

# Interaction of Dibucaine with the Transmembrane Domain of the Ca<sup>2+</sup>-ATPase of Sarcoplasmic Reticulum†

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**ABSTRACT:** The site of interaction of dibucaine with the Ca<sup>2+</sup>-ATPase of rabbit sarcoplasmic reticulum, an ion-transporting membrane protein, was investigated by determining the effect of dibucaine on the denaturation of the transmembrane domain and the aqueous domain containing, respectively, the high-affinity Ca<sup>2+</sup> binding sites and the site of ATP hydrolysis. In the absence of Ca<sup>2+</sup>, a single irreversible denaturation transition with  $T_m \cong 49^\circ\text{C}$  is observed for the Ca<sup>2+</sup>-ATPase by differential scanning calorimetry (DSC). In the presence of Ca<sup>2+</sup>, but not Mg<sup>2+</sup>, Sr<sup>2+</sup>, or Ba<sup>2+</sup>, a new high-temperature transition is observed that has been shown to be due to stabilization of the transmembrane region [Lepock, J. R., Rodahl, A. M., Zhang, C., Heynen, M. L., Waters, B., & Cheng, K. H. (1990) *Biochemistry* 29, 681–689]. The maximum stabilization corresponds to a shift in  $T_m$  of 13.8 °C, and Hill analysis indicates that the Ca<sup>2+</sup> binding site yielding stabilization has a  $K_d = 2.5 \times 10^{-4}$  M with a cooperativity ( $n$ ) of 1. Thus, stabilization is due to Ca<sup>2+</sup> binding not to the high-affinity sites but to one of the previously observed sites of low or intermediate affinity, which must be located in the transmembrane or stalk subdomains. Dibucaine has little effect on the  $T_m$  of the aqueous domain, but it decreases the  $T_m$  of the transmembrane domain with  $K_d \cong 4.1 \times 10^{-4}$  M and a cooperativity of approximately 1.6, implying that destabilization is due to the binding of dibucaine to sites of intermediate or moderately high affinity. These sites could be located on the Ca<sup>2+</sup>-ATPase or possibly at the lipid–protein interface. The decrease in  $T_m$  of the transmembrane domain is due to either destabilization of the native state, stabilization of the denatured state, or an increase in the rate of the irreversible step of denaturation. These results using DSC are consistent with the sensitization to thermal inactivation of Ca<sup>2+</sup> uptake by dibucaine but a lack of effect on the thermal inactivation of ATP hydrolysis. Thus, a general site of interaction of local anaesthetics such as dibucaine with integral membrane proteins may be through the transmembrane region.

There are two major models of anaesthetic action. The lipid hypothesis proposes that anaesthetics act by altering the physical properties of the lipid bilayer of cellular membranes (Richards, 1980). A second model, the protein hypothesis, explains anaesthetic action as resulting from a direct interaction of anaesthetic molecules with proteins (Franks & Lieb, 1982). It is generally assumed that either of these initial events leads to an alteration in neuronal ion channel activity, which is supported by observations such as the activation of potassium flow in neurons of *Lymnaea stagnalis* by isoflurane (Franks & Lieb, 1988). Since membrane proteins apparently are involved, it is very difficult, if not impossible, to distinguish lipid from protein effects.

A necessary condition for direct interaction of anaesthetics with proteins is that anaesthetics bind to proteins, which has been shown to occur for a few proteins such as BSA (Ray et al., 1966; Dubois & Evers, 1992) and hemoglobin (Kiebs et al., 1966). More studies exist examining the effects of anaesthetics on protein activity. The best and most extensively studied protein is the water soluble enzyme luciferase (Johnson et al., 1954; Ueda & Kamaya, 1973). Anaesthetics inhibit luciferase activity in a competitive manner by binding to a well-defined site on the enzyme and preventing the binding

of luciferin (Middleton & Smith, 1976). A large number of anaesthetics inhibit luciferase activity at physiologically relevant concentrations, demonstrating that anaesthetics can interact with a soluble protein, but in general a poor correlation exists between anaesthetic potency and degree of inhibition (Curry et al., 1990).

The hydrophobicity of anaesthetics increases their concentration in the vicinity of the transmembrane region of membrane proteins that are known to contain bilayer-spanning helices. Thus, a logical site of action would be transmembrane domains, with the most likely candidates including ion transport proteins such as channels (Franks & Lieb, 1988, 1991). Local anaesthetics have been shown to interact with some membrane proteins. These include the acetylcholine receptor (Blanchard et al., 1979), cytochrome oxidase (Casanova et al., 1985), and the F<sub>1</sub>-ATPase (Kresheck et al., 1985). In addition, it has been shown by differential scanning calorimetry that the denaturation temperature ( $T_m$ ) of the Ca<sup>2+</sup>-ATPase of sarcoplasmic reticulum (SR) is lowered slightly by dibucaine and tetracaine, indicating an interaction between local anaesthetics and this membrane protein (Gutiérrez-Merino et al., 1989). The interaction could be a direct binding of the anaesthetic to the protein or, as previously suggested (Gutiérrez-Merino et al., 1989), a disruption of the lipid annulus. The Ca<sup>2+</sup>-ATPase is a suitable protein to use as a model of the interaction of anaesthetics with membrane proteins since it is an ion-transporting protein, and it contains a number of transmembrane helices, as do ion channels.

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The Ca<sup>2+</sup>-ATPase of SR transports two calcium ions during each kinetic cycle, obtaining energy from the hydrolysis of one ATP. Predictions have been made that the protein is composed of five domains of specific secondary structure (MacLennan et al., 1985; MacLennan, 1990). The ATP binding, phosphorylation, and energy transduction domains are exposed to the aqueous cytoplasm. These three cytoplasmic domains are connected through a stalk domain to the transmembrane domain, which is thought to consist of 10 transmembrane helices. There is evidence that the high-affinity Ca<sup>2+</sup> binding sites are located in the transmembrane domain (Clarke et al., 1989). Ca<sup>2+</sup> transport is associated with a conformational change induced in the protein by the hydrolysis of ATP, and Ca<sup>2+</sup> translocation is thought to occur through a central channel that forms between the transmembrane helices (MacLennan, 1990). The structure of the Ca<sup>2+</sup>-ATPase at 14 Å resolution obtained by cryo-electron microscopy supports this model and indicates that the transmembrane region consists of three segments, one inclined at approximately 40° from vertical (Toyoshima et al., 1993).

The Ca<sup>2+</sup>-ATPase has been shown to consist of two weakly interacting domains by DSC and fluorescence spectroscopy (Lepock et al., 1990). Domain I, the cytoplasmic domain, contains the ATP binding subdomain and denatures or unfolds at 50 °C while domain II, containing the transmembrane subdomain and possibly the stalk subdomain, denatures at 50 °C in EGTA, the same  $T_m$  as for the cytoplasmic domain, but is stabilized by Ca<sup>2+</sup> and denatures at approximately 59 °C in 1 mM Ca<sup>2+</sup>. The stabilization of the transmembrane domain by Ca<sup>2+</sup> allows one to simultaneously determine the interaction of compounds, such as anaesthetics, with both the aqueous, cytoplasmic domain and the bilayer-spanning, transmembrane domain since each domain has a distinct denaturation peak in the DSC profile.

In addition to anaesthesia, local anaesthetics sensitize cells to heat shock (Yatvin, 1977). Since protein denaturation appears to be responsible for heat-induced killing, this suggests that anaesthetics might be able to destabilize proteins and promote their denaturation.

The interaction of the local anaesthetic dibucaine with the Ca<sup>2+</sup>-ATPase was investigated by determining the effect of dibucaine on the  $T_m$ 's of both the cytoplasmic and transmembrane domains by DSC. In addition, the effect of dibucaine on the thermal inactivation of ATP hydrolysis and Ca<sup>2+</sup> transport was measured to investigate the interaction of dibucaine with the regions of the protein responsible for these activities. We show that dibucaine interacts with the transmembrane domain of the Ca<sup>2+</sup>-ATPase and destabilizes this domain, which may be a general mechanism of interaction of anaesthetics with transmembrane proteins, but that dibucaine has little effect on the cytoplasmic domain.

## MATERIALS AND METHODS

**Sarcoplasmic Reticulum Isolation.** Sarcoplasmic reticulum (SR) was isolated from the hind legs and back muscles of New Zealand white rabbits, essentially as described by Eletr and Inesi (1972). The light SR (LSR) fraction was obtained using a sucrose density gradient, as described by Campbell et al. (1980). The purity of the Ca<sup>2+</sup>-ATPase of each isolation of LSR was 85–95%, as determined by SDS-PAGE. The remaining protein was mostly calsequestrin. Protein concentration was measured by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

Dibucaine as the free base was purchased from Sigma Chemical Co., and solutions were prepared by adding the

buffer used for DSC (see below) at low pH, before adjustment to pH 7, to evaporated aliquots of an ethanolic stock solution of dibucaine and heating to about 90 °C to facilitate solubilization. The free calcium concentration was buffered by a CaCl<sub>2</sub>/EGTA solution and determined with the aid of a computer program using previously reported association constants (Fabiato & Fabiato, 1978).

**Differential Scanning Calorimetry.** Differential scanning calorimetry (DSC) was used to measure the transition temperatures ( $T_m$ ) of the two domains of the Ca<sup>2+</sup>-ATPase. The LSR samples were suspended in 10 mM TES, 100 mM KCl, 5 mM DTT, CaCl<sub>2</sub>/EGTA, and 0–4 mM dibucaine at pH 7 and at a protein concentration of 4 mg/mL. DSC scans of specific heat ( $C_p$ ) vs temperature were obtained with a Microcal-2 calorimeter interfaced to a DEC Pro 380 computer. The sample and reference were degassed on ice under vacuum for 5 min before loading the cells (1.21 mL). When equilibration at about 4 °C was reached, the scan was run to 100 °C, the temperature was cooled back to 4 °C, and a rescan was obtained. The scan rate for all scans was 1 °C/min.

The baseline was corrected by first subtracting the rescan, which showed no evidence of reversibility. A second correction for the increase in specific heat ( $\Delta C_p$ ) upon denaturation was made by employing a factor proportional to the extent of the transition, as previously described (Lepock et al., 1990).

The corrected curves were fit by an equation that predicts the shape of the DSC profile, assuming that each transition is two-state  $N \xrightarrow{k} D$ , that the rate constant  $k$  obeys the Arrhenius relation

$$k(T) = e^{A - E_A/RT}$$

and that denaturation is pseudo-first-order

$$\frac{df_d(t)}{dt} = k(T)(1 - f_d(t))$$

where  $E_A$  is the activation energy describing the temperature dependence of  $k$ ,  $A$  is the frequency constant,  $T$  is the temperature,  $f_d$  is the fraction denatured, and  $t$  is the time.  $T$  varies at a constant rate,  $T(t) = T_0 + vt$ , where  $T_0$  is the initial temperature and  $v$  is the scan rate. The resulting equation was solved to obtain an approximate solution for  $f_d$ , as previously described (Lepock et al., 1990).

The excess specific heat is given by

$$C_p(T) = \Delta H_{cal} k(T)(1 - f_d(t))$$

where  $\Delta H_{cal}$  is the apparent calorimetric enthalpy. This equation was used to generate the DSC profile of  $C_p$  vs  $T$ , and the best fit values of  $E_A$ ,  $A$ , and  $\Delta H_{cal}$  were obtained using a Simplex nonlinear curve-fitting algorithm. Denaturation of the Ca<sup>2+</sup>-ATPase is irreversible; thus,  $\Delta H_{cal}$  is just the heat absorbed and cannot be interpreted as the enthalpy of a reversible process. The transition temperature ( $T_m$ ) is defined, as for reversible denaturation, as the temperature at which half of the protein is denatured; however, the meaning of  $T_m$  for an irreversible process is not the same since it is dependent on both temperature and time of exposure and varies as a function of the scan rate (Sanchez-Ruiz et al., 1988).

**Calcium Uptake and ATP Hydrolysis Assays.** Calcium uptake was measured by monitoring the absorbance of arsenazo III at 660 nm, as described by Herbet et al. (1977), in a solution of 100 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM MgSO<sub>4</sub>, 50 μM

CaCl<sub>2</sub>, 0.4 mM ATP, 100 μM arsenazo III, and LSR 14 μg of protein/mL at pH 7. For ATPase activity measurements, the absorbance of NADH at 340 nm was monitored using an ATP-regenerating, coupled enzyme system similar to that described by Warren et al. (1974): 100 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM MgSO<sub>4</sub>, 50 μM CaCl<sub>2</sub>, 1 mM ATP, 125 μg/mL phosphoenol pyruvate, 8.5 μg/mL lactic dehydrogenase, 5 μg/mL pyruvate kinase, 300 μg/mL NADH, and LSR 3–14 μg of protein/mL at pH 7. Ca<sup>2+</sup> uptake and ATPase activity were initiated by adding either ATP or LSR, respectively, and the absorbance was measured as a function of time at 3 s intervals at room temperature (25 °C) using an HP8451 diode array spectrophotometer. Activity was determined from the slope of the linear portion of this curve.

**Thermal Inactivation.** The LSR at 4 mg of protein/mL was heated in the same solution that was used for DSC measurements. Samples were exposed at the elevated temperature (24 to 50 °C, depending on dibucaine concentration) for different time intervals (incubation time), and inactivation was stopped by removing aliquots and adding them to the appropriate activity solution at 20 °C, which immediately diluted the dibucaine by a factor of 100. Activity was measured as indicated above. The rate of inactivation at the elevated temperature ( $k_i$ ) was obtained from a least-squares analysis of the plot of ln(activity) vs time of incubation. An Arrhenius plot of the rate of inactivation was used to obtain the activation energy ( $E_A$ ) and the constant  $A$ , from which the inactivation temperature ( $T_i$ ) was calculated using the same method described above for obtaining  $T_m$  for the denaturation profiles of  $C_p$  vs temperature (Lepock et al., 1990).

**Ca<sup>2+</sup> Leakage.** Ca<sup>2+</sup> leakage at 20 °C was measured after heating or treating LSR with dibucaine, followed by passively loading the LSR with <sup>45</sup>Ca essentially as described by Meissner et al. (1986). LSR at 4 mg/mL was either heated at 45 °C in inactivation buffer for 195 s, treated with 1 mM dibucaine, or treated with 1 mM dibucaine followed by heating at 45 °C for 195 s. The LSR was concentrated to 10 mg/mL by centrifugation followed by resuspension in loading medium (1 mM <sup>45</sup>CaCl<sub>2</sub>, 100 mM KCl, and 10 mM TES at pH 7.0) containing no dibucaine. Calcium efflux was determined by measuring the calcium retained as a function of time after dilution (1:100) into release medium (100 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, and 10 mM TES at pH 7.0) at room temperature. Aliquots of 1 mL were removed as a function of time and filtered through 0.45-μm HAWP Millipore filters. The filters were washed with 10 mL of stop medium (release medium containing 10 μM Ruthenium Red) and counted in CytoScint (ICN). The decay curves of Ca<sup>2+</sup> retained as a function of time were fit with the multiexponential function  $A(t) = A_1e^{-k_1t} + A_2e^{-k_2t} + A_0$ , where  $A_1$  and  $A_2$  are fractional components with rate constants of leakages  $k_1$  and  $k_2$ , respectively.  $A_0$  is a minor component with a leakage rate too small to be measured over the time course of the experiment (10 min).

## RESULTS

**Differential Scanning Calorimetry of the Ca<sup>2+</sup>-ATPase.** A corrected DSC scan of sarcoplasmic reticulum membranes (LSR fraction) in the solution given in the Materials and Methods section containing 1 mM free Ca<sup>2+</sup> is shown in Figure 1. This scan is representative of those obtained from different isolations. Two major endothermic peaks are observed in 1 mM Ca<sup>2+</sup>, one centered at about 50 °C and a second peak centered at about 59 °C. Denaturation is highly irreversible, and it has not been possible to obtain any evidence for the

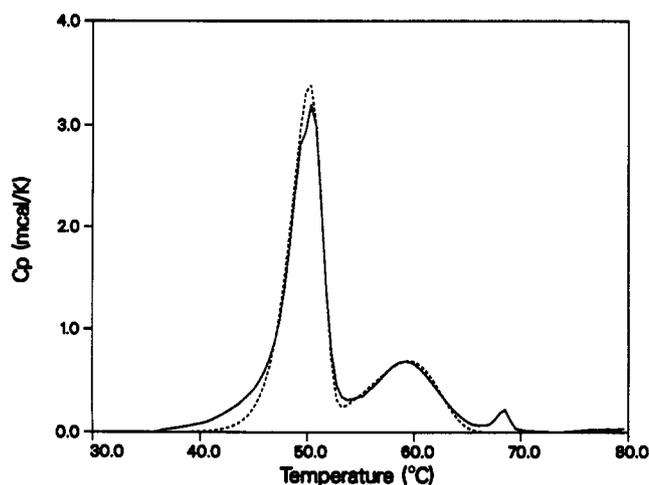


FIGURE 1: DSC profile (excess  $C_p$  vs temperature) of LSR (4.8 mg of protein) in 1 mM Ca<sup>2+</sup> with the baseline corrected. The dotted line represents the best fit assuming two irreversible transitions with  $T_m$ 's of 49.8 and 58.6 °C.

existence of a reversibly denatured fraction or intermediate. The baseline was corrected and the best fit for the two transitions obtained as described in the Materials and Methods. The third peak at 68 °C in Figure 1 is not present in all preparations, and its identity is unknown. The first peak ( $T_m = 50$  °C) is perturbed by an exothermic transition, usually causing a doublet appearance as shown in Figures 2, 4, 5, and 6 or occasionally a distortion in the region of maximum  $C_p$  as shown in Figure 1. There is considerable evidence that this is an exotherm and that the first peak does not represent two closely spaced transitions (Lepock et al., 1990). The aggregation of the unfolded Ca<sup>2+</sup>-ATPase is probably involved in the exothermic process (Gutierrez-Merino et al., 1988). The portion of the curve of excess  $C_p$  vs temperature corresponding to the exotherm was not used in curve fitting, and all calculations of transition temperatures were made from the fitted curves, not the original scans.

These results indicate that the Ca<sup>2+</sup>-ATPase denatures through two steps in 1 mM Ca<sup>2+</sup>. Two explanations are possible: (a) the protein undergoes two sequential conformational changes or (b) two discrete domains unfold independently. Through DSC measurements alone it is not possible to distinguish between these possibilities since denaturation is irreversible. Site specific fluorescence studies have permitted identification of these transitions as the denaturation of two independent domains: domain I ( $T_m = 50$  °C), which includes the ATP binding subdomain and probably the phosphorylation and transduction subdomains, and domain II ( $T_m = 59$  °C), which includes the transmembrane subdomain, containing the high-affinity Ca<sup>2+</sup> binding sites and probably the stalk subdomain (Lepock et al., 1990). Domain I can be specifically probed through the fluorescence properties of FITC-labeled Ca<sup>2+</sup>-ATPase and domain II by the measurement of intrinsic protein fluorescence (Trp residues). In the presence of Ca<sup>2+</sup>, each of these domains can be monitored using DSC alone.

**Stabilization of the Transmembrane Domain by Ca<sup>2+</sup>.** DSC scans of the Ca<sup>2+</sup>-ATPase at different free calcium concentrations are shown in Figure 2. The presence of Ca<sup>2+</sup> preferentially stabilizes domain II, whose transition temperature increases with increasing calcium concentration. The height of the first peak varies considerably, primarily due to slight shifts in the location of the exotherm, making an accurate determination of  $T_m$  from the original scan difficult. Each peak was fit as shown in Figure 1, and the best fit curves were used to determine the activation energy ( $E_A$ ),  $A$ , apparent

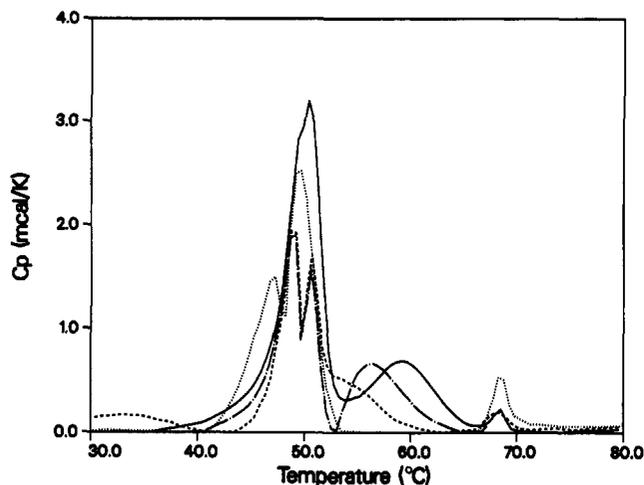


FIGURE 2: Effect of  $\text{Ca}^{2+}$  on the DSC profiles (excess  $C_p$  vs temperature) of the  $\text{Ca}^{2+}$ -ATPase of LSR. The scans (corrected for the baseline) correspond to the following free calcium concentrations (mM): 0 (---), 0.1 (---), 0.32 (-·-·), and 1 (—).

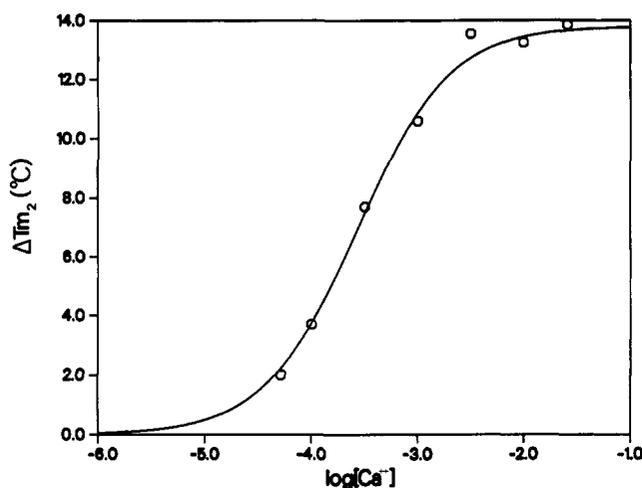


FIGURE 3: Dependence of the shift in the denaturation temperature ( $\Delta T_{m2}$ ) on the concentration of free  $\text{Ca}^{2+}$ . The solid line represents the best fit to the Hill equation, with the parameters  $pK_d = 3.6$ ,  $n = 1.0$ , and  $\Delta T_{m2}^{\text{max}} = 13.8$  °C.

$\Delta H_{\text{cal}}$ , and  $T_m$ . Between 0 and 10 mM  $\text{Ca}^{2+}$  there is no significant change in  $E_A$  ( $587 \pm 19$  kJ/mol) for peak 1. The value of  $T_{m1}$  increases slightly from  $48.8 \pm 0.1$  °C to  $49.9 \pm 0.2$  °C.  $\text{Ca}^{2+}$  also has no effect on the  $E_A$  of peak 2 ( $301 \pm 11$  kJ/mol); however, there is a large increase in  $T_{m2}$  from  $48.2 \pm 0.2$  to  $61.7 \pm 0.6$  °C. The apparent calorimetric enthalpies also remain constant at  $11.3 \pm 1.0$  and  $4.7 \pm 0.9$  J/g for peaks 1 and 2, respectively.

The apparent dissociation constant ( $K_d$ ) for  $\text{Ca}^{2+}$  binding was estimated from the plot of  $\Delta T_{m2}$  as a function of free  $\text{Ca}^{2+}$  concentration, where  $\Delta T_{m2}$  is the shift in the temperature of denaturation of domain II.  $\Delta T_{m2}$  was calculated taking the  $T_m$  in the absence of  $\text{Ca}^{2+}$  (1 mM EGTA) as the reference. A form of the Hill equation

$$\Delta T_{m2} = \frac{\Delta T_{m2}^{\text{max}} [\text{Ca}^{2+}]^n}{(K_d)^n + [\text{Ca}^{2+}]^n}$$

where  $n$  is the degree of cooperativity and  $\Delta T_{m2}^{\text{max}}$  is the maximum temperature shift, was fit to the experimental data using the Simplex nonlinear curve-fitting algorithm, as was done for fitting the DSC curves. Figure 3 shows the best fit to the average values of the experimental results of  $\Delta T_{m2}$

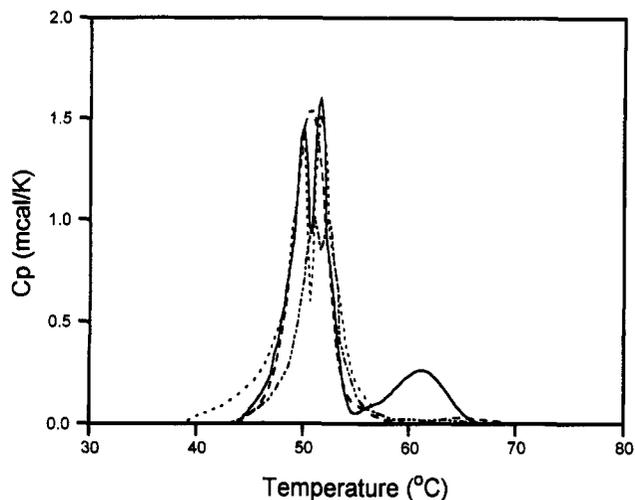


FIGURE 4: DSC profiles (excess  $C_p$  vs temperature) of LSR (4.8 mg of protein) in 1 mM  $\text{CaCl}_2$  (—), 1 mM  $\text{MgCl}_2$  (---), 1 mM  $\text{SrCl}_2$  (---), and 1 mM  $\text{BaCl}_2$  (-·-·).

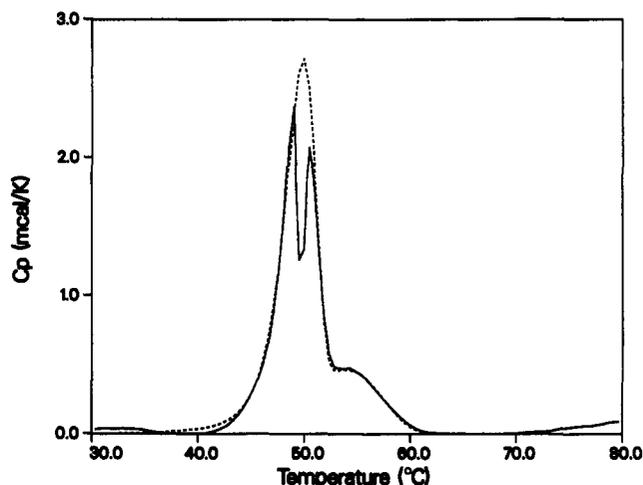


FIGURE 5: DSC profile (excess  $C_p$  vs temperature) of LSR (4.8 mg of protein) in 1 mM  $\text{Ca}^{2+}$  and 0.5 mM dibucaine. The dotted line represents the best fit to the experimental curve, assuming two irreversible transitions with  $T_m$ 's of 49.4 and 52.8 °C.

obtained from different preparations of LSR. This fit gives a value of 1.0 for the coefficient  $n$  of the Hill equation, indicating an absence of cooperativity, and an apparent dissociation constant  $K_d$  of  $2.5 \times 10^{-4}$  M ( $pK_d = 3.6$ ).

The stabilization of domain II is specific for  $\text{Ca}^{2+}$ . No stabilization is detected in the presence of 1 mM  $\text{MgCl}_2$ ,  $\text{SrCl}_2$ , or  $\text{BaCl}_2$  (Figure 4).

**Destabilization of the Transmembrane Domain by Dibucaine.** Figure 5 shows a typical scan and the best fit for a sample of LSR in 1 mM  $\text{Ca}^{2+}$  and 0.5 mM dibucaine. Dibucaine shifts the  $T_m$  of both transitions to lower values; however, the  $T_m$  of domain II, containing the transmembrane domain, is much more sensitive to dibucaine. Concentrations in excess of 1 mM are required to significantly lower  $T_{m1}$ , while  $T_{m2}$  is lowered 6.5 °C by 0.5 mM dibucaine.

The concentration of  $\text{Ca}^{2+}$  (1 mM) used is higher than that in most cells, but under these conditions domain II is stabilized and consequently the transitions of the two domains in the  $\text{Ca}^{2+}$ -ATPase are resolved, while in the absence of  $\text{Ca}^{2+}$  both transitions would be superimposed and the protein would appear to denature as a single unit. Thus, in this way it is possible to determine which domain of the protein is preferentially affected by the presence of dibucaine.

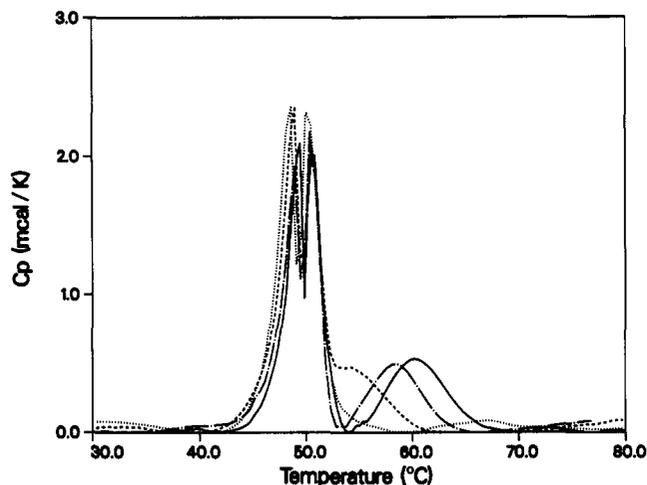


FIGURE 6: Effect of dibucaine on the DSC profiles (excess  $C_p$  vs temperature) of the Ca<sup>2+</sup>-ATPase of LSR in 1 mM Ca<sup>2+</sup>. The scans (corrected for the baseline) correspond to the following dibucaine concentrations (mM): 0 (—), 0.125 (---), 0.5 (· · ·), and 1 (- · - ·).

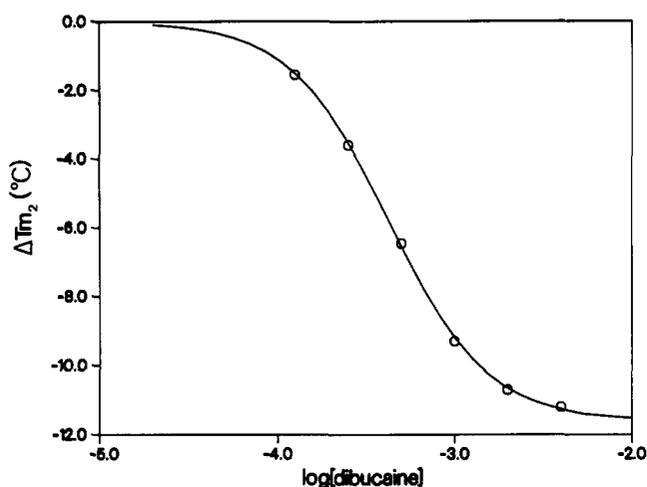


FIGURE 7: Dependence of the shift in the denaturation temperature ( $\Delta T_{m2}$ ) on the concentration of dibucaine. The solid line represents the best fit to the Hill equation, with the parameters  $pK_d = 3.4$ ,  $n = 1.6$ , and  $\Delta T_{m2}^{max} = -11.6$  °C.

DSC scans of LSR in 1 mM Ca<sup>2+</sup> with the baseline corrected at different dibucaine concentrations are shown in Figure 6. As in the case of Ca<sup>2+</sup>, dibucaine causes no significant change in the parameters  $E_A$ ,  $A$ , and the apparent calorimetric enthalpy ( $\Delta H_{cal}$ ) for either transition. These parameters were determined as before from the best fits for each peak (not shown).

Figure 7 gives the dependence of the shift in the denaturation temperature  $T_{m2}$  ( $\Delta T_{m2}$ ) as a function of dibucaine concentration. Since dibucaine is adsorbed by sarcoplasmic reticulum membranes (Volpe et al., 1983; Gutierrez-Merino et al., 1989), for further interpretation of this curve, the free concentration of the drug must be calculated by using the partition coefficient ( $K_p$ ) between the aqueous phase and the sarcoplasmic reticulum membranes. Moreover, under the experimental conditions used (pH 7), dibucaine exists in the neutral and cationic forms since its  $pK_a$  in aqueous solution is approximately 8.5 (Volpe et al., 1983). Most reported values of the partition coefficient  $K_p$  are for pure lipid membranes (Papahadjopoulos et al., 1975; Volpe et al., 1983). In SR membranes it may be lower due to the presence of proteins that can make membrane lipids less accessible. A value of  $K_p = 0.75$  has been reported (Gutierrez-Merino et al., 1989) for dibucaine in a similar buffer at similar ionic strength (0.1

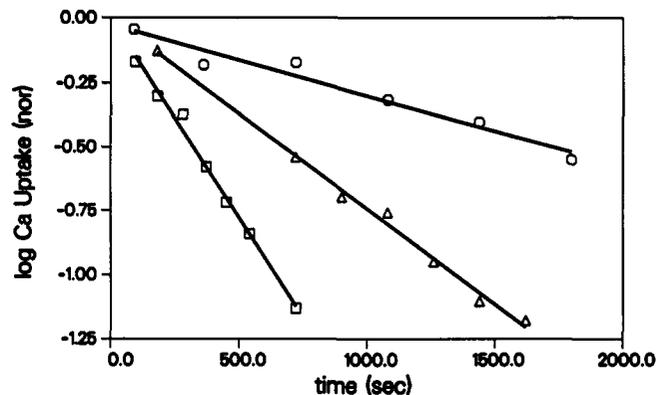


FIGURE 8: Thermal inactivation of Ca<sup>2+</sup> uptake of LSR in 1 mM Ca<sup>2+</sup> and 0, 0.5, and 1.0 mM dibucaine. The normalized rate of Ca<sup>2+</sup> uptake at 20 °C is plotted following exposure to the elevated temperature for the time indicated. The symbols correspond to the following dibucaine concentrations and exposure temperatures: control, 47 °C (○); 0.5 mM, 47 °C (Δ); and 1 mM, 45 °C (□).

M) and at a pH of about 7. This value was constant in the range from 0 to 6.5 mg of protein/mL, up to a total anaesthetic concentration of 1 mM.

The apparent dissociation constant  $K_d$  for dibucaine binding and the degree of cooperativity can be determined following the same procedure used earlier for Ca<sup>2+</sup> binding, but now taking into account the partition coefficient of the drug ( $K_p = 0.75$ ). For calculating the free dibucaine concentration,  $K_p$  was considered constant up to 4 mM dibucaine. Even if  $K_p$  is lower for such high concentrations due to saturation, there is not a substantial difference in the fitting results since the dependence of  $T_{m2}$  upon dibucaine concentration also shows saturation at that level. The best fit to the Hill equation gives an apparent dissociation constant of  $4.1 \times 10^{-4}$  M ( $pK_d = 3.4$ ) and  $n = 1.6$ . However, these values are dependent on the value of  $K_p$ . If  $K_p$  is constant in the whole range below saturation,  $n$  will remain the same and  $pK_d$  will be larger for larger values of  $K_p$ , but if  $K_p$  is not constant in that range, then both values will be altered slightly.

**Potential by Dibucaine of the Thermal Inactivation of Ca<sup>2+</sup> Uptake and ATPase Activity.** Evidence of the site of interaction of dibucaine with the Ca<sup>2+</sup>-ATPase can also be obtained from a determination of the sensitivity to thermal inactivation of Ca<sup>2+</sup> uptake and ATPase activity since these activities can be uncoupled from one another, implying that at least one component of the mechanism required for Ca<sup>2+</sup> translocation resides in a part of the protein distinct from the ATP binding subdomain (McIntosh & Berman, 1978; Lepock et al., 1990; Cheng & Lepock, 1992). Thermal inactivation of Ca<sup>2+</sup> uptake and ATPase activity in the presence of 1 mM Ca<sup>2+</sup> at different dibucaine concentrations was determined by measuring activity at 20 °C after exposure to inactivating temperatures as a function of time. Representative curves of the thermal inactivation of Ca<sup>2+</sup> uptake of LSR in 1 mM Ca<sup>2+</sup> are shown in Figure 8. Dibucaine increases the rate of thermal inactivation of Ca<sup>2+</sup> uptake by a considerable extent. The inactivation curve in 1 mM dibucaine shown in Figure 8 was obtained at 45 °C since inactivation was too rapid at 47 °C, the temperature used for the control, to measure accurately. The inactivation rates ( $k_i$ ) were obtained from curves of this type by linear regression analysis. Arrhenius plots of the rates of inactivation of Ca<sup>2+</sup> uptake for different dibucaine concentrations (0, 0.5, 1.0, and 2.0 mM) are shown in Figure 9. Similar measurements were also made of the inactivation of ATPase activity as a function of dibucaine concentration.

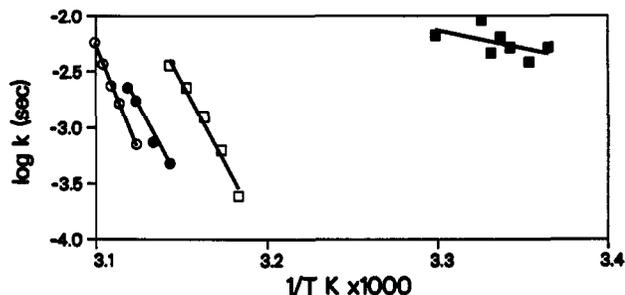


FIGURE 9: Arrhenius plots of the rate of inactivation ( $k_i$ ) of  $\text{Ca}^{2+}$  uptake at different dibucaine concentrations. The symbols correspond to control (O), 0.5 mM (●), 1 mM (□), and 2 mM (■) dibucaine.

The values of  $E_A$  obtained by linear regression analysis of the Arrhenius plots for inactivation of  $\text{Ca}^{2+}$  uptake and ATPase activity are given in Table 1. Dibucaine shifts the Arrhenius plots to the right (Figure 9), implying sensitization of  $\text{Ca}^{2+}$  uptake to thermal inactivation. Up to 1 mM dibucaine there is little change in  $E_A$ , indicating that the mechanism of inactivation is the same for heating in the absence of dibucaine as well as in the presence of low dibucaine concentrations. However, at 2 mM dibucaine  $E_A$  drops from approximately 600 to 62 kJ/mol, indicating a change in the mechanism of inactivation.

An inactivation temperature, corresponding to a transition temperature, can be calculated from the Arrhenius parameters  $E_A$  and  $A$  (Lepock et al., 1990).  $T_i$  is defined as the temperature at which half of the activity is lost if the temperature were to be increased at a constant rate, in this case 1 °C/min. If inactivation is caused by denaturation, then  $T_i$  and  $T_m$  should be identical. The values of  $T_i$  for inactivation of  $\text{Ca}^{2+}$  uptake and ATPase activity in the presence of dibucaine are given in Table 1.

Dibucaine has little effect on the thermal inactivation of ATPase activity (Table 1). There is no change in  $E_A$ , and only slight decreases in  $T_i$  at 1 and 2 mM dibucaine of  $0.8 \pm 0.6$  and  $1.6 \pm 0.7$  °C, respectively. This corresponds to the decrease in the denaturation temperature ( $T_{m1}$ ) of domain I. Thus, there is only a weak interaction with the cytoplasmic domain, which contains the site for ATP hydrolysis. In contrast, dibucaine has a much larger effect on the  $T_i$  for  $\text{Ca}^{2+}$  uptake, which is decreased by  $4.1 \pm 0.9$  °C at 1 mM dibucaine. This indicates that the combination of dibucaine and high temperature induces an irreversible change in the SR that inhibits the uptake of  $\text{Ca}^{2+}$ .

How does dibucaine inhibit  $\text{Ca}^{2+}$  uptake? When dibucaine is present in the uptake solution,  $\text{Ca}^{2+}$  uptake is inhibited, with half-inhibition at approximately 0.7 mM and complete inhibition at 1 mM (Figure 10). Inhibition of  $\text{Ca}^{2+}$  uptake in the presence of dibucaine has been observed previously at these same concentrations (de Boland et al., 1975; Nash-Adler et al., 1980). At 20 °C, inhibition of  $\text{Ca}^{2+}$  uptake is reversible. If the SR is incubated in the presence of dibucaine but then added to uptake solution lacking dibucaine, there is no inhibition of  $\text{Ca}^{2+}$  uptake (Figure 10). This corresponds to how the experiments summarized in Figure 9 and Table 1 were done.

$\text{Ca}^{2+}$  efflux increases in the presence of dibucaine, and this apparently is the mechanism of inhibition of uptake (de Boland et al., 1975; Nash-Adler et al., 1980). However, when SR is heated in the presence of dibucaine, inhibition is irreversible and is not reversed when dibucaine is removed.

The effect of 1 mM dibucaine and heating at 45 °C on  $\text{Ca}^{2+}$  permeability was determined by treating with dibucaine and heat, washing by centrifugation to remove the dibucaine to

correspond to the  $\text{Ca}^{2+}$  uptake experiments, and passively loading the SR vesicles with  $^{45}\text{Ca}$ . Calcium leakage in EGTA was then monitored as a function of time (Figure 11). The release curves can be fit with a double exponential, giving release rates of  $k_1 = 1.04 \pm 0.26 \text{ min}^{-1}$  and  $k_2 = 0.26 \pm 0.05 \text{ min}^{-1}$  (three experiments). These are similar to values previously obtained (Lepock et al., 1990). Neither treatment with 1 mM dibucaine at room temperature nor heating at 45 °C for 195 s significantly affected the rate of leakage. However, heating for 195 s at 45 °C after treatment with 1 mM dibucaine decreased the initial level of uptake by a factor of 2 (approximately from 12 to 5 nmol of  $\text{Ca}/\text{mg}$  of protein), increased  $k_1$  by a factor of 2 to  $2.10 \pm 0.30 \text{ min}^{-1}$  but had no effect on  $k_2$  ( $0.30 \pm 0.09 \text{ min}^{-1}$ ). Since the SR is passively loaded, the initial level of uptake must be the same for each sample, indicating that there must be increased leakage from the SR heated in dibucaine that is too rapid to measure by the slow filtration method. Thus, treatment with 1 mM dibucaine alone has no effect on  $\text{Ca}^{2+}$  leakage if the dibucaine is removed or diluted out, but heating in the presence of dibucaine causes an irreversible change in the SR leading to increased  $\text{Ca}^{2+}$  leakage.

Leakage is not caused by interaction of dibucaine with the cytoplasmic domain of the  $\text{Ca}^{2+}$ -ATPase since ATPase activity is unaffected, but must occur because of interactions deeper within the membrane. There are two possible modes of leakage: (1) through the lipid bilayer or (2) through the protein or at the protein-lipid interface.

Increased  $\text{Ca}^{2+}$  efflux after heating in the presence of dibucaine does not appear to be due simply to an increase in the passive permeability of the lipid bilayer. Efflux from SR is several orders of magnitude faster than that from dibucaine-treated liposomes made from SR lipids (de Boland et al., 1975). In addition, the activation energy for efflux from liposomes is approximately 9.7 kcal/mol (de Boland et al., 1975), compared to the activation energy of 150 kcal/mol (640 kJ/mol) for inactivation of  $\text{Ca}^{2+}$  uptake in SR. This indicates that  $\text{Ca}^{2+}$  efflux through the lipid bilayer is not the rate-limiting step for uptake following heat inactivation in the presence of dibucaine.

At 2 mM dibucaine there is a dramatic decrease in  $T_i$  for  $\text{Ca}^{2+}$  uptake, implying much greater sensitization, and a large decrease in the  $E_A$  for inactivation. This implies a change in the mechanism of inactivation, consistent with an increase in the permeability of the lipid bilayer limiting uptake at higher dibucaine concentrations.

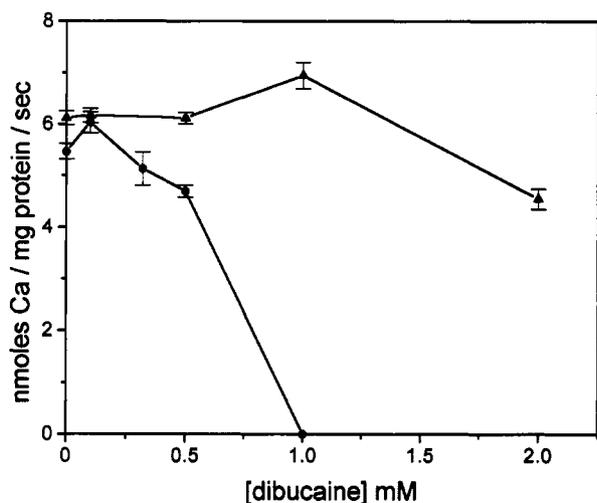
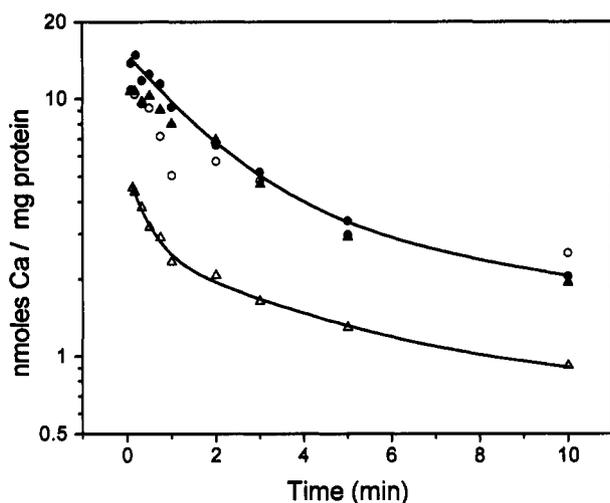
Thus, dibucaine-potentiated inactivation of  $\text{Ca}^{2+}$  uptake at dibucaine concentrations of 1 mM or less appears to require protein components of the SR, suggesting the possibility of an irreversible conformational change in the  $\text{Ca}^{2+}$ -ATPase resulting in efflux through the protein or at the lipid-protein interface. There is no straight forward way of distinguishing between these two modes of leakage; however, both imply an interaction of dibucaine at the transmembrane level.

## DISCUSSION

The interaction of dibucaine with the  $\text{Ca}^{2+}$ -ATPase of rabbit sarcoplasmic reticulum has been investigated by determining its effect on the thermal denaturation of this membrane protein. The denaturation of the  $\text{Ca}^{2+}$ -ATPase has been studied previously by DSC, several site specific fluorescent probes, and inactivation of both ATPase activity and  $\text{Ca}^{2+}$  uptake. In the absence of  $\text{Ca}^{2+}$ , there is only one transition detectable by DSC, with  $T_m \approx 49$  °C (Lepock et al., 1990). This is consistent with the transition at 45–55 °C detected by Gutierrez-Merino

Table 1: Activation Energies ( $E_A$ ) and Inactivation Temperatures ( $T_i$ ) for the Thermal Inactivation of the Ca<sup>2+</sup>-ATPase and the Transition Temperature ( $T_{m1}$ ) of Domain I in the Presence of Dibucaine

dibucaine (mM)	Ca <sup>2+</sup> uptake		ATPase activity		DSC $T_{m1}$ (°C)
	$E_A$ (kJ/mol)	$T_i$ (°C)	$E_A$ (kJ/mol)	$T_i$ (°C)	
0	685 ± 58	49.8 ± 0.6	637 ± 35	51.2 ± 0.4	50.2 ± 0.3
0.5	532 ± 76	49.5 ± 0.8	561 ± 93	51.3 ± 1.0	49.8 ± 0.6
1	554 ± 65	45.7 ± 0.7	632 ± 41	50.4 ± 0.4	49.9 ± 0.5
2	62 ± 15	9.2 ± 3.1	665 ± 49	48.6 ± 0.5	49.0 ± 0.2

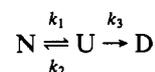
FIGURE 10: Rates of Ca<sup>2+</sup> uptake of LSR following treatment with dibucaine for 195 s at 20 °C with (●) and without (▲) dibucaine in the uptake solution.FIGURE 11: Ca<sup>2+</sup> efflux from LSR in 1 mM EGTA as a function of time at 20 °C. The lines are the best fit curves, and the symbols represent the experimental points. The conditions are as follows: circles, no dibucaine; triangles, 1 mM dibucaine; closed symbols, 20 °C for 195 s; open symbols, 45 °C for 195 s.

et al. (1989) and appears to be the main denaturation event; the inactivation of ATPase activity occurs with the same  $T_i$ . However, Ca<sup>2+</sup> uptake in EGTA is inhibited at a much lower temperature,  $T_i \approx 37$  °C, and correlates with an irreversible conformational change detectable with a fluorescent analog of dicyclohexylcarbodiimide [*N*-cyclohexyl-*N'*-[4-(dimethylamino)- $\alpha$ -naphthyl]carbodiimide (NCD-4)] that is thought to label the region of the Ca<sup>2+</sup> binding sites (Cheng & Lepock, 1992). The identity of the conformational change at  $T_m \approx 37$  °C is unknown.

The Ca<sup>2+</sup>-ATPase is stabilized against the thermal inactivation of Ca<sup>2+</sup> uptake in the presence of Ca<sup>2+</sup> (McIntosh & Berman, 1978). In addition, Ca<sup>2+</sup> stabilizes the Ca<sup>2+</sup>-ATPase

against thermal denaturation. Two transitions are detected in 1 mM Ca<sup>2+</sup> by DSC: one at  $T_m \approx 50$  °C, which includes denaturation of the ATP binding domain, and another at  $T_m \approx 58$  °C, which includes the transmembrane domain (Lepock et al., 1990). Inactivation of Ca<sup>2+</sup> uptake is dramatically stabilized in Ca<sup>2+</sup> and occurs simultaneously with the inactivation of ATPase activity, with a  $T_i$  equal to that of the  $T_m$  for the denaturation of domain I ( $\sim 50$  °C).

All three detectable transitions in the Ca<sup>2+</sup>-ATPase are highly irreversible and have been modeled as  $N \xrightarrow{k} D$ , where N is the native state, D the denatured or inactive state, and  $k$  the rate constant for denaturation (Lepock et al., 1990; Cheng & Lepock, 1992). However, the native state for most proteins is thought to be the lowest energy state, suggesting that denaturation should be reversible in the absence of a subsequent irreversible process. Thus,



where U is a reversibly unfolded or denatured state, should be a more accurate model (Lepock et al., 1992). The rate constant  $k_3$  then gives the rate of the irreversible process.

Ca<sup>2+</sup> stabilizes the Ca<sup>2+</sup>-ATPase, but denaturation is still irreversible. The denaturation of the transmembrane subdomain becomes resolvable by DSC due to the shift in  $T_{m2}$  to higher temperatures, which is dependent on Ca<sup>2+</sup> concentration with a  $pK_d$  of 3.6 and a cooperativity ( $n$ ) of 1, suggesting that the non-cooperative binding of Ca<sup>2+</sup> is sufficient for stabilization of the transmembrane subdomain.

Various classes of binding sites for Ca<sup>2+</sup> are present on the Ca<sup>2+</sup>-ATPase of SR characterized by affinity, capacity, degree of cooperativity, and specificity. High-affinity sites with  $pK_d$ 's ranging between 6.3 and 6.9 are highly specific for Ca<sup>2+</sup> and exhibit cooperative binding with  $n$  approximately 2 (Watanabe et al., 1981; Meltzer & Berman, 1984). Additional sites of intermediate and low affinity are also present in the protein. Binding to these sites is non-cooperative, with  $pK_d$  values ranging from 4.3–4.8 for intermediate sites to approximately 3 for low-affinity sites (Watanabe et al., 1981; Inesi & Kurzmack, 1983). The latter are nonspecific, and Ca<sup>2+</sup> binding can be blocked by MgCl<sub>2</sub> and KCl. Moreover, binding to high-affinity sites activates ATP hydrolysis, while binding to low-affinity sites shields inhibition of the ATPase. The intermediate sites are apparently unrelated to the regulation of enzyme activity.

The absence of cooperativity and the  $pK_d$  value of 3.6 estimated for Ca<sup>2+</sup> binding from the shift in  $T_{m2}$  suggest that Ca<sup>2+</sup> stabilization of the transmembrane domain of the Ca<sup>2+</sup>-ATPase is achieved through binding to non-cooperative low- or intermediate-affinity sites. These sites must be located in the transmembrane or stalk subdomains, which contain numerous Asp and Glu residues that are usually involved in Ca<sup>2+</sup> binding (MacLennan et al., 1985; Clarke et al., 1989).

Dibucaine has little effect upon the aqueous ATP binding domain, as determined by either DSC or inactivation of

ATPase activity, but lowers  $T_{m2}$  with a  $pK_d$  of approximately 3.4 and a cooperativity of approximately 1.6. Destabilization of a protein or a protein domain as detectable by a decrease in  $T_m$  could occur by three general mechanisms: (1) a destabilization (decrease in the free energy  $\Delta G$ ) of the native state N, (2) a stabilization of the reversibly unfolded or denatured state U, or (3) an increase in the rate constant  $k_3$  for the irreversible step in the three-state scheme of irreversible denaturation shown earlier.

Binding of a ligand to a protein usually stabilizes rather than destabilizes the native state (Brandts et al., 1989). However, either transformation to a less stable state or disruption of stabilizing interactions (e.g., lipid-protein interactions) could lead to a decrease in  $T_m$  through destabilization of the native state.

Tighter binding of dibucaine to the reversibly denatured compared to the native state would reduce  $T_{m2}$  by stabilizing the denatured state. In general, it is difficult to distinguish between this mechanism and destabilization of the native state. In addition, a quantitative analysis of this possibility is complicated by the additional step required to account for the irreversibility of denaturation. However, this introduces a third possibility: a decrease in the apparent  $T_m$  of an irreversible process by increasing the rate ( $k_3$ ) of the irreversible step. Simulations have shown that this can lead to a reduction in  $T_m$  of several degrees, depending upon the relative values of the rates of reversible unfolding ( $k_1$ ) and the irreversible step ( $k_3$ ) (Lepock et al., 1992).

Dibucaine also increases the rate of thermal inactivation of  $Ca^{2+}$  uptake and increases  $Ca^{2+}$  efflux, but has little effect on the thermal inactivation of ATPase activity. This supports the DSC evidence that dibucaine interacts at the bilayer or the transmembrane level, either directly with the  $Ca^{2+}$ -ATPase or with annular lipids surrounding the protein (Gutierrez-Merino et al., 1989).

From these experiments, it is not possible to identify the specific mechanism of destabilization; however, these results indicate a much stronger interaction of dibucaine with the transmembrane domain rather than with the aqueous regions of the protein. The observation by Gutierrez-Merino et al. (1989) that tetracaine also lowers the overall  $T_m$  of the  $Ca^{2+}$ -ATPase of SR, although they were not able to distinguish the separate domains, indicates that other local anaesthetics interact with the  $Ca^{2+}$ -ATPase, possibly through the transmembrane domain.

Local anaesthetics are also very potent sensitizers of cells to heat shock or hyperthermia (Yatvin, 1977), and they induce heat shock protein synthesis (Nover, 1991). This has been interpreted as evidence for the involvement of membrane targets in heat killing, which is also supported by the reduction in hyperthermic killing by high pressure (Minton et al., 1980). The effect of dibucaine on the  $Ca^{2+}$ -ATPase suggests that local anaesthetics may potentiate hyperthermic killing and induce heat shock protein synthesis by sensitizing transmembrane proteins to thermal denaturation.

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