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Biochimica et Biophysica Acta 1233 (1995) 7–13

BBA Biochimica et Biophysica Acta

Use of thermal analysis to distinguish magnesium and calcium stimulated ATPase activity in isolated transverse tubules from skeletal muscle

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Received 23 February 1994; accepted 25 September 1994

Abstract

The presence of calcium stimulated adenosine triphosphatase (Ca^{2+},Mg^{2+}-ATPase) activity in isolated transverse tubule (t-tubule) membranes is distinguished from magnesium adenosine triphosphatase (Mg^{2+}-ATPase) activity on the basis of differing thermal stabilities. The Mg^{2+}-ATPase is the major protein component of the t-tubule membrane, and it can be difficult to discriminate between the low levels of Ca^{2+} stimulated ATPase activity found in isolates of t-tubules compared to the much higher Mg^{2+}-ATPase activity. Thermal analysis reveals different inactivation temperatures (T_i) for the proteins responsible for ATP dependent calcium transport ($T_i = 49°C$) and Mg^{2+}-ATPase activity ($T_i = 57°C$) in isolated t-tubule membranes. The differential scanning calorimetry profile of t-tubule membranes consists of three major components with transition temperatures (T_m) of 51°C, 57°C and 63°C. Denaturation of the component with $T_m = 57°C$ correlates with inactivation of Mg^{2+}-ATPase activity, and denaturation of the $T_m = 51°C$ component correlates with the inactivation of Ca^{2+},Mg^{2+}-ATPase activity and calcium transport. The functions of the t-tubule membrane component or components that denature with $T_m = 63°C$ have yet to be identified. The lack of stimulation of calcium transport in isolated t-tubules by oxalate, the impermeability of isolated t-tubules to oxalate, and experiments performed on t-tubules with defined amounts of sarcoplasmic reticulum (SR) added suggest that contamination of the isolated t-tubules by SR is unlikely to account for the level of Ca^{2+},Mg^{2+}-ATPase activity detected. The presence of a Ca^{2+},Mg^{2+}-ATPase in the t-tubule membrane would provide a mechanism that may be involved in the partial removal of calcium that is accumulated in the junctional space during muscle relaxation or calcium that is released from the terminal cisternae of sarcoplasmic reticulum during excitation-contraction coupling.

Keywords: ATPase, Ca^{2+},Mg^{2+}-; ATPase, Mg^{2+}-; t-Tubule; Skeletal muscle; DSC

1. Introduction

Transverse tubule (t-tubule) membranes from skeletal muscle are invaginations of the cell membrane (sarcolemma (SL)) which act as a conduit for the muscle action potential to the fiber interior and serve as the site for coupling the action potential to the rapid release of Ca^{2+} from the terminal cisternae of the sarcoplasmic reticulum (SR). The receptor for the Ca^{2+} blocker dihydropyridine (DHP) is enriched 50-fold in the t-tubule membrane with respect to SL [1]. The DHP receptor acts as a voltage dependent Ca^{2+} channel in other cellular systems, but Ca^{2+} influx is not necessary for contraction in skeletal muscle [2]. However, it has been shown that DHP receptors purified from the t-tubule membrane from skeletal muscle and reconstituted into phospholipid vesicles give functional Ca^{2+} channels with appropriate pharmacological properties [3]. Although the receptors are voltage sensitive, only a few percent are activated during voltage clamp stimuli and function as Ca^{2+} channels [4].

There is evidence that some Ca^{2+} influx occurs during contraction of skeletal muscle [2,5]. In addition, Dunn [6] obtained a voltage dependent Ca^{2+} efflux from highly purified inside out t-tubule vesicles which was modulated by Ca^{2+} channel blockers and activators. The function of any Ca^{2+} influx is unknown, but Ca^{2+} homeostasis requires a mechanism of active transport out of the cell. Several studies suggest that ATP dependent and other Ca^{2+} translocation mechanisms are present in the SL [7–9]

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SSDI 0005-2736(94)00243-6

and the t-tubule membrane [10–12]. These may be involved in the regulation of intracellular Ca^{2+}.

A major problem in studying calcium transport and in measuring possible Ca^{2+},Mg^{2+}-ATPase activity in the t-tubule membrane is the large enzymatic activity of the Mg^{2+}-ATPase compared to the small Ca^{2+},Mg^{2+}-ATPase activity associated with highly purified t-tubule membrane preparations [13–15]. In the present study we differentiate between the Mg^{2+}-ATPase and Ca^{2+},Mg^{2+}-ATPase activities in highly purified t-tubule membranes by means of their differing thermal stabilities and associate the inhibition of each activity with specific peaks in the denaturation profile as determined by differential scanning calorimetry.

2. Material and methods

2.1. Isolation of t-tubules and sarcoplasmic reticulum

T-tubule membranes were isolated from New Zealand white rabbits by minor modification of the differential sedimentation procedure previously described by Rosemblatt et al. [16]. When required, isolated t-tubule membranes were incubated in a Ca^{2+} loading solution in the presence of 5 mM potassium oxalate, and an additional centrifugation through a 25%/45% discontinuous sucrose gradient was done to remove Ca^{2+}-oxalate loaded vesicles [14]. Heavy (HSR) and light (LSR) sarcoplasmic reticulum were isolated essentially as described by Campbell et al. [17].

2.2. Thermal inactivation

Isolated t-tubule membranes at 1.5–2 mg/ml were heated in 0.3 M sucrose, 20 mM Tes, and 1 mM DTT, pH 7.0 in a water bath at a heating rate of 1°C/min. Samples were removed at time intervals of 1 min from 30°C to 70°C and placed on ice until all samples were collected. Each sample was kept on ice approximately the same amount of time before the assays of ATPase activity (Mg^{2+} or Ca^{2+},Mg^{2+}) or Ca^{2+} uptake were made at room temperature. An inactivation curve was generated by plotting activity as a fraction of control activity (no heating) vs. temperature. The inactivation temperature (T_i), defined as the temperature of half inactivation, corresponds to the temperature recorded at half the area of the deconvoluted peaks obtained from the derivative of the inactivation curves. For all the inactivation experiments the curves are not symmetrical; therefore T_i does not correspond to the peak temperature.

2.3. Differential scanning calorimetry

Calorimetric experiments were performed with a high resolution Microcal MC2 differential scanning calorimeter (DSC) interfaced to a DEC 380 computer with an automatic data collection system as has been described previously [18]. For DSC experiments, t-tubule membranes were suspended in the same solution used for thermal inactivation. An initial DSC scan of t-tubule membranes (8 to 10 mg/ml protein) was obtained at a scanning rate of 1°C/min from 10 to 100°C. The sample was immediately cooled to 10°C followed by a rescan to 100°C. Denaturation was completely irreversible after scanning to 100°C. Intrinsic baseline curvature was corrected by subtracting the rescan. The shift in specific heat on denaturation (ΔC_p) was corrected as described previously [18].

Both the DSC scans and derivative curves of inactivation of ATPase activity were deconvoluted using a recursive minimization routine assuming irreversible denaturation and inactivation as described previously [18]. This procedure requires that denaturation and inactivation can be approximated by a two state reaction of the form $N \xrightarrow{k} D$ obeying pseudo-first-order kinetics, where the temperature dependence of the rate constant k is given by the Arrhenius relation. The fraction of each component denatured or inactivated (f_D) is given by

$$f_D[T(t)] = 1 - \exp\left\{ \frac{-RT_c^2}{E_A v} \exp\left(\frac{E_A}{RT_c^2}(T - T_c) \right) \right\}$$

where T_c is the temperature at which $k = 1$, E_A the activation energy, and v the scan rate. The derivative of f_D as a function of temperature (df_D/dT) is proportional to the excess C_p which is also proportional to the temperature derivative of activity. The transition temperature (T_m) is defined as the temperature of half denaturation, and corresponds to the temperature at half the area under the individual peaks after the DSC scans are deconvoluted.

2.4. ATPase activity measurements

ATPase activities at 25°C were determined by an enzyme-coupled assay essentially as described by McClure [19] containing 1 mM ouabain. This assay follows the formation of ADP by observing the change in absorbance at 340 nm as NADH is oxidized by the coupling system. The Mg^{2+}-ATPase activity was measured in the presence of EGTA (1 mM), and the Ca^{2+},Mg^{2+}-ATPase activity is the difference between the activity measured with $CaCl_2$ (0.1 mM) and the activity measured with EGTA. Parallel measurement of activity by inorganic phosphate release were determined colorimetrically [20] for some experiments and gave similar results to activity determined by the coupled enzyme system.

2.5. Calcium uptake

Calcium transport was measured at 25°C in a solution containing 0.1 mM $CaCl_2$, 0.1 M KCl, 5 mM $MgCl_2$, 20 mM Tes, 1 mM ATP and 0.07 mM Arsenazo III from the change in absorbance at 660 nm both in the presence and

absence of 5 mM potassium oxalate [21]. The rate of uptake was calculated from the linear change in absorbance during the first five minutes of the assay.

2.6. [^{14}C]Oxalate uptake

[^{14}C]Oxalate uptake by isolated t-tubules and SR was measured as a function of time at room temperature in the solution for Ca^{2+} uptake described above containing ^{14}C-labelled potassium oxalate. Isolated membranes at a concentration of 0.05 mg/ml were added to the oxalate uptake solution containing 0.1 mM $CaCl_2$, 0.1 M KCl, 20 mM Tes, 5 mM $MgCl_2$, 5 mM potassium oxalate, 5 μCi/ml [^{14}C]oxalate and 1 mM ATP, pH 7.0. Aliquots of 0.5 ml were filtered through Millipore filters (0.45 μm), and the filters were washed with a 20-fold volume of ice-cold solution containing 10 mM EGTA, 5 mM $MgCl_2$, 20 mM Tes, pH 7.0 (quench solution) before counting.

3. Results

3.1. Thermal inactivation of ATPase activity

Isolated t-tubule membranes, which are oriented primarily inside-out [22,23], have a high level of Mg^{2+}-ATPase activity which is stimulated slightly in the presence of 0.1 mM $CaCl_2$. The average Mg^{2+}-ATPase activity was 2.26 $\pm$ 0.23 μmol/mg per min. Any purported ecto Mg^{2+}-ATPase activity in sealed inside-out vesicles would not have been measured by the assay used [24]. The activity of the Ca^{2+},Mg^{2+}-ATPase, which is the difference between Ca^{2+}, Mg^{2+} and Mg^{2+} stimulated ATPase activities, was 0.140 $\pm$ 0.008 μmol/mg per min as determined from 13 isolations. The extent of stimulation by Ca^{2+} was nearly constant for each isolation (6.2 $\pm$ 0.6%), although the level of Mg^{2+}-ATPase activity was somewhat variable. This Ca^{2+},Mg^{2+}-ATPase activity could be due to a distinct ATPase or to stimulation of the Mg^{2+}-ATPase by calcium.

This was investigated by measuring the thermal inactivation of both Mg^{2+} and Ca^{2+},Mg^{2+}-ATPase activities. Irreversible inactivation occurred at temperatures in excess of 40°C and was complete after heating to 65–70°C. A controlled heating rate of 1°C/min was used since irreversible enzyme inactivation is dependent on both temperature and time of exposure, necessitating carefully controlled heating for quantitative comparison of inactivation [18].

To better visualize the transition between the active $\rightarrow$ inactive form of the enzymes, the first derivative of the inactivation curves (Fig. 1) was calculated and plotted as a function of temperature (Fig. 2A, B and C). The inactivation temperature (T_i) was calculated for each component from these curves. Only one transition with $T_i = 57.1°C \pm 0.3$ (4 experiments) was found when the ATPase activity was measured in the presence of EGTA, the usual condition for measuring Mg^{2+}-ATPase activity (Fig. 2A). However, two transitions with T_i values of 49.3°C $\pm$ 0.3 (peak A, 3 experiments) and 57°C (peak B) were observed when 0.1 mM $CaCl_2$ was added to the reaction solution to measure the combined Mg^{2+} and Ca^{2+},Mg^{2+}-ATPase activities (Fig. 2B). The single peak in Fig. 2A at $T_i = 57°C$ represents the Ca^{2+} independent Mg^{2+}-ATPase activity. In the presence of Ca^{2+}, a second component shows up with a lower transition temperature of $T_i = 49°C$. The ATPase activity of the second component, which is proportional to the area under the peak, corresponds to about 10% of the total ATPase activity as determined by deconvolution of the peaks in Fig. 2B. This is consistent with the 6% stimulation of ATPase activity on addition of 0.1 mM Ca^{2+}.

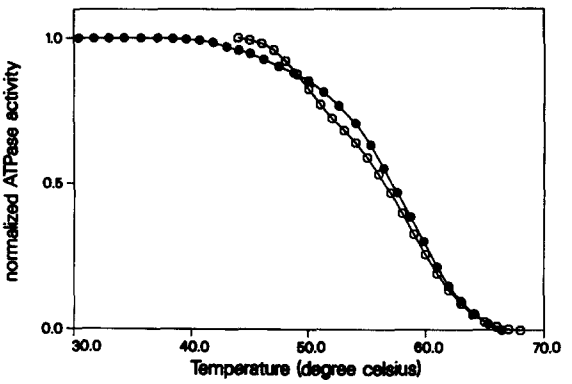

Fig. 1. Thermal inactivation of ATPase activity of t-tubule membranes. Normalized ATPase activity in the presence of 1 mM EGTA (●, Mg^{2+} stimulated) and 0.1 mM $CaCl_2$ (Ca^{2+},Mg^{2+} stimulated, ○) is plotted as a function of temperature increased at 1°C/min.

Contribution to the overall ATPase activity by the Na^+/K^+-ATPase was minimized by conducting all ATPase assays in the presence of 1 mM ouabain and having only trace amounts of Na^+ and K^+ present. In any case, background Na^+/K^+-ATPase activity would have no effect on the Ca^{2+},Mg^{2+}-ATPase and Mg^{2+}-ATPase inactivation temperatures.

The inactivation curve of Mg^{2+}-ATPase activity can be fit well by the curve (dotted line in Fig. 2A) predicted for a single irreversible transition of the form $N \xrightarrow{k} D$ where N and D are the native and denatured forms, respectively, with an activation energy (E_A) of 190 kJ/mol for the rate constant of inactivation k. The inactivation curve of combined Mg^{2+} and Ca^{2+},Mg^{2+}-ATPase activity requires two transitions with E_A values of 427 kJ/mol for inactivation of the Ca^{2+},Mg^{2+}-ATPase activity and 190 kJ/mol for inactivation of the Mg^{2+}-ATPase activity.

Thermal inactivation of ATP dependent Ca^{2+} transport has a T_i equal to that for Ca^{2+},Mg^{2+}-ATPase activity (49.0°C $\pm$ 0.2 (3 experiments)). Fig. 2C shows the derivative of the normalized activity as a function of temperature. There is complete inactivation above 53°C, a temperature at which there is little inactivation of the Mg^{2+}-

ATPase. The E_A obtained from curve fitting is 543 kJ/mol, similar to that for inactivation of the Ca^{2+},Mg^{2+}-ATPase activity. Thus, Mg^{2+}-ATPase activity in the presence of 0.1 mM Ca^{2+} in isolated t-tubules membranes consists of two components with the more thermally sensitive component responsible for Ca^{2+} transport.

The values for the activities plotted in Figs. 1 and 2 were normalized to those of the unheated control. The average control values were 0.140 μmol/mg per min for

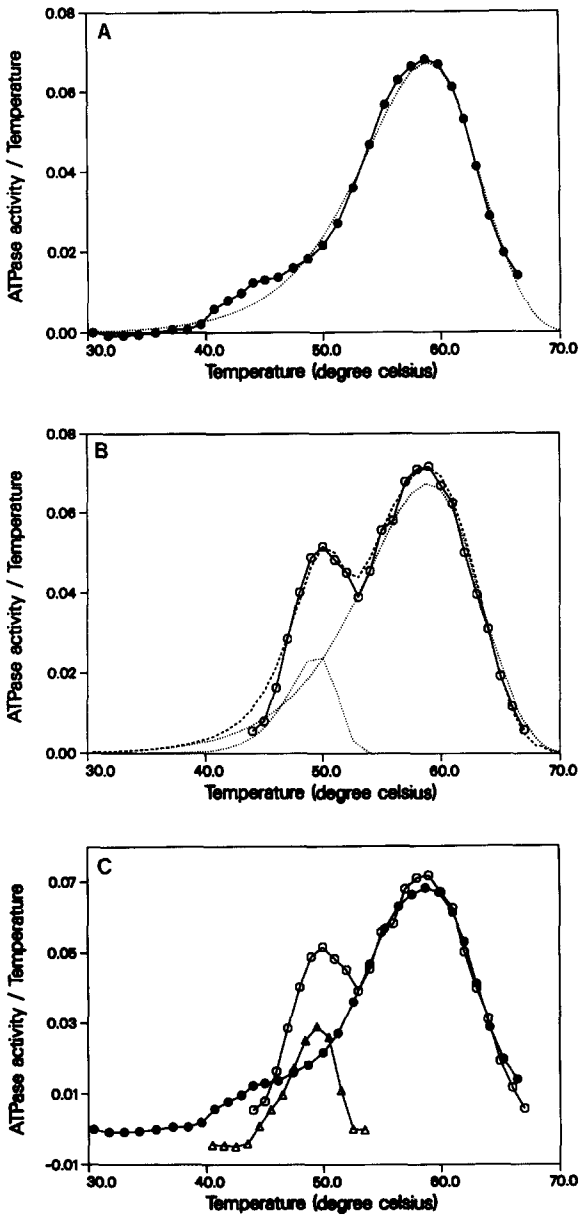

Fig. 2. First derivative of the inactivation of ATPase activity as a function of temperature. The experimental curves are given by the solid lines, the best fit theoretical curves for each component (labelled A and B) by the dotted lines, and the sum of the two components (Fig. 2B) by the dashed line. (A) Inactivation of ATPase activity in the presence of EGTA (●, Mg^{2+}-ATPase activity). (B) Inactivation of Ca^{2+},Mg^{2+}-stimulated ATPase activity (○) and the best two component fit. (C) Comparison of the inactivation of ATP dependent Ca^{2+} uptake (▲) and Ca^{2+},Mg^{2+}-ATPase (○) and Mg^{2+}-ATPase (●) activities.

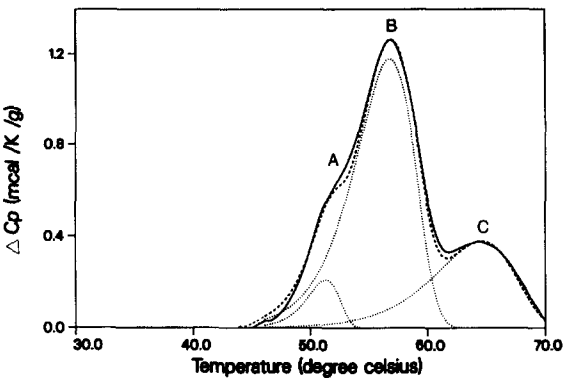

Fig. 3. Differential scanning calorimetry profile (C_p (excess) vs. temperature) of t-tubule membranes. The solid, dotted, and dashed lines represent the original profile, the individual components obtained by deconvolution, and the sum of the components, respectively. Values for the transition temperatures are (means ± S.E. taken from three separate membrane preparations): $T_m(A) = 51.3 \pm 0.6$°C, $T_m(B) = 57.1 \pm 0.1$°C and $T_m(C) = 63.2 \pm 0.6$°C.

Ca^{2+},Mg^{2+}-ATPase activity and 0.146 ± 0.54 μmol/mg per min (4 experiments) for Ca^{2+} uptake. These give a coupling ratio of approximately one which indicates that nearly all the Ca^{2+} uptake was into well sealed vesicles.

3.2. *Differential scanning calorimetry of t-tubule membranes*

The thermal denaturation profile of isolated t-tubule membranes obtained by differential scanning calorimetry (DSC) at a heating rate of 1°C/min is shown in Fig. 3. Protein thermal denaturation is an endothermic process that shows up as an excess specific heat (C_p). The transition temperature (T_m) and activation energy of each component were determined by deconvolution of the profile as was done for the derivative curves of inactivation. Denaturation was completely irreversible after scanning to 100°C.

Three major transitions labelled A (T_m = 51.3°C ± 0.6), B (T_m = 57.1°C ± 0.1) and C (T_m = 63.2°C ± 0.6) were consistently present. Transition B, the major denaturation event of the t-tubule membrane, correlates with the inactivation temperature observed for Mg^{2+}-ATPase activity (T_i = 57°C). The activation energy of denaturation (E_A) obtained by deconvolution and curve fitting of this component is 360 kJ/mol, somewhat larger than that obtained for inactivation of Mg^{2+}-ATPase activity. The denaturation of component A (T_m = 51°C) correlates with the T_i of inactivation of ATP dependent Ca^{2+} transport and Ca^{2+},Mg^{2+}-ATPase activity. The E_A for denaturation of component A (635 kJ/mol), which is difficult to determine accurately since it is such a minor component, is larger than that for component B and corresponds to that for inactivation of ATP dependent Ca^{2+} uptake (543 kJ/mol) as determined from Fig. 2.

The correspondence of the DSC profile with the thermal inactivation scans clearly shows that inactivation of Ca^{2+}

independent Mg^{2+}-ATPase activity is due to denaturation of a membrane component at $T_m = 57°C$ while inactivation of Ca^{2+},Mg^{2+}-ATPase activity and Ca^{2+} uptake is due to denaturation of a component with lower thermal stability at $T_m = 49–51°C$. The identity of the peak with $T_m = 63°C$ is unknown.

3.3. Level of contamination by sarcoplasmic reticulum

Since SR has a high Ca^{2+}-ATPase activity, it is important to obtain evidence regarding the degree of contamination of the isolated t-tubules by SR. Oxalate has been previously reported to strongly stimulate Ca^{2+} uptake in SR [14], while it has no effect on Ca^{2+} uptake in t-tubules [25]. As one assay for the amount of functional SR contamination, we measured the amount of oxalate stimulated Ca^{2+} uptake of the isolated t-tubules. Because of the possibility that broken vesicles of SR may be present and contribute to the Ca^{2+}-ATPase activity observed, we always measured both Ca^{2+}-ATPase activity and non-oxalate stimulated Ca^{2+} transport since broken vesicles can not contribute to Ca^{2+} uptake. Coupling ratios of Ca^{2+} transported to ATP hydrolyzed of approximately one were always found, implying little contribution to Ca^{2+},Mg^{2+}-ATPase activity from leaky vesicles (results given above).

Oxalate fails to stimulate Ca^{2+} uptake in isolated t-tubules (Table 1). For the isolation used in Table 1, Ca^{2+} uptake was 0.128 μmol/mg protein per min and there was no detectable increase in Ca^{2+} uptake in 5 mM potassium oxalate. Addition of very small amounts of SR gave a measurable stimulation of Ca^{2+} uptake in the presence of oxalate. For example, addition of 0.62% SR (by weight SR protein/t-tubule protein) increased Ca^{2+} accumulation in oxalate over that of t-tubules by 0.031 μmol/mg protein per min. This is measurable and is approximately equal to the maximum level of oxalate stimulated Ca^{2+} accumulation found for any isolation used.

All t-tubule isolations were checked for oxalate stimulated Ca^{2+} uptake. When even very low levels were found, the isolation was further purified by loading the vesicles with Ca^{2+} in oxalate and removing the calcium oxalate loaded vesicles through a second discontinuous sucrose gradient as described in Materials and methods. No detectable oxalate stimulated Ca^{2+} uptake remained after this procedure. Thus, contamination by functional, sealed SR appears to be very small, probably no more than a few tenths of a percent, using oxalate stimulated Ca^{2+} uptake as an assay for contamination.

The level of contamination required to account for the measured Ca^{2+},Mg^{2+}-ATPase activity and Ca^{2+} uptake can be easily calculated. The activity of the Ca^{2+},Mg^{2+}-ATPase from heavy SR is 0.907 ± 0.010 μmol/mg SR protein per min (3 experiments) as determined using the same procedure as for the t-tubules. Thus, the level of Ca^{2+},Mg^{2+}-ATPase activity found in the isolated t-tubules (0.140 μmol/mg per min) would require 15% contamination (weight SR protein/t-tubule protein) since this would give a Ca^{2+},Mg^{2+}-ATPase activity of 0.140 μmol/mg total protein per min. The necessary level of contamination (15%) is 2.5-times greater than the level of stimulation of t-tubule ATPase activity by Ca^{2+} (6.2%) due to the higher activity of the t-tubule Mg^{2+}-ATPase (2.26 μmol/mg per min) compared to the heavy SR Ca^{2+},Mg^{2+}-ATPase (0.907 μmol/mg per min). If the contamination is solely due to light SR, which is almost pure Ca^{2+},Mg^{2+}-ATPase, a lower level of SR could account for the Ca^{2+},Mg^{2+}-ATPase activity since light SR has a higher specific Ca^{2+},Mg^{2+}-ATPase activity. Light SR has a Ca^{2+},Mg^{2+}-ATPase activity of approx. 2.0 μmol/mg per min [26]. Thus, about 6% contamination (by weight) of this fraction could account for the level of Ca^{2+},Mg^{2+}-ATPase activity measured in isolated t-tubules. This level of contamination (6–15%) is much greater than that which causes a detectable increase in the level of oxalate stimulated Ca^{2+} uptake. Thus, it appears difficult to explain the level of Ca^{2+},Mg^{2+}-ATPase activity measured in the isolated t-tubules by contamination with sealed SR.

Leaky SR would contribute to Ca^{2+},Mg^{2+}-ATPase activity but would not be able to accumulate Ca^{2+}. Thus, the Ca^{2+},Mg^{2+}-ATPase activity of t-tubules (0.140 μmol/mg per min) can not be due exclusively to the presence of broken or leaky SR vesicles since a comparable level of Ca^{2+} uptake (0.140 μmol/mg per min) to Ca^{2+},Mg^{2+}-ATPase activity is observed. Hence, contamination by

Table 1
Contribution of SR contamination to ATP dependent Ca^{2+} uptake of isolated t-tubule membranes

Sample	Ca^{2+} uptake (μmol/mg protein per min)
t-tubules, no oxalate [a]	128
t-tubules, oxalate [a]	128
t-tubules, oxalate, SR (0.04%) [b]	138
t-tubules, oxalate, SR (0.62%) [b]	159
t-tubules, oxalate, SR (1.25%) [b]	178
t-tubules, oxalate, SR (2.5%) [b]	209

[a] Ca^{2+} uptake was measured in the presence or absence of 5 mM potassium oxalate.
[b] t-Tubule membranes were mixed with increasing amounts of SR (0.04 to 2.5% weight SR protein/weight t-tubule protein), and Ca^{2+} uptake measured in the presence of oxalate.

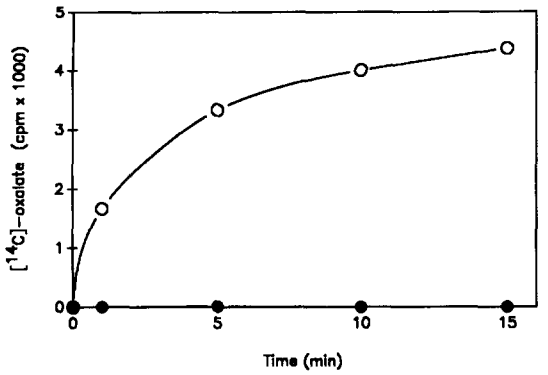

Fig. 4. [^{14}C]Oxalate permeability of t-tubule and SR membranes. [^{14}C]Oxalate uptake by SR membranes (○) and t-tubule membranes (●) as a function of time.

neither sealed nor leaky SR appears able to account for the measured levels of Ca^{2+},Mg^{2+}-ATPase activity and Ca^{2+} uptake.

The lack of effect of oxalate on Ca^{2+} transport in t-tubule vesicles can be explained by the impermeability of t-tubules to oxalate (Fig. 4). Oxalate incorporation in SR as a function of time is apparent within the first minute of the reaction, while t-tubule vesicles have no detectable oxalate incorporation for times as long as 15 min. This lack of oxalate uptake by isolated t-tubules can be used both as a criterion for contamination and as a means of separation of t-tubules and calcium oxalate loaded SR.

4. Discussion

Several studies have suggested that the Ca^{2+}-ATPase and Mg^{2+}-ATPase activities in t-tubules and in other membrane systems [7,27] are expressions of the same protein. Such a view is based on reports that either Ca^{2+} or magnesium at saturating concentrations produce similar maximal activities with no additive effect [3]; however, there are other reports that ATP dependent Ca^{2+} transport in t-tubule membranes occurs at a rate that does not correspond to the activity of the Mg^{2+}-ATPase, which is 10-fold higher than the activity of Ca^{2+} transport [22,25].

In this study we show that t-tubule membranes isolated from skeletal muscle contain a functional Ca^{2+},Mg^{2+}-ATPase activity associated with Ca^{2+} transport that can be separated from the Ca^{2+} independent Mg^{2+}-ATPase on the basis of differing thermostability. The thermostability, defined as the temperature of half inactivation at a heating rate of 1°C/min, of the different ATPase activities in t-tubule membranes shows that Ca^{2+},Mg^{2+}-ATPase activity is inactivated with a $T_i = 49$°C and corresponds to the denaturation of a protein component related to ATP dependent Ca^{2+} transport which is also inactivated at 49°C. The small amount of Ca^{2+},Mg^{2+}-ATPase activity, less than 10% of the total as determined by deconvolution of the curves of the first derivative of inactivation, can easily be masked by the greater activity of the Mg^{2+}-ATPase when comparing only activity measurements.

The denaturation of the Ca^{2+},Mg^{2+}-ATPase of SR has previously been investigated and has been shown to proceed through two steps [18]. The nucleotide binding domain unfolds with a T_m of 49–51°C, depending on Ca^{2+} concentration, and the transmembrane domain and Ca^{2+} binding domain unfolds at 58–62°C in excess Ca^{2+}. Ca^{2+} uptake and ATPase activity is inactivated with a T_i of 49–51°C in SR. Thus, it is not possible to distinguish between the Ca^{2+},Mg^{2+} stimulated ATPase activities of SR and t-tubules on the basis of thermal stability.

The inactivation temperatures of the various activities correspond to denaturation temperatures of resolvable peaks in the DSC profile, consistent with the proposal that inactivation of ATPase activity and Ca^{2+} transport are due to denaturation of specific proteins. The good correlation between inactivation and denaturation implies that denaturation is irreversible, even at temperatures of 50–60°C. However, it is possible that Ca^{2+} is an activator of Ca^{2+} transport by the Mg^{2+}-ATPase and that this process is inactivated with a T_i of 49°C. This is unlikely, but if true the component responsible for activation could be identified by determining the component denatured at $T_m \sim 50$°C.

The temperature dependence of both inactivation and denaturation is approximated by the Arrhenius relation since the theoretical fits, based on this assumption, to the experimental curves are very good. In general, there is a good correlation between the activation energies for denaturation and inactivation, except for the Mg^{2+}-ATPase. The E_A from the DSC scan is 360 kJ/mol compared to 190 kJ/mol as determined by inactivation. This could be explained if we consider that although the Mg^{2+}-ATPase comprises a large fraction of the t-tubule membrane protein, about 26–30% [13,15], many other proteins are present and their denaturation must contribute to the DSC profile at some point, possibly in the temperature range of 50–60°C, influencing the apparent E_A for component B. Thus, peak B on the DSC scan may be due to the denaturation of more than one protein.

The extreme thermal sensitivity of Mg^{2+}-ATPase activity previously reported [14,15], which is indicative of a T_i as low as 26°C, is inconsistent with our results. Damage to SR during isolation and other treatments (e.g., long storage at 4°C, exposure to room temperature, and bubbling with oxygen) that presumably result in oxidation give SR with a reduced ability to accumulate Ca^{2+} and a Ca^{2+}-ATPase activity that is far more thermally sensitive (results not shown). Thus, the greater thermal sensitivity previously reported for the Mg^{2+}-ATPase may be explained by protein damage.

The ability of t-tubule membranes to transport Ca^{2+} via the Ca^{2+},Mg^{2+}-ATPase would have an important physiological implication as has been previously proposed [10]. The large surface area of t-tubule system, plus the proximity to the interior sarcoplasma, would enable these mem-

branes to provide a mechanism of controlling intracellular Ca^{2+} in association with that of the SR and SL. Fluxes of Ca^{2+} are reported to exist across t-tubule membranes which would require the presence of Ca^{2+} in the t-tubule lumen [28]. Direct measurements of Ca^{2+} concentration in the t-tubule have not been made, however elevation of t-tubule luminal Ca^{2+} has been postulated to occur during repetitive muscle activity [10]. The presence of a Ca^{2+},Mg^{2+}-ATPase would supply a mechanism that could actively transport Ca^{2+} to the t-tubule lumen and, thus, be involved in fatigue by causing either a failure of the t-tubule action potential [29,30] or by raising the mechanical threshold for excitation/contraction coupling [10].

Acknowledgements

This work was supported by grants from the PHS/NIH (CA40251) and the Natural Sciences and Engineering Research Council of Canada.

References

[1] Fosset, M., Jaimovich, E., Delpont, E. and Lazdunski, M. (1983) J. Biol. Chem. 258, 6086–6092.
[2] Gonzalez-Serratos, H., Valle-Aguilera, R., Lathrop, D.A. and Garcia, M.C. (1982) Nature 298, 292–294.
[3] Curtis, B. and Catterall, W.A. (1986) Biochemistry 25, 3077–3083.
[4] Schwartz, L.M., McCleskey, E.W. and Almers, W. (1985) Nature 314, 747–751.
[5] Bianchi, C.P. and Shanes, A.M. (1959) J. Gen. Physiol. 42, 803–815.
[6] Dunn, S.M. (1989) J. Biol. Chem. 264, 11053–11060.
[7] Blaustein, M.P. (1982) Ann. NY Acad. Sci. 402, 457–458.
[8] Michalak, M., Famulski, K. and Carafoli, E. (1984) J. Biol. Chem. 259, 15540–15547.
[9] Seiler, S. and Fleischer, S. (1982) J. Biol. Chem. 257, 13862–13871.
[10] Bianchi, C.P. and Narayan, S. (1982) Science 215, 295–296.
[11] Bianchi, C.P. and Narayan, S. (1982) Can. J. Physiol. 60, 503–507.
[12] Brandt, N., Caswell, A.H. and Brunschwig, J.P. (1980) J. Biol. Chem. 255, 6290–6298.
[13] Sabbadini, R. and Dhams, S. (1989) J. Bioenerg. Biomembr. 21, 163–212.
[14] Sabbadini, R.A. and Okamoto, V.R. (1983) Arch. Biochem. Biophys. 223, 107–119.
[15] Moulton, M.P., Sabbadini, R.A., Norton, K.C. and Dahms, A.S. (1986) J. Biol. Chem. 261, 12244–12251.
[16] Rosemblatt, M., Hidalgo, C., Vergara, C. and Ikemoto, N. (1981) J. Biol. Chem. 256, 8140–8148.
[17] Campbell, K.P., Franzini-Armstrong, C. and Shamoo, A.E. (1980) Biochim. Biophys. Acta 602, 97–105.
[18] Lepock, J.R., Rodhal, A.M., Zhang, C., Heynen, M.L., Waters, B. and Cheng, K.-H. (1990) Biochemistry 29, 681–689.
[19] McClure, W.R. (1969) Biochemistry 8, 2782–2786.
[20] Lin, T. and Morales, M. (1977) Anal. Biochem. 77, 10–17.
[21] Herbette, L., Marquardt, J., Scarpa, A. and Blasie, J.K. (1977) Biophys. J. 20, 245–275.
[22] Hidalgo, C., Parra, C., Riquelme, G. and Jaimovich E. (1986) Biochim. Biophys. Acta 855, 79–88.
[23] Horgan, D.J. and Kuypers, R. (1988) Arch. Biochem. Biophys. 260, 1–9.
[24] Saborido, A., Moro, G., and Megias, A. (1991) J. Biol. Chem. 266, 23490–23498.
[25] Hidalgo, C., Gonzalez, M.E. and Garcia, A.M. (1986) Biochim. Biophys. Acta 854, 279–286.
[26] Borsotto, M., Norman, I.R., Fosset, M. and Lazdunski, M. (1984) Eur. J. Biochem. 142, 449–455.
[27] Zhao, D. and Dhalla, N.S. (1988) Arch. Biochem. Biophys. 268, 40–48.
[28] Almers, W., Fink, R. and Palade, P.T. (1981) J. Physiol. 312, 177–207.
[29] Howell, J.N. and Oetliker, H. (1987) Can. J. Physiol. Pharmacol. 65, 691–696.
[30] Gonzalez-Serratos, H., Somlyo, A.V., McClellan, G., Shuman, H., Borrero, L.M. and Somlyo, A.P. (1978) Proc. Natl. Acad. Sci. USA 75, 1329–1333.