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# Ligand and lipid domain stabilization of a membraneous $Ca^{2+}$-ATPase during hyperthermia

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**The susceptibility of the membranous $Ca^{2+}$-ATPase of sarcoplasmic reticulum to enzymatic inactivation at hyperthermic temperatures was investigated. Inactivation produced a break in the Arrhenius plot at 45–46 °C and was accompanied by an increased mobility of spin label, covalently attached to the $Ca^{2+}$-ATPase. MgADP and MgATP exerted a markedly stabilizing effect on inactivation, both at pH 7.0 and in acidic media. By contrast, high-affinity $Ca^{2+}$ or $Mg^{2+}$ binding only moderately stabilized $Ca^{2+}$-ATPase (inactivation rates were decreased 2–3-times), and this effect was non-additive, i.e., only observed in the absence of the other divalent cation. But withdrawal of $K^+$ and $Na^+$ gave rise to a pronounced destabilization that could be reversed efficiently by high concentrations of $Ca^{2+}$ or $Mg^{2+}$. These results are compared with a previous study on detergent solubilized $Ca^{2+}$-ATPase (Møller, J.V., Lind, K.E. and Andersen, J.P. (1980) J. Biol. Chem. 255, 1912–1920) which showed the enzyme to be markedly stabilized by $Ca^{2+}$ as well as by nucleotide. It is concluded that, due to the presence of nucleotide, inactivation of $Ca^{2+}$-ATPase is not likely to occur during malignant hyperthermia and that the native environment of the lipid bilayer provides stabilization of the membrane-embedded and $Ca^{2+}$-translocating domain of the $Ca^{2+}$-ATPase.**

## Introduction

The essential role of myoplasmic $Ca^{2+}$ in regulating the contraction of skeletal muscle is dependent on a balance between release and uptake processes by the sarcoplasmic reticulum. This is evidenced by the occurrence of the malignant hyperthermia syndrome, a recessively inherited and multigenic muscular disease, commonly occurring in swine [1,2] and, less frequently, in man [3,4]. The primary cause of the disease probably is a defect in the excitation-contraction coupling, which in the presence of provoking agents such as volatile anesthetics (e.g., halothane) and muscle relaxants (e.g., succinylcholine) leads to increased release of $Ca^{2+}$ from the sarcoplasmic reticulum [5,6]. This view is supported to some extent by an increased $Ca^{2+}$ release from in vitro preparations of 'heavy' sarcoplasmic reticulum vesicles [7,8], while decreased $Ca^{2+}$ uptake by sarcoplasmic reticulum vesicles as a pathogenic factor is con-

Abbreviations: Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $C_{12}E_8$, octaethylene glycol dodecylmonoether.

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troversial [8,9], and probably secondary to hyperthermia of the affected muscles [10,11]. An effect on cellular membranes has been suggested as one plausible mechanism for the cytotoxic effects of hyperthermia [12,13]. As part of an investigation into the effect of elevated temperature on cellular membranes, we present in this communication the results of a study on the effect of hyperthermia on sarcoplasmic reticulum $Ca^{2+}$-ATPase protein. The results obtained show interesting analogies as well as differences with previous studies performed on detergent-solubilized $Ca^{2+}$-ATPase [14], pointing towards a stabilizing effect of the lipid environment on the enzyme by the membrane-associated domain of the peptide chain.

## Materials and Methods

Sarcoplasmic reticulum was isolated from rabbit skeletal muscle according to the method of De Meis and Hasselbach [15], and $Ca^{2+}$-ATPase vesicles, completely leaky to $Ca^{2+}$ [16], were prepared by extraction with a low concentration of deoxycholate according to method 2 of Meissner et al. [17]. To study temperature inactivation, a small vesicle sample of 15–20 mg protein/ml, suspended in 1 mM Hepes (pH 7.5) and 0.3 M sucrose, was added at a final concentration of 0.5 mg protein/ml to a medium which had been thermoequilibrated at the desired temperature (37–51° C). Aliquots were withdrawn from the hyperthermic mixture at timed intervals and immediately mixed with 2.5 ml medium at 25° C. Activity was measured after ammonium molybdate precipitation, using ascorbic acid as reducing agent [18] or by enzymatic spectrophotometry [14], using phospho*enol*pyruvate and pyruvate kinase (from rabbit muscle, Type III, Sigma, St. Louis, MO) as an ATP-generating system *. The hyperthermic medium usually contained 110 mM KCl, 40 mM Tris, 20 mM NaCl, 5 mM $MgCl_2$, 0.125 mM $CaCl_2$ and 0.1 mM EDTA, and was adjusted to pH 7.0 with HCl at the temperature used. For measurement of inactivation as a function of pH, 40 mM Tris was replaced by 10 mM Pipes buffer. Other changes in medium composition are described in the legends to the figures. Inactivation rate constants were evaluated from linear logarithmic plots of remaining enzyme activity as a function of time. The protective effect of stabilizing ligands was analysed by Penzer plots [19]:

$$1/(k - k_L) = 1/(k - k_{min}) + K_D/(k - k_{min}) \cdot 1/[L] \quad (1)$$

where $k$ is the inactivation rate constant in the absence of stabilizing ligand, $k_L$ in the inactivation rate constant at ligand concentration [L], $K_D$ is the dissociation constant of the enzyme-ligand complex, and $k_{min}$ is the inactivation rate constant in the presence of a saturating concentration of ligand.

For spin label studies, preparations of $Ca^{2+}$-ATPase were incubated with a sulfhydryl reactive spin label, 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl, obtained from Syva, Palo Alto, CA. Labeling was carried out at a ratio of 2 mol spin label/mol $Ca^{2+}$-ATPase. Unbound label was removed by washing of the preparation. Measurements were performed at room temperature in an SE/X 28 ESR spectrometer produced by Wroclaw Technical University, Poland, operating at the X-band. Rotational correlation times ($\tau_c$) of the weakly immobilized component of bound spin label were calculated from

$$\tau_c = 8.4\, \Delta W_{w+1} \left( (h_{w+1}/h_{w-1})^{1/z} - 1 \right) \cdot 10^{-10} \text{ s} \quad (2)$$

where $\Delta W_{w+1}$ is the width of the low-field peak of the weakly immobilized signal, and $h_{w+1}$ and $h_{w-1}$ are the heights of the low-field and high-field peaks, respectively, of the weakly immobilized signal (see Fig. 3).

## Results

The purified $Ca^{2+}$-ATPase preparation used for these experiments is completely leaky to $Ca^{2+}$ [16], which is advantageous because activity measurements can be used to follow directly the in-

* In the latter method the medium also contained 0.15 mM NADH and lactate dehydrogenase, and ATPase activity was monitored from absorbance changes at 340 nm, resulting from oxidation of NADH by liberated pyruvate.

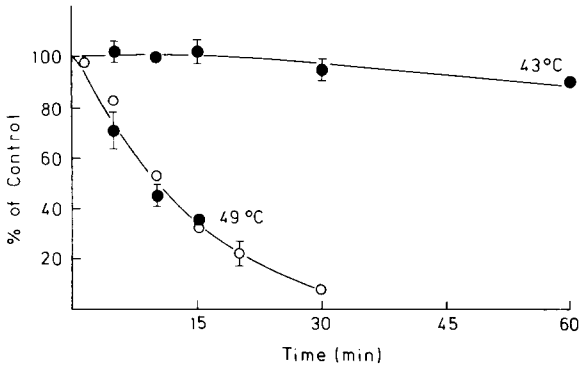


Fig. 1. Retention of $Ca^{2+}$-ATPase activity as a function of time during hyperthermia. $Ca^{2+}$-ATPase vesicles were incubated at 43 and 49 °C at a protein concentration of 0.5 mg/ml in a medium containing 110 mM KCl, 40 mM Tris (pH 7.0), 20 mM NaCl, 5 mM $MgCl_2$, 0.125 mM $CaCl_2$ and 0.1 mM EDTA for the indicated periods of time. Activity was measured after dilution of aliquots of the heated samples with cold (25 °C) medium. ●, Data obtained by measurement of inorganic phosphate liberation; ○, data obtained by NADH oxidation in the coupled assay system. Data are given relative to that of an unheated sample (approx. 4 μmol/mg per min). Bars indicate S.D. of 5–16 determinations (data without bars mean that the S.D. was smaller than or similar to the size of the symbol).

activation process without having to consider the inhibitory effect of a high intravesicular concentration of $Ca^{2+}$ on activity [20,21]. Fig. 1 shows the time course of inactivation at two hyperthermic temperatures (43 and 49 °C). After incubation for 15 min at 43 °C in standard medium at pH 7.0, containing $Ca^{2+}$ and $Mg^{2+}$, no change in activity is observed. However, the activity slightly declines after 1 h, to 90.6 ± 2.6% of the control value. At 49 °C activity rapidly decreases exponentially as a function of time. The inactivation at several temperatures is compared in an Arrhenius plot analysis in Fig. 2, from which it can be seen that there is a discontinuity at around 46 °C, which thus corresponds to a critical temperature for the hyperthermic inactivation process.

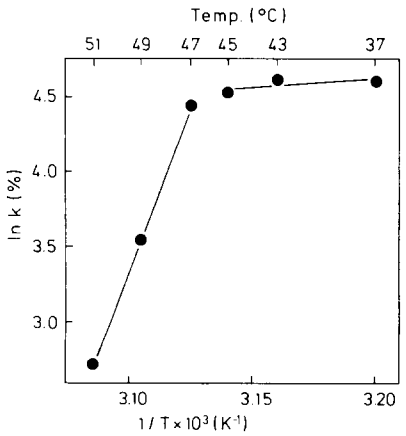


Fig. 2. Arrhenius plot of inactivation data obtained at various temperatures. The activity remaining after 15 min incubation was used as a measure of the inactivation rate.

Hyperthermic inactivation of $Ca^{2+}$-ATPase is accompanied by structural changes, as evidenced by the use of $Ca^{2+}$-ATPase, modified with spin label at a molar ratio of 2 : 1. The short chain label used exhibits a typical two-component ESR spectrum, consisting of a strongly immobilized and a weakly immobilized component (Fig. 3). The latter component is calculated to represent approx. 9% of the label. The rotational correlation time of weakly immobilized spin label is calculated to be

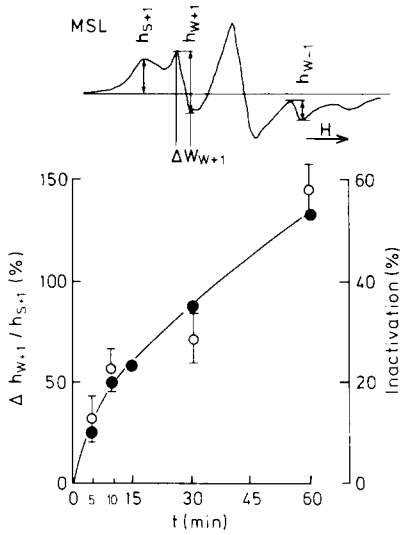


Fig. 3. Effect of hyperthermia on mobility of spin label, covalently attached to the $Ca^{2+}$-ATPase. Vesicles were labelled with spin label (MSL) at a molar ratio of $Ca^{2+}$-ATPase to label of 2 : 1 as described in Materials and Methods, and ESR spectra were recorded before and after incubation at 47 °C as a function of time. The upper curve shows the ESR spectrum of the unheated sample, and the presence of the weakly ($w+1$) and strongly ($s+1$) immobilized $z+1$ component. During hyperthermia the ratio of the heights of weakly and strongly immobilized component ($h_{w+1}/h_{s+1}$) increases. Changes in this empirical parameter of increased spin label mobility during hyperthermia is recorded in the lower figure (●, and left ordinate). The simultaneously occurring enzymatic inactivation is also shown (○, and right ordinate).

$13.5 \cdot 10^{-10}$ s. Hyperthermic inactivation leads to an increase of weakly immobilized spin label, and, as can be seen from the lower part of Fig. 3, this increased mobility is correlated with the extent of $Ca^{2+}$-ATPase inactivation. Furthermore, the rotational correlation time of weakly immobilized spin label is decreased (to about $0.65 \cdot 10^{-10}$ s after 60 min at 47 °C). Overall, the data thus indicate increased spin label mobility after hyperthermic inactivation.

*Effect of substrates*

In the following experiments a temperature of 49 °C was used to study the effect of adenine nucleotides and $Ca^{2+}/Mg^{2+}$ on $Ca^{2+}$-ATPase inactivation. Fig. 4 demonstrates that high (millimolar) concentrations of MgADP offer complete protection of the enzyme from inactivation. A Penzer plot analysis of the data (Fig. 5A) suggests that the protective effect is accounted for by binding of MgADP with an apparent dissociation constant of 0.23 mM. For MgATP a more pronounced stabilizing effect was observed, leading to an apparent $K_D$ of 0.04 mM. The experiments of Fig. 5B were carried out in a $Ca^{2+}$-chelated medium to prevent phosphorylation of the $Ca^{2+}$-ATPase. When the effect of MgATP was tested in a $Ca^{2+}$-containing standard medium (supplemented with ATP-generating system) the concentration dependence was more complex: At low MgATP levels (20–100 $\mu$M) the inactivation rate was reduced by about 70%, but remained constant as a function of nucleotide concentration, while at higher nucleotide levels (0.5–1 mM) the inactivation rate was reduced towards zero (not shown). These results probably indicate that phosphorylation per se affords partial protection against inactivation, but that full protection requires additional binding of MgATP in the phosphorylated state. This conclusion is supported by direct evidence of low-affinity binding and modulation of $Ca^{2+}$-ATPase by MgATP after phosphorylation [22].

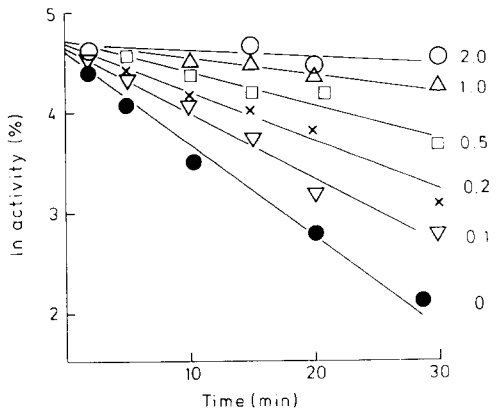


Fig. 4. Activity of $Ca^{2+}$-ATPase as a function of MgADP concentration at 49 °C. The media contained 110 mM KCl, 40 mM Tris (pH 7.0), 20 mM NaCl, 5 mM $MgCl_2$, 0.125 mM $Ca^{2+}$, 0.1 mM EDTA, and the following concentrations of MgADP: ○, 2.0; △, 1.0; □, 0.5; X, 0.2; ▽, 0.1; and ●, 0 mM. Activity was measured by the coupled assay system. The ordinate shows the ln of the remaining activity (%), relative to that of an unheated sample.

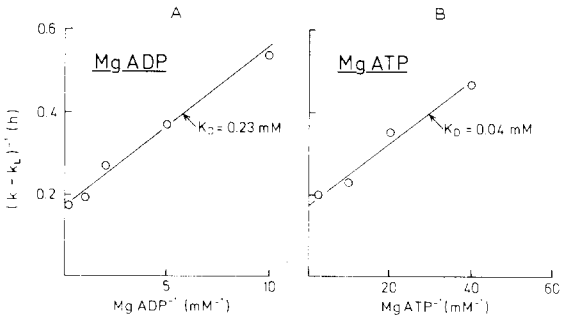


Fig. 5. (A) Penzer plot of the data on retention of activity by MgADP at 49 °C in the standard medium at pH 7.0. (B) Penzer plot of similar data on MgATP in the absence of $Ca^{2+}$ (medium composition: 110 mM KCl, 40 mM Tris (pH 7.0), 20 mM NaCl, 5 mM $MgCl_2$ and 0.5 mM EGTA).

Withdrawal of $Ca^{2+}$ from the standard medium (by substitution of 0.125 mM $Ca^{2+}$ with 0.5 mM EGTA) did not affect the inactivation rate constants, which became $5.33 \pm 0.2$ $h^{-1}$ (S.D. of four experiments), compared with $5.55 \pm 0.17$ (four experiments) in the $Ca^{2+}$-containing standard medium. On the other hand, removal of both $Ca^{2+}$ and $Mg^{2+}$ (by substitution of 0.215 mM $Ca^{2+}$ and 5 mM $Mg^{2+}$ with 0.5 mM EDTA) increased the inactivation rate to $13.6 \pm 0.6$ $h^{-1}$. The $Mg^{2+}$ concentration dependence of this effect is shown in Fig. 6, from which it can be seen that activity is increased towards the same level as observed in the standard medium by a high concentration of $Mg^{2+}$ (9.5 mM). Any further increase in $Mg^{2+}$ concentration does not produce more stabilization. A Penzer plot analysis indicates binding of $Mg^{2+}$ with a dissociation con-

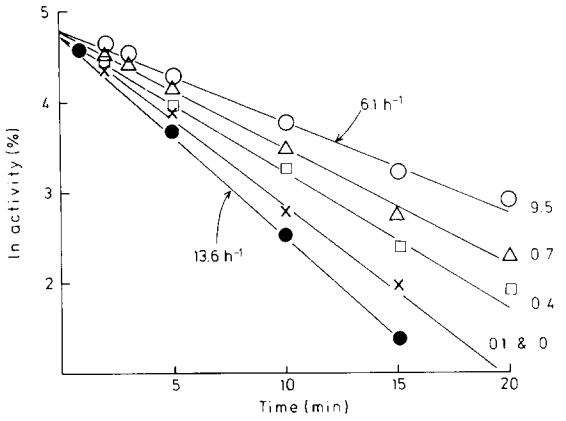


Fig. 6. Effect of free concentration of $Mg^{2+}$ on activity retention at 49 °C in the absence of $Ca^{2+}$. The medium had the following composition: 110 mM KCl, 40 mM Tris (pH 7.0), 20 mM NaCl, 0.5 mM EDTA, and various concentrations of $Mg^{2+}$: ○, 10; △, 1.2; □, 0.9; X, 0.6; and ●, 0 mM. The ordinate shows the ln of the remaining activity (%) measured by the coupled assay relative to that on an unheated sample. The free (unchelated) concentrations of $Mg^{2+}$ are indicated in the figure.

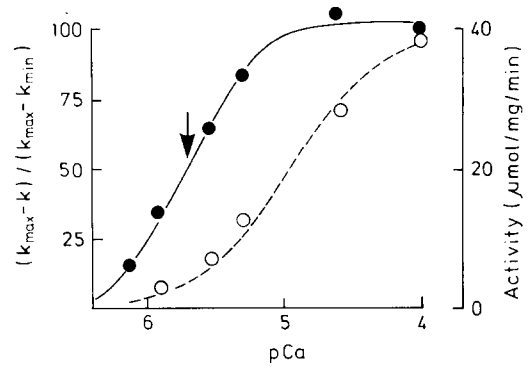


Fig. 7. Effect of p*Ca* on retention of activity and $Ca^{2+}$-ATPase activity at 49 ° C. Inactivation of $Ca^{2+}$-ATPase (●, left ordinate) was followed in the absence of $Mg^{2+}$ in a medium containing 110 mM NaCl, 40 mM Tris (pH 7.0), 20 mM KCl, 0.5 mM EGTA and 0–0.6 mM $Ca^{2+}$. The inactivation rate in the absence of added $Ca^{2+}$ ($k_{max}$) was 13.3 $h^{-1}$, while $k_{min}$ (at 0.6 mM $Ca^{2+}$, corresponding to p*Ca* 4) was 7.5 $h^{-1}$ (mean of two experiments). $Ca^{2+}$-ATPase activity at 49 °C (○) was measured in media containing 110 mM KCl, 40 mM Tris (pH 7.0, 20 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.25–0.6 mM $Ca^{2+}$, 1 mM phospho*enol*pyruvate, $Ca^{2+}$-ATPase (0.0052 mg/ml), 5 mM MgATP, 1 mM phospho*enol*pyruvate, pyruvate kinase (0.1 mg protein/ml), 0.15 mM NADH, and lactate dehydrogenase. Calculations of p*Ca* were based on an apparent stability constant of $0.8 \cdot 10^6$ $M^{-1}$, calculated from measurements by Dupont [37] and corrected for the effect of temperature [38]. The arrow indicates p*Ca* for half-maximal effect on retention of activity.

stant of 0.4 mM (not shown). Furthermore, in the absence of $Mg^{2+}$ low concentrations of $Ca^{2+}$ exert a stabilizing effect on the $Ca^{2+}$-ATPase (Fig. 7). Thus, $Ca^{2+}$ and $Mg^{2+}$ stabilize the $Ca^{2+}$-ATPase moderately in the absence of one another. The protective effect of $Ca^{2+}$ is characterized by a Hill coefficient of 1.8 and a high affinity (p*Ca* = 5.7 for 50% protection), suggesting that it is a consequence of cooperative binding at the translocation site. In fact, protection occurs at a lower concentration of free $Ca^{2+}$ than is required for activation of ATP hydrolysis at 49 °C in the standard medium (Fig. 7). This can be accounted for by an antagonistic effect of high concentrations of $Mg^{2+}$ in the standard medium (5 mM) on $Ca^{2+}$-ATPase activity [23].

*Other medium effects*

Omission of $K^+$ and $Na^+$ from the medium markedly destabilizes the $Ca^{2+}$-ATPase, resulting in an increase in the inactivation rate constant to about 80–90 $h^{-1}$. This effect can be reversed by the addition of $Ca^{2+}$ and $Mg^{2+}$ (Fig. 8). High-affinity binding of $Ca^{2+}$ approximately halves the inactivation rate constant, but further stabilization is achieved by higher concentrations of $Ca^{2+}$. The

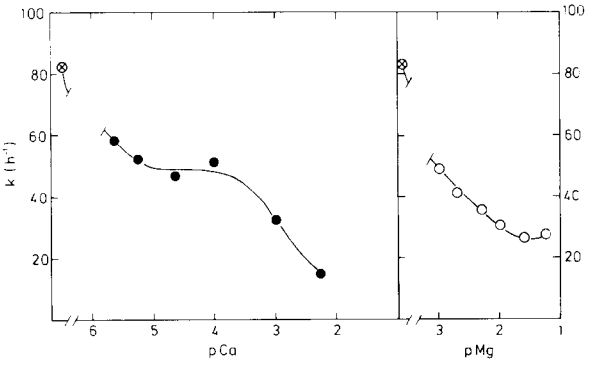


Fig. 8. Effect of $Ca^{2+}$ and $Mg^{2+}$ on retention of activity at 49 °C in the absence of $K^+$ and $Na^+$. Rate constants for inactivation were measured in media containing 150 mM choline chloride, 40 mM Tris (pH 7.0), 0.5 mM EGTA and various $Ca^{2+}$ (●) and $Mg^{2+}$ (○) concentrations to give the indicated p*Ca* and p*Mg* values. Activity in absence of added $Ca^{2+}$ and $Mg^{2+}$ is shown by ⊗.

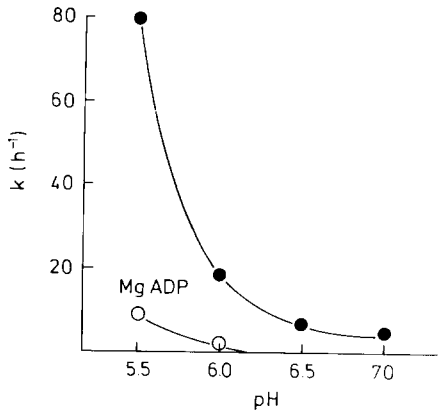


Fig. 9. Effect of pH on retention of activity at 49 °C. ●, Rate constants for inactivation in media containing 110 mM KCl, 10 mM Pipes, 20 mM NaCl, 5 mM $MgCl_2$, 0.125 mM $Ca^{2+}$ and 0.1 mM EDTA, titrated with HCl to give pH 5.5, 6.0, 6.5 or 7.0. ○, Rate constants for inactivation in media containing, in addition, 5 mM MgADP.

stabilizing effect therefore includes binding of $Ca^{2+}$ at secondary, low-affinity binding sites [24]. Similar observations are noted by addition of $Mg^{2+}$, except that the protective effect levels off at around 25 mM.

During a malignant hyperthermia attack there is a marked decrease in pH [1,25]. Fig. 9 shows that the inactivation rate is markedly increased at pH 5.5–6.0, but under these conditions the presence of a high concentration of MgADP also affords significant protection.

## Discussion

The present study shows that the application of hyperthermic temperatures (43–51 °C) to the in vitro system of isolated membranous $Ca^{2+}$-ATPase leads to inactivation of the enzyme. However, addition of ATP and ADP dramatically protects the enzyme against inactivation, so that at millimolar levels of these nucleotides there is complete retention of enzyme activity, even after storage at 49 °C and pH 7.0 for 1 h. Complete protection by ATP apparently is dependent on binding of nucleotide in addition to phosphorylation of $Ca^{2+}$-ATPase, which only affords incomplete protection against inactivation. In as much as sarcoplasmic reticulum membranes in the natural environment are exposed to millimolar concentrations of ATP plus ADP, enzymatic inactivation is therefore not expected to contribute to a rise in myoplasmic $Ca^{2+}$ during malignant hyperthermia. Only in extreme conditions, with depletion of energy-rich phosphate and lactacidosis, leading to rigor mortis, could the $Ca^{2+}$-ATPase possibly be considered to be susceptible to inactivation. It should be pointed out that retention of enzyme activity does not exclude the possibility of other damage to cellular membranes, resulting in increased permeability [13]. Thus, there is clear evidence that malignant hyperthermia is accompanied by leakage of creatine kinase and other enzymes to the blood from muscle tissue [1,26].

Inactivation of the $Ca^{2+}$-ATPase was associated with an increased mobility of spin label, covalently attached to the $Ca^{2+}$-ATPase. This phenomenon is generally observed after hyperthermic inactivation of membrane proteins [27]. The mobile component in the ESR spectrum existing before hyperthermic inactivation may be attributed to modification of superficial sulfhydryl groups with spin label, while the immobilized component may be considered to represent internal sites that are exposed during denaturation.

From the structural point of view the sarcoplasmic reticulum $Ca^{2+}$-ATPase, with a molecular weight of 110000 [28], is composed of a large cytoplasmic head and a membrane-associated domain [29]. From sequence determinations and other studies, it has been concluded that the cytoplasmic head contains the nucleotide binding and phosphorylation site, while $Ca^{2+}$ binding and translocation takes place in the membrane-associated domain [30,31]. After solubilization with the non-ionic detergent octaethylene glycol dodecyl monoether ($C_{12}E_8$), $Ca^{2+}$-ATPase can be prepared in a relatively stable form which is also protected against inactivation by ATP and ADP [14]. However, after detergent solubilization to monomer the enzyme is markedly destabilized (by a factor of approx. $10^2$) by removal of $Ca^{2+}$ [14]. In the present study we found that membraneous $Ca^{2+}$-ATPase was destabilized by a factor of maximally 2–3 by removal of $Ca^{2+}$, and only provided that $Mg^{2+}$ was absent as well. Detergent-solubilized $Ca^{2+}$-ATPase is destabilized during enzymatic turnover and differs in this respect qualitatively from membranous $Ca^{2+}$-ATPase, which

was found to be partially stabilized by phosphorylation. Both $Ca^{2+}$ depletion and enzymatic turnover are considered to be associated with a transition of the enzyme from the $E_1$ state of the $E_2$ state [32]. Thus, the enzyme is particularly vulnerable to inactivation in the $E_2$ state after detergent solubilization in monomeric form and removal of lipid [14,33]. When contrasted with the pronounced stabilization of membranous and detergent-solubilized [14] $Ca^{2+}$-ATPase by nucleotide, it seems probably that especially the normal phospholipid environment of membranous $Ca^{2+}$-ATPase protects the membrane-associated domain against inactivation. For the closely related membranous enzyme, $(Na^+ + K^+)$-ATPase, a smaller difference between hyperthermic inactivation of membranous enzyme in the $E_1$ and $E_2$ state is also apparent as compared to the detergent-solubilized form [34[. However, a significant difference between the two ATPase pumps is that for $(Na^+ + K^+)$-ATPase it is the $E_1$ state that is most susceptible to hyperthermic inactivation, both in the membranous form and after detergent solubilization [34].

According to the mechanism for $Ca^{2+}$ translocation proposed by Brandl et al. [30], hydrophobic amino acid residues of the $Ca^{2+}$-binding domain are exposed during phosphorylation and transport, accompanied by further insertion of the protein into the membrane to shield the exposed residues from contact with medium. For detergent-solubilized $Ca^{2+}$-ATPase uncompensated exposure of hydrophobic amino acid residues may account for the characteristic destabilization in the $E_2$ state. Another factor that may stabilize the membrane-embedded polypeptide chain is the relatively higher viscosity of lipid as compared to that of the bound detergent layer and surrounding medium [31].

It appears from our findings that $Mg^{2+}$ can substitute for the moderately protective effect of $Ca^{2+}$ on enzyme activity. Binding of $Mg^{2+}$ at the translocation site and partial conversion towards an $E_1$-like state has previously been suggested, on the basis of intrinsic fluorescence and phosphorylation studies with inorganic phosphate [35,36]. However, the $Mg^{2+}$ dissociation constant calculated from the present data (0.4 mM) is lower than that expected for binding of $Mg^{2+}$ at the translocation site [35,36]. A similar dissociation constant is also found for $Mg^{2+}$ protection of $C_{12}E_8$ solubilized enzyme (Møller, J.V., unpublished observation). It is therefore probable that the $Mg^{2+}$ effect is exerted by binding of the divalent cation to the enzyme at another location (phosphorylation site?) than the translocation site.

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