; Villalobo, 1990; Zhou et al., 1991) and upregulation of heat shock proteins (Lepock, 2005). Although this phenomenon might represent an important factor in the regulation of ion homeostasis, insights are needed to fully understand its role in cell function, and also to better comprehend changes in catalytic performance of these enzyme systems along their biochemical pathways throughout evolution.

If we accept the thesis that ectothermic organisms have enzymes with higher catalysis and lower substrate activation values than their homologous enzymes from mammals (Hochachka and Somero, 2002), we find one of the most interesting puzzles in biology. Assuming that mammals evolved from ectothermic ancestors that already presented highly efficient catalysis, how do we explain the apparent loss of enzyme activity

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during the course of cell evolution? The analysis of this phenomenon has mainly focused on the study of the basic properties of the cell membrane, the fundamental elements of protein structure (Perutz, 1978; Low et al., 1973), and the reshaping of many biochemical pathways (Raymond and Segrè, 2006). Interestingly, these properties playing a key role in molecular evolution, have been modulated by temperature, one of the most important environmental factors controlling the activity and evolution of organisms (Low et al., 1973; Brock, 1985). Elevated temperatures known to produce modifications of the physical properties of membranes in prokaryotes, are mainly developed through an alteration on the relative content of saturated versus unsaturated fatty acids (Sinensky, 1974), which in turn might promote thermal stability in these organisms (McElhaney, 1985). Although prokaryote membranes do not contain sterols and their membrane composition in general is quite different from that of eukaryotes, when cholesterol is introduced to prokaryote membranes, the physical changes observed during the acclimation to elevated temperatures are remarkably similar to changes observed with unmodified mammalian cell membranes (Dahl and Dahl, 1988). A modification in the cholesterol concentration of these membranes, promote more ordered and less permeable membranes; characteristics proposed to regulate the survival sensitivity of mammalian cells to hyperthermic cell killing (Cress and Gerner, 1980). Although it is known that cholesterol is not the only factor involved in the phenomenon of thermostability, it has been suggested that this sterol promotes an important degree of protection against denaturation, and confers thermostability to membrane proteins such as the plasma membrane Ca^{2+},Mg^{2+}-ATPase as the catalytic entity of the plasma membrane Ca^{2+} pump (Cheng et al., 1988; Mas-Oliva and Santiago-García, 1990; Ortega et al., 1996). Cholesterol has been shown to stabilize and modulate the Ca^{2+}-ATPase from sarcoplasmic reticulum (Cheng et al., 1988), the acetylcholine receptor (Artigues et al., 1989), the $GABA_A$ receptor (Sooksawate and Simmonds, 2001), the oxytocin receptor (Klein et al., 1995) the UDP-glucoronyl transferase (Rotenberg and Zakim, 1991) and serotonin (1A) receptors (Pucadyl et al., 2005). Although specific cholesterol-rich membrane domains involved in numerous cellular functions have been associated to the modulation of membrane-activity (Pang et al., 2005; Almeida et al., 2005) including the one shown by the plasma membrane Ca^{2+}-ATPase (PMCA) (Sepúlveda et al., 2006; Tang et al., 2006), the molecular basis for this complex process although actively investigated, is still a matter of active research. In this study we report the effect of cholesterol upon the activity and thermal stability of the plasma membrane Ca^{2+},Mg^{2+}-ATPase contained in cardiac muscle cells, as an example of the powerful adaptive properties cholesterol might have conferred cell membranes along evolution.

2. Materials and methods

2.1. Cardiac muscle microsomal membranes

The plasma membrane enriched fraction was isolated from calf ventricular muscle based on a procedure previously reported by us (Ortega et al., 1996; Santiago-García et al., 2000). Briefly, bovine cardiac tissue was cleaned and finely minced with cold scissors, homogenized with a blender twice for 2 min and homogenized 5 cycles with an Ultra-Turrax homogenizer (Janke and Kunkel) for 5 s 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 was centrifuged at 8700 ×*g* for 30 min and the supernatant was separated. The pellets were blender homogenized for 2 min with 5–6 volumes of buffer A, recentrifuged at 8700 ×*g* for 30 min, and the supernatants were mixed and centrifuged at 22,000 ×*g* for 60 min. Pellets from this spin were homogenized with a Potter pestle in 5–6 volumes of a buffer containing 20 mM malic acid (pH 6.8), 0.6 M KCl (buffer B) and the suspension centrifuged at 33,000 ×*g* for 60 min. Pellets were resuspended and homogenized with a Potter pestle in 5–6 volumes of a buffer containing 10 mM HEPES–KOH (pH 7.4), and 2.0 mM EDTA; the suspension was mixed for 10 min with a magnetic stirrer and centrifuged at 50,000 ×*g* for 30 min. Pellets were resuspended in 2 vol. of a buffer containing 50 mM Tris-malate (pH 7.4), 0.5 mM $MgCl_2$, 50 μM $CaCl_2$, and 2 mM DTT (buffer C) separated in aliquots, and stored at −70 °C. All reagents and salts employed in this study were of the highest quality and purchased from Sigma Chem. Co. (St. Louis, MO., USA).

2.2. Cholesterol enrichment of isolated membranes

Cholesterol incorporation was achieved by incubating membranes in plasma medium enriched with cholesterol, (Ortega and Mas-Oliva, 1984, 1986). Briefly, inactivated blood plasma was diluted 1:10 in 55 mM NaH_2PO_4 (pH 7.1) and 30 mg of cholesterol previously diluted in 1 mL of 2% DMSO added for each 10 mL of diluted plasma. The mixture was centrifuged at 10,000 ×*g* for 10 min at 4 °C and the supernatant recovered. Cholesterol was incorporated by incubating the cholesterol-treated plasma with isolated plasma membranes at 37 °C overnight, centrifuged at 30,000 ×*g* for 30 min at 4 °C, and resuspended in buffer C. Native plasma membranes were employed as control.

2.3. Membrane cholesterol determination

The concentration of cholesterol in the different plasma membrane fractions was determined using a colorimetric assay kit employing an enzymatic system based on the oxidation of cholesterol (Boehringer, Mannheim) as previously reported (Ortega and Mas-Oliva, 1984, 1986; Ortega et al., 1996).

2.4. Ca^{2+},Mg^{2+}-ATPase activity measurements

For ATPase activity measurements, 15–30 μg aliquots of control and cholesterol-enriched membranes contained in 20 mM MOPS–KOH (pH 7.4), 130 mM KCl were incubated in a circulating bath for the indicated times at different temperatures in a reaction medium containing: 20 mM MOPS–KOH (pH 7.4), 130 mM KCl, 100 μM $CaCl_2$, 5 mM $MgCl_2$ and 3 mM ATP–Tris (pH 7.4). According to this reaction mixture, the free calcium

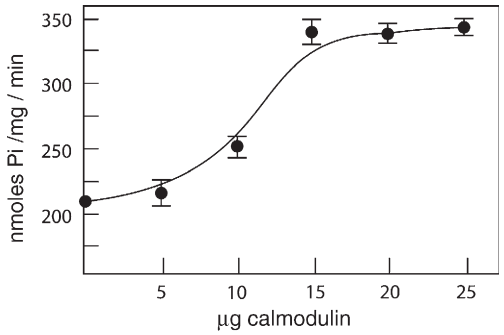

Fig. 1. Effect of calmodulin upon the hydrolytic activity of the Ca^{2+},Mg^{2+}-ATPase present in cardiac microsomes. The assays were performed in duplicates and SD from four independent experiments is presented.

concentration without the use of EGTA corresponds to 63.0 µM (Fabiato, 1988). After incubation, the reaction was stopped with the malachite acid reagent and liberated inorganic phosphate measured as described by Lanzetta et al. (1979). When calmodulin stimulation was studied, the assays were performed with 50 µg of protein in a medium containing 10 mM $MgCl_2$, 100 µM $CaCl_2$, 6 mM ATP, 50 mM Tris–maleate (pH 7.4) at 37 °C (Oliva-Ramírez, 1996), the free calcium concentration corresponds to 61.5 µM. Under the conditions employed in this study, nonenzymatic cleavage of ATP measured in control samples was negligible. Protein was measured using the Lowry method (Lowry et al., 1951). Inhibitors of the sarcoplasmic reticulum Ca^{2+}-ATPase and F_1F_o ATPase, thapsigargin and NaN_3 respectively, were used employing the same reaction medium at 37 °C.

2.5. Western blot analysis

In order to show the degree of sarcolemmal enrichment in our microsomal preparation, Western blot assays were performed using anti-PMCA (5F10 clone, Affinity Bioreagents) and anti-SERCA2 (C20, Santa Cruz Biotechnology, Inc.) antibodies. Twenty µg of solubilized protein as well as positive controls for each antibody were transferred into nitrocellulose membranes (0.45 µm). Sarcoplasmic reticulum membranes from cardiac muscle were isolated following the procedure described by Ortega et al., 2000. Ca^{2+}-ATPase purification from human erythrocytes has been carried out according to procedure previously described (Niggli et al., 1981). Non-specific sites were blocked in 2.5% non-fat milk in TBST buffer (150 mM NaCl, 0.05% Tween 20, 20 mM Tris–HCl pH 7.5) for 1 h and incubations performed at 37 °C. Membranes were incubated with the antibodies 5F10 and C20 for 1 h in 1:1500 and 1:1000 dilutions respectively and washed for 45 min in TBST. Finally, membranes were incubated with the corresponding secondary antibodies for 1 h and washed for 45 min in TBST. Blots were developed with Immobilon Western System (Millipore Co.) and exposed to X-OMAT film.

3. Results and discussion

As previously shown before by us (Oliva-Ramírez, 1996), assays to establish the degree of sarcolemmal enrichment show

Table 1
Effect of inhibitors upon the Ca^{2+},Mg^{2+}-ATPase activity measured in a cardiac muscle microsomal fraction

	(nmoles/mg/min) $\bar{x}\pm$SD
Control	99.40±8.28
+DMSO	95.80±6.04
+TG (1 µM)	93.58±8.83
+NaN_3 (1 mM)	51.48±3.60

DMSO, dimethyl sulphoxide; TG, thapsigargin (dissolved in 0.5% DMSO); NaN_3, sodium azide; SD, standard deviation of four independent experiments.

that the microsomal preparation employed in this study basically consist of a calmodulin free preparation (Fig. 1). The addition of exogenous calmodulin to the reaction medium stimulates the Ca^{2+},Mg^{2+}-ATPase close to 80% demonstrating the important sarcolemmal enrichment in the microsomal fraction (Fig. 1). As shown in Table 1, the use of thapsigargin, a well accepted inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase, does not present any significant effect upon the Ca^{2+}, Mg^{2+}-ATPase activity assayed on this membrane fraction. In contrast, N_aN_3 inhibiting the F_1F_o ATPase shows the presence of internal mitochondrial membranes in the fraction. Western blot analysis employing anti-PMCA and anti-SERCA2 antibodies corroborates the sarcolemmal enrichment and the almost negligible presence of sarcoplasmic reticulum in the preparation (Fig. 2), in accordance to the results employing thapsigargin.

The plasma membrane Ca^{2+},Mg^{2+}-ATPase activity shows that increased reaction temperatures result in increased enzyme–substrate collisions only offset by the increasing rate of denaturation (Fig. 3). Sensitivity to a heat challenge is clearly different depending on the cholesterol content of the membranes. ATPase in membranes containing high cholesterol concentrations appears to be a more thermal-resistant protein, since the activity of the enzyme is higher at temperatures above 42 °C, compared to control membranes. The cholesterol-induced ATPase inhibition found at temperatures below 42 °C is apparently lost when the assay is performed at higher temperatures

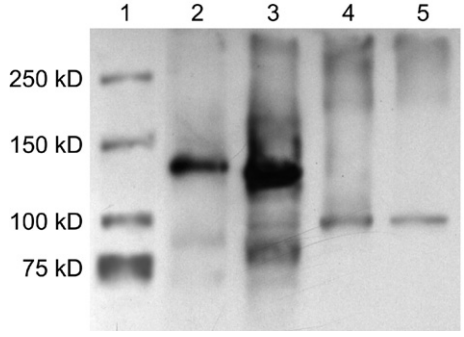

Fig. 2. Western blot analysis showing the presence of the sarcolemmal Ca^{2+}, Mg^{2+}-ATPase and sarcoendoplasmic Ca^{2+}ATPase (SERCA) in the cardiac muscle microsomal fraction. Lane 1, molecular weight markers. Lane 2, Ca^{2+}-ATPase purified from human erythrocytes used as a positive control for the 5F10 antibody. Lane 3, microsomal fraction employed in this study exposed to the 5F10 antibody. Lane 4, microsomal fraction employed in this study exposed to the C20 antibody. Lane 5, sarcoplasmic reticulum membranes isolated from rabbit cardiac muscle exposed to antibody C20 used as a positive control.

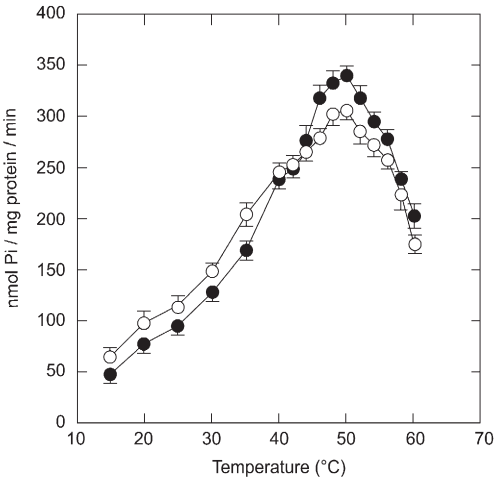

Fig. 3. Ca^{2+},Mg^{2+}-ATPase activity as a function of temperature and membrane cholesterol. Native (○, 15 μg cholesterol/mg protein), and cholesterol-enriched membranes (●, 35 μg cholesterol/mg protein). Ca^{2+},Mg^{2+}-ATPase activity was measured employing 15–30 μg of membrane protein and incubated during 5 min at the indicated temperatures. SD of four independent experiments.

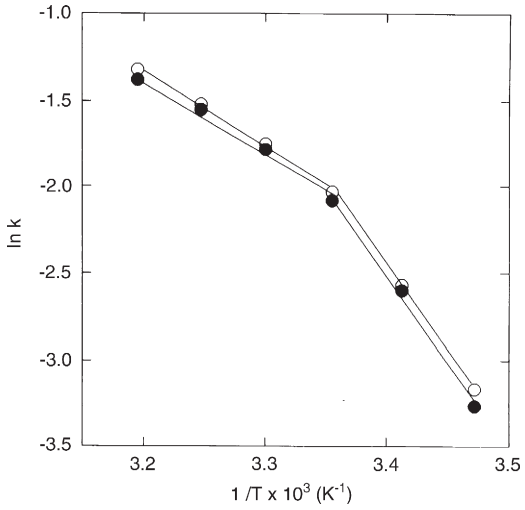

Fig. 5. Arrhenius plots obtained from data presented in Fig. 2 (○) native and (●) cholesterol-enriched membranes.

where a higher cholesterol content of plasma membrane might keep more ATPase units in an active conformation under a better protected environment working against thermal denaturation. The estimation of ΔH for denaturation processes presents many difficulties, nevertheless if we choose the basic denaturation reaction native → random coil, it is still possible to estimate several parameters. For instance, the transfer of aliphatic hydrocarbon moieties contained in a protein from a nonpolar medium (the interior of a membrane) to a medium containing water, is considered an exothermic process. While valyl, lucyl and isoleucyl side chains correspond to ~−2 kcal/mol at 25 °C, for aromatic side chains may be close to zero. Moreover, for the breaking of internal interpeptide hydrogen bonds we would get ~−1.35 kcal/mol of peptide groups exposed (Tanford, 1970).

To further test the cholesterol effect on the activity and thermal stability of the enzyme, we carried out experiments at different temperatures as a function of time. Our data show that at low temperatures (15–40 °C), enzyme activity in cholesterol-enriched membranes is lower than the activity found with control membranes (Fig. 4). From the results shown in Fig. 4A and B employing both membrane conditions, control and high cholesterol content respectively, the initial criterion we employ to quantify catalytic performance is the catalytic rate constant of the reaction, the rate at which the substrate is converted to product per active site per unit time at a specific temperature (Hochachka and Somero, 2002). The calculated rate constant values at the 15–40 °C range present small differences according to the slightly lower ATPase activity consistently found with the cholesterol enriched membranes. Arrhenius plots obtained from these data show that activation energies (E_a) for the Ca^{2+},Mg^{2+}-ATPase present a biphasic behavior (Fig. 5). The transition temperature corresponds to 25 °C and the E_a values to 9.1 kcal/mol above, and 19.3 kcal/mol below the break temperature for the control membranes. In the same fashion, E_a values obtained for the cholesterol-enriched membranes correspond to 8.7 kcal/mol above, and 20.4 kcal/mol below the

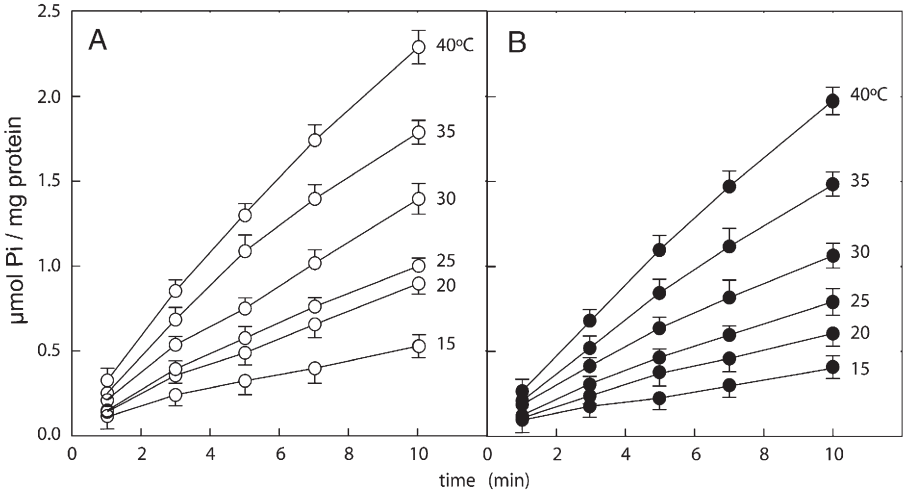

Fig. 4. Ca^{2+},Mg^{2+}-ATPase activity measured as a function of membrane cholesterol and incubation time at the indicated temperatures. Panel A. Native membrane (15 μg cholesterol/mg protein). Panel B. Cholesterol-enriched membranes (35 μg cholesterol/mg protein). SD of three independent experiments.

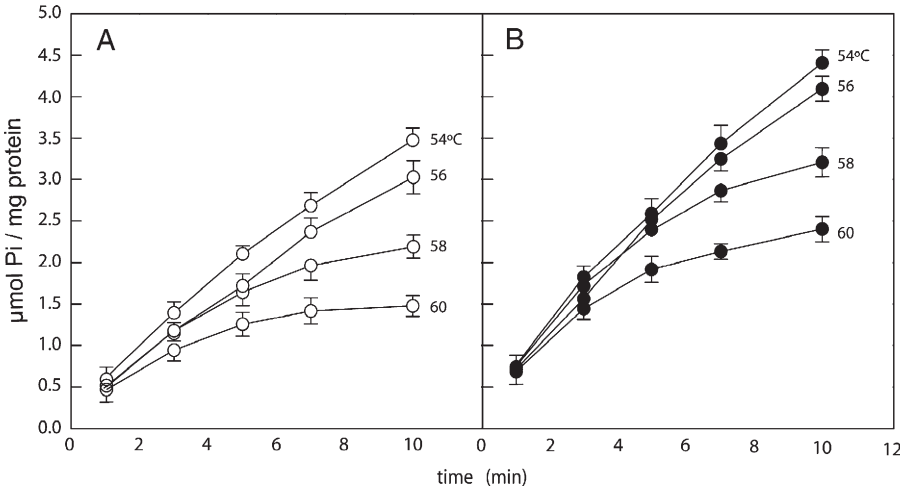

Fig. 6. Ca^{2+},Mg^{2+}-ATPase activity measured as a function of membrane cholesterol and incubation at the indicated temperatures. Panel A. Native membranes (15 µg cholesterol/mg protein). Panel B. Cholesterol-enriched membranes (35 µg cholesterol/mg protein). SD of three independent experiments.

break temperature. Although under these conditions there are slight changes in the E_a for the Ca^{2+},Mg^{2+}-ATPase, the differences between the two conditions are negligible. Moreover, despite the differences in cholesterol content between control and cholesterol incorporated membranes, the transition temperature is kept constant (Fig. 5). The results obtained under these conditions suggest that cholesterol does not have an effect upon the transition temperature of membrane lipids. Therefore, a possible explanation for the changes in activity observed at temperatures below 40 °C caused by changing the concentration of membrane cholesterol, could have been given as a direct effect of cholesterol upon the Ca^{2+},Mg^{2+}-ATPase.

On the other hand, time course experiments performed at high temperatures (54–60 °C) show that the ATPase activity present in cholesterol-incorporated membranes is higher than the one in control membranes (Fig. 6). Arrhenius plots obtained from rate constants calculated from data presented in Fig. 4 gave E_a values of 58 kcal/mol for the control membranes and 45 kcal/mol for the cholesterol enriched ones (Fig. 7). These results suggest that at high temperatures, energy required for enzyme activation in high cholesterol content membranes is lower compared to control membranes. However, due to the increase in the inactivation process as the temperature is raised, E_a values could represent the energy of the inactivation process. Since the inactivation process is higher for the Ca^{2+},Mg^{2+}-ATPase present in control or low cholesterol membranes, the activation energy is also high.

Results presented in this study suggest that cholesterol might render more ATPase units active at high temperatures, where the catalytic efficiency of the ATPase is given by the cholesterol/enzyme ratio. Since enzyme inactivation and denaturation increase as temperature is raised, E_a values that represent the minimal level of energy required for the reaction to occur, are shown to be lower in cholesterol incorporated membranes. Therefore, as the reaction temperature is increased, the active/inactive ratio shown by the ATPase seems to be proportional to the content of cholesterol associated to membranes. Since under conditions employed in this study the phenomenon appears to be independent of the physical state of the membrane, most probably we are dealing with a direct effect of cholesterol upon the enzyme, as previously suggested by us (Mas-Oliva and Santiago-García, 1990), in accordance to direct binding of cholesterol to other membrane proteins such as glycophorin (Yeagle, 1984), band 3 protein from human erythrocytes (Klappauf and Schubert, 1977) and the acetylcholine receptor (McNamee and Fong, 1988). Moreover, the association of cholesterol with the acetylcholine receptor has been shown to be necessary for an adequate function and suggested that 32 of its 44 lipid molecules that form its lipid ring are occupied by cholesterol (McNamee and Fong, 1988). Highly temperature tolerant enzymes of the domain Archaea present extremely low catalytic activities when catalytic measurements are made at moderate temperature. To understand this phenomenon it is necessary not only to analyze the kinetic data based on protein structure, but also on microenvironment singularities (Hochachka and Somero, 2002). In our hands, the presence of cholesterol at the membrane at higher concentration than normal, might be considered one of these microenvironment singularities acting upon the catalytic rate constant of the ATPase.

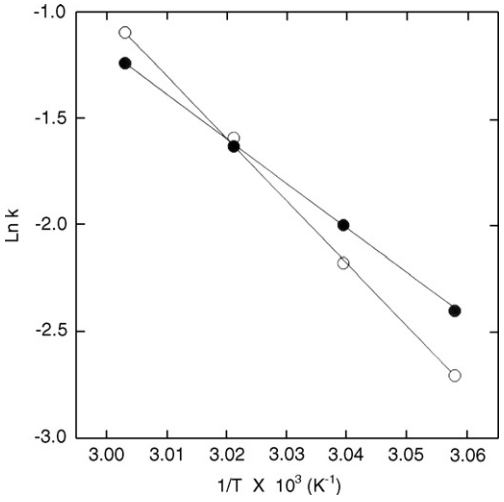

Fig. 7. Arrhenius plots obtained from data presented in Fig. 4 (○) native and (●) cholesterol-enriched membranes.

An increase in cholesterol content results in an increase in the packing order and a decrease in the rotational rate of fatty acyl chains of membranes. This enhanced packing and reduced motional flexibility of the fatty acyl chains may decrease the frequency and/or amplitude of the thermal induced folding/unfolding process of critical segments that might provoke general stabilization of a membrane protein (Cheng et al., 1988). Furthermore, evidence suggests that the cholesterol effect on the activity and stability of the Ca^{2+},Mg^{2+}-ATPase could be extended and shared to several other membrane proteins (Cheng et al., 1988; Artigues et al., 1989; Mitchell et al., 1990; Rotenberg and Zakim, 1991).

While inclusion of cholesterol to lipid bilayers has been shown to produce changes in membrane thickness (McIntosh, 1978), cholesterol depletion of the plasma membrane has been reported to induce solid-like regions (Nishimura et al., 2006). Moreover, considering the influence of cholesterol on the physical properties of membranes, it has been proposed that cholesterol induced thickness changes are accomplished without microviscosity being affected (Bloom and Mouritsen, 1988). In this respect, fatty acids shorter or longer than 18 carbons produce a decrease of SERCA1 activity (Johannsson et al., 1981; Starling et al., 1993). Parallel to thickness changes, the presence of cholesterol in model membranes has been shown to reduce the hydration and water penetration into the bilayer (Simon et al., 1982). The normal arrangement of hydrogen bonding between water and the different phospholipids surrounding the proteins may also be disrupted by the introduction of cholesterol to the bilayer. Since thermostability is thought to be dominated by hydrophobic bonds that are formed by the energetically favorable dehydration of solvated hydrophobic side chains (Matsumura et al., 1988; Vriend et al., 1991), a change of this nature modifying the interaction of boundary lipids with the ATPase might cause a change in the normal equilibrium between conformations (Lee, 1987), thus favoring thermoresistance. In accordance with previous results from our laboratory (Santiago-García et al., 2000), the present evidence suggests that changes in protein conformation that render Ca^{2+},Mg^{2+}-ATPase molecules in a more stable conformation, reflects high thermal protein stability. Moreover, the presence of an important number of plasma membrane of Ca^{2+}-ATPase isoforms of diverse cell types (Delgado-Coello et al., 2003, 2006), will have to be taken into account in terms of possible specific cholesterol sensitivities associated to each one of them (Sepúlveda et al., 2006).

It has been proposed that due to the evolution of sterols and related precursors such as triterpenes in an oxygen-rich atmosphere, the modification of many membrane properties made it possible for new evolutionary driving forces in eukaryotes (Rohmer et al., 1979; Bloom and Mouritsen, 1988, 1995) such as the one discussed in this study associated to the presence of cholesterol in the cell membrane. Recent evidence proposes that the appearance of molecular oxygen as part of many biochemical pathways, developed the ability for key enzymatic reactions to be replaced in aerobic cells (Raymond and Blankenship, 2004).

Since an increase in thermal stability of membrane proteins is most probably directly related to the influence of cholesterol upon their molecular conformations, based on this and previous studies we conclude that the presence of cholesterol in biological membranes promotes an "optimum" environment for protein stability. From the evolutionary point of view this is of importance, since proteins from the same family located in the membrane of prokaryotes where there are no sterols, are known to function in a more efficient way but in a less complex micro environment (Cavalier-Smith, 2002; Bapteste and Brochier, 2004), phenomenon that might promote shorter protein half-lives and the need to maintain protein synthesis at maximum.

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