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Calcium transport in transverse tubules isolated from rabbit skeletal muscle

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Isolated transverse tubule vesicles free of sarcoplasmic reticulum transport calcium with high affinity in the presence of ATP. The calcium transport by transverse tubules differs from calcium transport by sarcoplasmic reticulum. It is not increased by oxalate or phosphate, it has a different temperature dependence, it is inhibited by sub-micromolar concentrations of orthovanadate, it is stimulated by calmodulin, and is inhibited by quercetin without causing calcium release. The rates of calcium transport by transverse tubules are two orders of magnitude lower than those of sarcoplasmic reticulum, suggesting that the calcium pump protein of transverse tubules is a minor component of the membrane. Addition of calmodulin to transverse tubule vesicles – treated with high salt in the presence of EGTA to remove endogenous calmodulin – caused a marked stimulation of transport rates at low concentrations of calcium, and decreased from 1.0 to 0.3 μM the calcium concentration at which half-maximal rates of transport were obtained. A role for the transverse tubule calcium pump in maintaining low sarcoplasmic calcium concentrations is proposed.

In the last few years, a great deal of research has been carried out on the calcium pumps present in plasma membranes. In addition to the well-studied calcium-pumping ATPase of red blood cells [1,2], other calcium pumps have been characterized [3]. Based on the finding that they all transport calcium with high affinity, a function of these enzymes in maintaining low intracellular calcium has been proposed.

The physiological action of calcium in triggering contraction in skeletal muscle requires maintaining an intracellular level lower than 10^{-6} M. There is continuous passive diffusion of calcium inside the muscle cells down its large electrochemical gradient. Furthermore, inward calcium currents have been described in frog and rat skeletal muscle [4–8]. This makes it likely that calcium entry during the action potential contributes to increase the intracellular calcium concentration. The sarcoplasmic reticulum system can only accumulate a finite amount of calcium, being a saturable compartment. Accordingly, the cell needs to pump calcium across the plasma membrane in order to regulate the intracellular calcium concentration.

In heart muscle, where there is a significant calcium entry into the cell in response to depolarization [9], two different sarcolemmal systems remove calcium from the cell; a sodium-calcium exchange system [10,11] and a calcium-pumping ATPase [12].

Several studies have shown that in skeletal muscle both T-tubules and sarcolemma participate in the regulation of the intracellular calcium concentration. Thus, sodium-calcium exchange activ-

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Abbreviation: T-tubule, transverse tubule.

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ity has been found in sarcolemmal preparations isolated from rabbit and pig skeletal muscle [13,14], but not in T-tubules isolated from rabbit muscle [15]. Furthermore, T-tubules isolated from rabbit skeletal muscle by mechanical disruption of triadic junctions transport calcium with low rates compared to the rates of calcium transport by sarcoplasmic reticulum [16], and recent reports indicate that sarcolemmal membranes isolated from rabbit and pig skeletal muscle [13,14] have a calmodulin-stimulated calcium pump.

We have described elsewhere [15] a procedure to isolate T-tubule membranes devoid of sarcoplasmic reticulum contamination. The isolated T-tubules transport calcium with an absolute requirement for Mg and display two K_m values for ATP, 5.5 μM and 0.3 mM. This work presents a further characterization of the calcium transport by isolated T-tubules and shows, in particular, that transport is stimulated by calmodulin and inhibited by orthovanadate and quercetin. A role of the T-tubule calcium pump in maintaining a low intracellular calcium concentration in muscle cells is proposed.

Materials and Methods

Isolation of T-tubules. Muscle microsomes, 400–600 mg, isolated from rabbit muscle as described [17], were loaded on top of each one of six discontinuous gradients formed by three 10-ml layers of 25, 27.5 and 35% (w/v) sucrose in 20 mM Tris-maleate (pH 7.0). After sedimentation in a Beckman SW27 rotor at 4°C for 16 h at 25000 rpm, T-tubules were found in the faint band formed on top of the 25% sucrose solution and mostly in the 25%/27.5% and 27.5%/35% interfaces. Light sarcoplasmic reticulum was found in the 27.5%/35% interface and occasionally in the lighter fractions as well. The rest of the sarcoplasmic reticulum was found as a large pellet at the bottom of the gradient. The lighter fractions collected either from the top of the 25% layer or from the 25%/27.5% sucrose interface were usually devoid of sarcoplasmic reticulum, as indicated by their lack of measurable Ca^{2+}-ATPase activity [15]. After collection from the gradients, all fractions were diluted with 20 mM Tris-maleate to a final sucrose concentration of approx. 0.3 M, and were sedimented at 150000 × g for 45 min. The resulting pellets were homogenized in a small volume of 0.3 M sucrose/20 mM Tris-maleate (pH 7.0) and were stored at −20°C. The concentration of sucrose solutions used to make gradients was always measured by refractometry. Protein concentration was determined according to Lowry et al. [18], using bovine serum albumin as standard.

Calcium transport. Unless otherwise indicated, calcium transport was measured at 25°C in a solution containing 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 M KCl, 20 mM Tris-maleate (pH 7.0), 5 mM ATP, 0.02–0.05 mg protein/ml and 1 μCi/ml $^{45}CaCl_2$. T-tubules were added to the reaction solution and, after 2 min incubation, the reaction was started by addition of ATP. The pH of the solution did not change after ATP addition. To stop the reaction, 0.5-ml fractions were added at different times to 0.5 ml of an ice-cold solution comprising 5 mM $MgCl_2$/10 mM EGTA/20 mM Tris-maleate (pH 7.0) (quench solution). Within 20 s of stopping the reaction, 0.5-ml fractions were filtered through Millipore filters (HA, 0.45 μm), previously washed with 10 ml of a solution comprising 5 mM $MgCl_2$/0.1 mM $CaCl_2$/0.1 M KCl/20 mM Tris-maleate (pH 7.0). The filters were washed three times using 5 ml of quench solution each time, dried and counted in a liquid scintillation counter. Controls without ATP were routinely carried out. The T-tubule fraction obtained from the 25%/27.5% sucrose interface was used in most experiments, and, less frequently, the fraction obtained from the top of the 25% sucrose layer.

Determination of free calcium concentration. A computer program was used to calculate $[Ca^{2+}]$, using the algorithm and binding constants for the EGTA and ATP complexes of Ca^{2+} and Mg^{2+} described elsewhere [19].

Reagents. ATP was obtained from Boehringer Mannheim. Calmodulin, quercetin and compound 48/80 were obtained from Sigma. Sodium orthovanadate was obtained from Baker; orthovanadate solutions were freshly made. $^{45}CaCl_2$ was obtained from New England Nuclear.

Results

Calcium transport by isolated T-tubules. T-tubule vesicles isolated by loading contaminating sarcop-

lasmic reticulum with calcium phosphate contain significant amounts of calcium [20] and display very low, if any, calcium transport compared to the values reported for T-tubules isolated by disruption of triadic junctions [16]. It is likely that during the calcium phosphate loading step T-tubules accumulate enough calcium, 100–200 nmol/mg protein [20] to inhibit further accumulation into the T-tubule vesicles. Using a simple modification of the isolation procedure, which avoids the calcium phosphate loading step, we have successfully separated T-tubules from light sarcoplasmic reticulum according to their density in sucrose gradients. The properties of these T-tubule membranes, including their high cholesterol content, high density of nitrendipine receptors, the presence of 80% or more of vesicles sealed with the inside-out orientation, and the lack of surface membrane contamination, have been described in detail elsewhere [21,22]. The T-tubules collected from the two lightest membrane fractions accumulated 110–140 nmol calcium/mg protein at 25°C. Rates of 7–10 nmol · mg^{-1} · min^{-1} were usually found at 25°C (Fig. 1). In the absence of ATP, negligible amounts of calcium were accumulated, indicating that the observed transport is the result of an ATP-dependent calcium pump. It was described previously that neither the rate nor the maximal accumulation of calcium of isolated T-tubules was stimulated by oxalate or phosphate [15], although the time-course of the transport reaction was not shown. An estimation of the contamination of T-tubules with sarcoplasmic reticulum can be obtained by comparing the time-course of the calcium transport reaction with and without oxalate. T-tubule vesicles free of sarcoplasmic reticulum display the same time-course of calcium transport with or without oxalate (Fig. 1), whereas vesicles contaminated with about 5% sarcoplasmic reticulum show a marked increase of transport following addition of oxalate (Fig. 1, inset). However, we have observed that after prolonged storage frozen preparations become increasingly permeable to oxalate, as determined by the increase in calcium accumulation observed after oxalate addition. Thus, to judge if T-tubules are contaminated with sarcoplasmic reticulum, it is necessary to use fresh T-tubule preparations.

The results shown above indicate that the rates

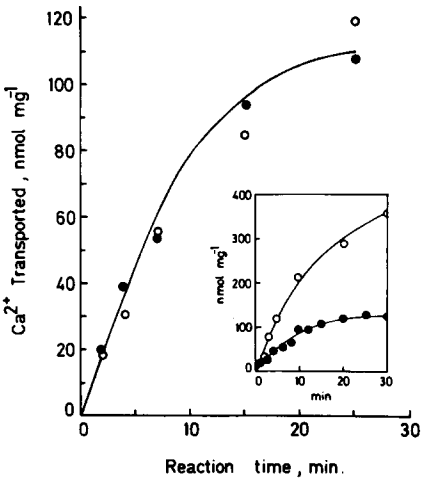

Fig. 1. Calcium transport by T-tubules. Lack of effect of oxalate. Calcium transport was measured at 25°C. A protein concentration of 0.05 mg/ml was used. Controls without ATP were routinely carried out; less than 5 nmol calcium/mg protein were accumulated after 25 min incubation in the absence of ATP. ●, Control; ○, plus 5 mM potassium oxalate. Inset: calcium transport by T-tubules contaminated with about 5% sarcoplasmic reticulum.

of calcium transport in T-tubules (10 nmol · mg^{-1} · min^{-1}) are 300–500-fold lower than in sarcoplasmic reticulum (3000–5000) nmol · mg^{-1} · min^{-1}). However, in the absence of precipitating anions both accumulate similar amounts of calcium, indicating that the transport that we have measured in the T-tubules represents a property of the majority of the vesicles present in the preparations.

Calcium transport in T-tubules displayed a different temperature dependence when compared to calcium transport in sarcoplasmic reticulum. Arrhenius plots of calcium transport rates showed a break temperature of 27–30°C (Fig. 2), with a very low activation energy above this temperature and an activation energy of 19 kcal · mol^{-1} below it. By comparison, calcium transport in sarcoplasmic reticulum shows a break at 20°C, (Fig. 2), with activation energies of 15–20 and of 27–30 kcal · mol^{-1} above and below the break temperature, respectively (Fig. 2; Ref. 23).

Inhibition of calcium transport by orthovanadate and quercetin

A variety of ATPases are inhibtied by ortho-

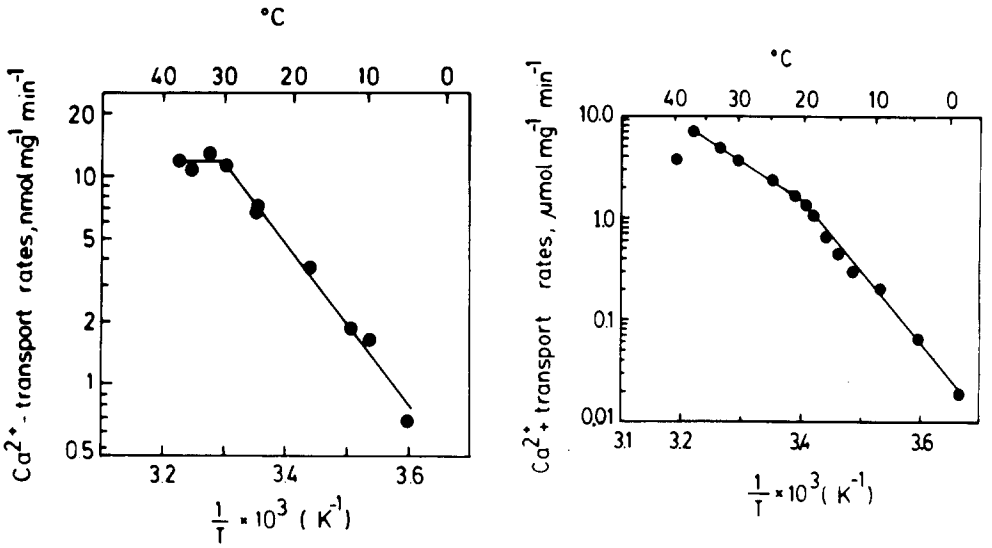

Fig. 2. Arrhenius plot of calcium transport rates by T-tubules (left) and sarcoplasmic reticulum (right). Calcium transport in T-tubules was measured as described in the text. Calcium transport in sarcoplasmic reticulum was measured in the presence of 5 mM potassium oxalate, at a protein concentration of 0.01 mg/ml, in a solution comprising 0.1 M KCl/5 mM $MgCl_2$/0.1 mM $^{45}CaCl_2$/20 mM Tris-maleate (pH 7.0). The transport reaction was initiated by addition of 5 mM Na_2ATP, and was stopped by filtration through Millipore filters (HA, 0.45 µm). The filters were washed and their radioactivity determined by liquid scintillation counting.

vanadate [24], although the concentrations of orthovanadate required for half-maximal inhibition vary considerably. Calcium transport in T-tubules was strongly inhibited by low concentrations of orthovanadate (Fig. 3); 50% inhibition of calcium transport rates was obtained at 0.5 µM orthovanadate. This result is similar to the inhibition caused by orthovanadate on calcium transport rates by heart and pig sarcolemmal membranes [25,14], where the half-maximal inhibition of transport was obtained at sub-micromolar orthovanadate. In contrast, much higher orthovanadate concentrations are required to inhibit the Ca^{2+}-ATPase of sarcoplassmic reticulum [26].

Quercetin is another compound that inhibits several ATPases. Addition of 30 µM quercetin to T-tubules caused 40% inhibition of calcium transport rates. Complete inhibition was observed at 100 µM quercetin (Fig. 4). Addition of 100 µM quercetin 4 min after starting the calcium transport reaction prevented further calcium accumulation without causing a decrease in the amount of calcium already taken up by the vesicles (Fig. 4). This result is in contrast to the effect of quercetin in sarcoplasmic reticulum, where, in addition to inhibiting calcium transport [27], quercetin seems to stimulate calcium release [28].

Calmodulin stimulation of calcium transport

A variety of plasma membrane calcium pumps are stimulated by calmodulin [3]. In order to determine whether calcium transport in T-tubules was stimulated by calmodulin, the effect of

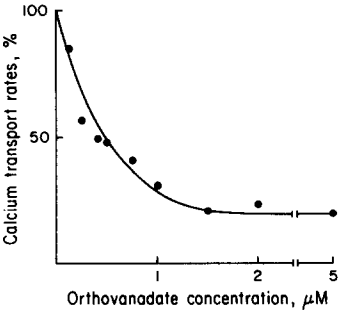

Fig. 3. Inhibition of calcium transport by orthovanadate. Calcium transport was measured at 25°C. The amount of calcium accumulated by the vesicles was measured as a function of time. A linear increase in the amount of calcium accumulated was obtained from 0 to 10 min, and transport rates were calculated as the average of 5–7 rate values.

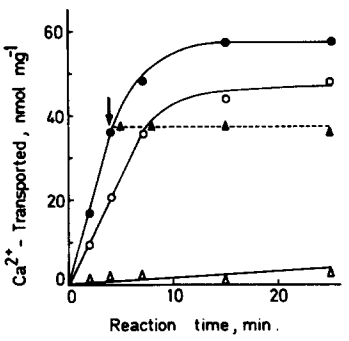

Fig. 4. Inhibition of calcium transport by quercetin. Calcium transport was measured at 25°C. Quercetin was added either before initiating the transport reaction with ATP (○, △), or 4 min after ATP addition (▲). ●, Control; ○, plus 30 μM quercetin; △, ▲, plus 100 μM quercetin.

calmodulin addition on calcium transport was investigated. Addition of 3 μg/ml (0.18 μM) calmodulin to T-tubule vesicles caused 37% stimulation of calcium transport rates (Table I). Since it is conceivable that the T-tubule vesicles contain endogenous calmodulin, addition of exogenous calmodulin might not be the best criterion to establish calmodulin effects. Accordingly, the effect of compound 48/80, a specific inhibitor of calmodulin [29], was investigated. A significant inhibition of calcium transport by 5 μg/ml of compound 48/80 was observed; the inhibition was completely reversed by addition of exogenous calmodulin (Table I). Thus, the limited stimulation of calcium transport by exogenous calmodulin is probably due to the presence of endogenous calmodulin in the T-tubules. In an attempt to remove endogenous calmodulin, T-tubules were incubated in high salt/EGTA solutions, as described in the legend to Fig. 5. This treatment produced a significant inhibition of calcium transport (Table I), which was reversed by addition of exogenous calmodulin. These combined observa-

TABLE I

CALCIUM TRANSPORT BY TRANSVERSE TUBULES: EFFECT OF CALMODULIN

Calcium transport was measured at 25°C, at a p*Ca* of 6.29. At this p*Ca* only 50% of maximal rates are obtained (Fig. 5). A concentration of calmodulin of 3 μg/ml and of compound 48/80 of 5 μg/ml was used. To remove endogenous calmodulin, vesicles were washed with EGTA as described in detail in the legend to Fig. 5. The amount of calcium accumulated by the vesicles was measured as a function of time and rates were calculated for each time point. Rates were constant up to 7 or 10 min. Values represent the averages ± S.D. of the rates. In parentheses are the numbers of time points used to calculate the average values.

Additions	Calcium transport rates (nmol/mg per min)
None	4.0 ± 0.2 (6)
Calmodulin	5.5 ± 0.3 (5)
Compound 48/80	2.0 ± 0.3 (6)
Compound 48/80 + calmodulin	4.9 ± 0.3 (5)
EGTA-washed vesicles	1.7 ± 0.3 (6)
EGTA-washed vesicles + calmodulin	4.7 ± 0.3 (5)

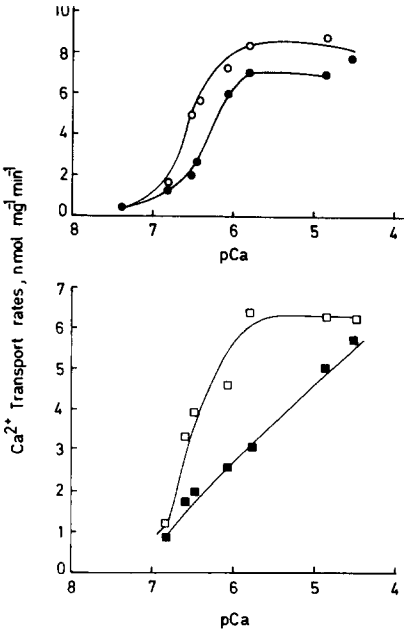

Fig. 5. (Top) Calcium transport by T-tubules as a function of $[Ca^{2+}]$ in the reaction solution. Calcium transport was measured in solutions containing variable amounts of $CaCl_2$ and EGTA. A protein concentration of 0.05 mg/ml was used. ○, Control; ●, plus 3 μg/ml exogenous calmodulin. (Bottom) Effect of calcium on transport rates measured with EGTA-washed T-tubule vesicles in the absence (■) or in the presence of 3 μg/ml calmodulin (□). Vesicles were washed with EGTA by incubation at 0°C for 60 min in 5 mM EGTA/20 mM Tris-maleate (pH 7.0) at a protein concentration of 0.3 mg/ml. The vesicles were subsequently diluted 2-fold with a solution of 1.2 M KCl/20 mM Tris-maleate (pH 7.0) incubated for an additional 30 min period, and collected by sedimentation at 150000 × *g*.

tions suggest that isolated T-tubules contain endogenous calmodulin which activates near-maximally calcium transport.

The effect of $[Ca^{2+}]$ on the stimulation of calcium transport by calmodulin was investigated by measuring transport rates as a function of calcium concentrations in control (unwashed) T-tubule vesicles and in vesicles washed with EGTA/high salt to remove endogenous calmodulin. It was found that in the absence of exogenous calmodulin, control T-tubules displayed measurable calcium transport rates even at $[Ca^{2+}]$ as low as $5 \cdot 10^{-8}$ M. Half-maximum rates were observed at $5 \cdot 10^{-7}$ M free calcium, indicating that the Ca^{2+} pump has a high affinity for calcium (Fig. 5, top). Half-maximal stimulation of the total amount of calcium transported by the vesicles by free calcium concentrations higher than 10^{-6} M was described by Brandt et al. [16], although the apparent difference with the present results might reflect differences inthe K_d for Ca-EGTA used. Addition of calmodulin to the unwashed T-tubules increased by 20% the maximal rate of calcium transport and shifted to $3.3 \cdot 10^{-7}$ M the $[Ca^{2+}]$ for half-maximal rate. The effects were more marked in the vesicles washed with EGTA (Fig. 5, bottom), where addition of calmodulin caused a decrease in the concentrations of calcium at which half-maximal rates of transport were observed, from a value of 10^{-6} M in the absence of calmodulin to a value of $3 \cdot 10^{-7}$ M in the presence of calmodulin. It is likely that the unwashed T-tubule preparation contained significant amounts of endogenous calmodulin, and thus was less stimulated by exogenous calmodulin than the washed preparations.

Discussion

The calmodulin-stimulated calcium pump of T-tubules described in this work has a high affinity for calcium and thus is a likely candidate to serve a physiological role in maintaining low intracellular resting calcium concentrations. The T-tubular calcium pump would thus be simular to the calcium pumps of nonexcitable cells such as the red blood cell [3], and to the calcium pumps present in squid axons [29], smooth muscle cell plasma membrane [30], and especially, to the calcium pumps present in cardiac sarcolemma [25] and in skeletal sarcolemma [14,13], both stimulated by calmodulin and inhibited by micromolar concentrations of orthovanadate.

The possibility that calcium transport in T-tubules was carried out by the Ca^{2+}- or Mg^{2+}-ATPase was considered previously [15]. However, this enzyme is not inhibited by micromolar concentrations of orthovanadate and is only slightly inhibited by 100 μM quercetin (data not shown), whereas calcium transport is completely abolished under these conditions. In addition, the Ca^{2+}- or Mg^{2+}-ATPase is not stimulated by calmodulin (data not shown). Thus, the present results do not seem to support a role of the Ca^{2+}- or Mg^{2+}-ATPase in calcium transport by T-tubules.

In other plasma membranes it has been shown that calcium transport is carried out by specific Ca^{2+}-ATPases that form phosphorylated intermediates upon addition of ATP [31]. Although we reported Ca^{2+}-ATPase activity in our initial T-tubule preparation [20], we have been unable to detect significant levels of Ca^{2+}-ATPase activity in the more purified T-tubule preparations used in this and in our previous work [15]. Assuming that one molecule of ATP is hydrolyzed per calcium ion transported, the Ca^{2+}-ATPase activity associated with calcium transport would be 10 nmol/mg per min, at 25°C. This value is 200–400-times lower than the rate of ATP hydrolysis by the Ca^{2+}- or Mg^{2+}-ATPase [15]. Hence, it will be difficult to detect the Ca^{2+}-ATPase activity associated with transport over the very high activity values of the Ca^{2+}- or Mg^{2+}-ATPase of our T-tubule preparations unless a specific inhibitor is found for the latter. Furthermore, the low rates of calcium transport suggest that the calcium pump of T-tubules is a minor membrane component. The inhibition by orthovanadate is consistent with the involvement of a phosphorylated intermediate in the transport reaction. Our inability to measure calcium-dependent, acid-stable phosphorylation of T-tubules [15] supports the proposal that the calcium pump protein is a minor component.

Preliminary experiments (Mickelson, J., Louis, C. and Hidalgo, C., unpublished observations), indicate that the T-tubules used in this work contain a minor membrane component (M_r approx. 180000) that can be specifically cross-linked to

calmodulin in a calcium-dependent fashion. Experiments are in progress to determine whether this component corresponds to the T-tubule calcium pump.

It was recently reported that T-tubules isolated by calcium phosphate loading had a Ca^{2+}-ATPase activity that was not stimulated by calmodulin or inhibited by calmidazolium, a potent calmodulin inhibitor [13]. The possibility that the Ca^{2+}-ATPase activity measured by these authors was due to minor sarcoplasmic reticulum contamination should be considered, since, as indicated by the authors, the calmodulin-dependent phosphorylation pattern of their T-tubule preparations correlates with the calmodulin-dependent phosphorylation pattern of sarcoplasmic reticulum.

Considering the resting intracellular calcium concentrations existing in muscle cells, the calcium pump of T-tubules should be able to transport calcium against a large electrochemical gradient. It is not known whether the T-tubular lumen contains calcium-binding proteins, or whether all the transported calcium diffuses out of the cell until it reaches equilibrium with the extracellular calcium concentration of about 2.5 mM. The isolated T-tubule vesicles used in this work, which are mostly sealed inside-out [21], when incubated at a free calcium concentration of 10^{-6} M accumulate up to 120–140 nmol/mg calcium. If none of this calcium is bound, it would correspond to an intravesicular calcium level of about 20 mM (assuming an intravesicular T-tubular lumen of 5 μl/mg, similar to that of sarcoplasmic reticulum vesicles). Thus, these results indicate that the T-tubule calcium pump has the ability to transport calcium against the large gradients found in the muscle cells. Furthermore, in view of the large area of T-tubule relative to the surface membranes, it is likely that the high-affinity calcium pump of T-tubules has a major role in maintaining the low intracellular calcium concentrations of resting muscle cells.

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