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Transverse tubules from frog skeletal muscle. Purification and properties of vesicles sealed with the inside-out orientation

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Transverse tubule vesicles were isolated from frog skeletal muscle by a procedure initially described by Rosemblatt et al. (J. Biol. Chem. 256, 8140–8148 (1981)) and later modified by Hidalgo et al. (J. Biol. Chem. 258, 13937–13945 (1983)). A large fraction of the isolated vesicles (80–90%) were sealed, as indicated by the detergent induced increase in $(Na^+ + K^+)$-ATPase activity and ATP-dependent ouabain binding. To determine the orientation of the sealed vesicles binding of digoxin, a lipid soluble derivative of ouabain, was measured. The same values of ATP-dependent digoxin binding were found with or without detergents, indicating that all the vesicles that are sealed have the ATP site accessible, and hence are sealed with the cytoplasmic side-out (inside-out orientation). The transverse tubule preparation isolated from frog muscle is highly purified, as indicated by its cholesterol content and its $(Na^+ + K^+)$-ATPase activity; negligible contamination with sarcoplasmic reticulum was observed, as indicated by the protein composition and the lack of measurable Ca^{2+}-ATPase activity of the isolated transverse tubules. High initial rates of Mg^{2+}-ATPase activity were found, with the peculiar property of being inhibited during the course of the reaction. Addition of lysophosphatidylcholine or saponin partially prevented the inhibition of Mg^{2+}-ATPase activity during the reaction.

Introduction

The transverse tubule (T-tubule) membrane of skeletal muscle plays a crucial role in the process of excitation-contraction coupling. The action potential elicited at the neuro-muscular junction propagates through the external plasma membrane into the T-tubules [1,2], where, by a mechanism yet not well understood, it triggers the release of calcium from the terminal cisternae of the sarcoplasmic reticulum [3]. The ensuing increase in intracellular calcium produces muscle contraction. Relaxation takes place following the decrease in myoplasmic calcium concentration brought about by the calcium pump of sarcoplasmic reticulum [3].

In view of its critical role in the physiology of muscle contraction, it is important to characterize the T-tubule membrane. Recent studies have established some of the enzymatic [4–8], transport [9,10] and pharmacological properties [11–13] of isolated T-tubule membrane preparations. How-

Abbreviations: T-tubules, transverse tubules; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

ever, these studies have been carried out with T-tubules isolated from rabbit or chicken skeletal muscle, whereas most of the physiology of the muscle cell has been studied in frog muscle. There are significant differences in the structural and electrical properties of T-tubules from mammalian and frog muscle. Thus, the T-tubule of mammalian muscle has a smaller lumen and a higher chloride conductance [14,15] than those described in frog muscle [16,17], which determines a shorter space constant for mammalian T-tubules [18]. Accordingly, with the aim of correlating properties of isolated membrane vesicles with the considerable information available on the physiology of frog muscle, we felt it was important to study the properties of the T-tubules isolated from the same source.

This paper describes the isolation, the lipid and protein composition and several ATPase activities of the T-tubule vesicles isolated from frog muscle, and provides, in addition, a characterization of the amount and orientation of sealed vesicles present in the preparation. The following paper [19] gives a description of some of the receptors for ion-channel blockers present in the T-tubules isolated from both frog and rabbit skeletal muscle.

Materials and Methods

Isolation of T-tubules. Three or four adult frogs (*Caudiverbera caudiverbera*) weighing from 400 to 700 g each, were routinely used. The animals were pithed and muscle was removed from the hind legs. 100–150 g of muscle was usually obtained. The muscle tissue was cleaned of blood vessels, connective tissue and large nerves, and was finely minced before homogenizing in a Waring blendor with 4 volumes of 0.1 M KCl/20 mM Tris-maleate (pH 7.0). All these steps were carried out at 4°C in a cold room. The microsomes were isolated by differential sedimentation, removing mitochondria by sedimentation at $10\,000 \times g$ for 30 min. To remove contractile proteins, the resulting suspension was made 0.6 M in KCl by addition of solid salt, and the microsomes were collected by sedimentation at $100\,000 \times g$. The microsomal pellet was washed twice; the first time with 0.1 M KCl/20 mM Tris-maleate (pH 7.0) and the second with 0.3 M sucrose/20 mM Tris-maleate (pH 7.0). A detailed description of this procedure was given by Fernández et al. [20] for rabbit muscle. The washed microsomal pellet was resuspended in 15 to 20 ml of 0.3 M sucrose/20 mM Tris-maleate (pH 7.0) and was loaded on top of three discontinuous sucrose gradients made of layers of equal volumes of 25%, 27.5% and 35% sucrose solutions (w/v, adjusted by refractometry). After overnight centrifugation at $100\,000 \times g$ at 4°C, bands were found on top of the 25% layer and at the 27.5%/35% interface, with a large pellet at the bottom of the tube. The lightest band contained T-tubules essentially free of sarcoplasmic reticulum contamination; some T-tubules and mostly light sarcoplasmic reticulum were found in the 27.5%/35% sucrose interface; the rest of the sarcoplasmic reticulum was found in the pellet. Only the lightest fraction (from the top of the 25% layer) was used in all the experiments described in this work. Bands were collected by aspiration, diluted 20–40-fold to a final concentration of 0.3 M sucrose with 20 mM Tris-maleate (pH 7.0), and sedimented at $100\,000 \times g$ for 60 min at 4°C. The resulting pellets were resuspended in a small volume of 0.3 M sucrose/20 mM Tris-maleate (pH 7.0), frozen rapidly by immersion in solid CO_2/acetone, and stored frozen at −20°C. The usual yield was 1 to 2 mg of T-tubule protein from 100 to 150 g of frog skeletal muscle.

T-tubules were isolated form rabbit skeletal muscle as described in detail elsewhere [8].

Determination of $(Na^+ + K^+)$-ATPase activity. The reaction was carried out at 37°C, in a solution containing 120 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 0.5 mM EGTA, 5 mM NaN_3, 30 mM imidazole-HCl (pH 7.5), 3 mM Na_2ATP, with or without 1 mM ouabain. The reaction was started by addition of protein (0.02–0.05 mg/ml), and was stopped by adding 0.5 ml of 10% SDS to 1.0 ml of reaction solution, followed by immediate cooling on ice. The reaction was stopped at different times, taking care to have at least five time points to establish a linear rate. This precaution was necessary since the Mg^{2+}-ATPase activity of the T-tubule vesicles is not linear with time but has fast initial rate that decays to a slower rate after the first 3–4 minutes of the initiation of the reaction. To unmask latent activity the reaction was carried out in the presence of saponin (0.2 mg per

ml); it was not necessary to preincubate the enzyme with saponin in order to unmask latent activity. The inorganic phosphate released was determined colorimetrically [21]. The $(Na^+ + K^+)$-ATPase activity was the difference between the activity measured with and without ouabain.

Determination of Ca^{2+}-ATPase and Mg^{2+}-ATPase activity. The reaction was carried out at 25°C, in a solution containing 100 mM KCl, 3 mM $MgCl_2$, 30 mM imidazole (pH 7.2), 5 μg per ml of ionophore A23187, 3 mM Tris-ATP, and either 50 μM $CaCl_2$ or 1 mM EGTA-Tris. The reaction was started by addition of membranes (0.02–0.05 mg of protein per ml) and was stopped at different times by addition of 10% SDS as described above for the $(Na^+ + K^+)$-ATPase. The Mg^{2+}-ATPase activity was the activity measured in the presence of EGTA. The Ca^{2+}-ATPase activity was the difference between the activity measured with $caCl_2$ and the activity measured with EGTA. The inorganic phosphate released was determined colorimetrically [21].

Binding assays. The binding of [^{3}H]ouabain was measured at 37°C as described [9], in a solution containing 120 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 40 mM Tris-HCl (pH 7.4), with or without 10 mM Na_2ATP, and variable concentrations of [^{3}H]ouabain (from 5 to 160 nM). A protein concentration of 0.01–0.03 mg per ml was used. After incubation for 60 min at 37°C, samples (0.5 ml) were collected by filtration through Millipore filters (HA, 0.45 μm), washing the filters with 3 ml of ice-cold reaction solution without [^{3}H]ouabain. Filters were dried and counted in a liquid scintillation counter. The non-specific binding (less than 5% in the K_d region) was determined in the absence of ATP and was subtracted from the total binding to determine the specific binding. The ATP-dependent binding of [^{3}H]digoxin was measured using identical conditions as described for [^{3}H]ouabain binding. In this case the non-specific binding was higher (15–20% of the total in the K_d region) than for [^{3}H]ouabain. To unmask latent binding sites for ouabain or digoxin, 0.2 mg of saponin per ml was added to the incubation solution. [^{3}H]Ouabain and [^{3}H]digoxin were obtained from New England Nuclear and Amersham Corp., respectively.

Other methods. SDS-polyacrylamide gel electrophoresis was carried out as described [22] using 8% to 10% gels. Cholesterol and phospholipid contents were measured as described previously [6]. Protein concentration was measured according to the method of Hartree [23] using bovine serum albumin as standard.

Results

Protein and lipid composition

The T-tubule membrane fraction isolated from frog skeletal muscle has a rather complex protein composition, as evidenced by its electrophoretic pattern in SDS-containing polyacrylamide gels (Fig. 1). The protein bands of the T-tubule membranes isolated from frog muscle have sub-unit molecular weights of 220000, 115000, 92000, 70000, 55000 (a broad band) and ten bands of molecular weights ranging from 50000 to 25000. Although they differ in a few bands, the protein composition of T-tubules isolated from frog is similar to the protein composition of T-tubules isolated from rabbit skeletal muscle (Fig. 1). These protein patterns are considerably more complex than the protein composition of sarcoplasmic reticulum isolated either from rabbit or from frog muscle, both of which are characterized by a relatively simple protein composition dominated by the 100000 band of the Ca^{2+}-ATPase (Fig. 1).

In contrast to sarcoplasmic reticulum, the functional activities of only a few of the polypeptides present in T-tubules have been identified [24]. Furthermore, several of the protein bands present in T-tubules isolated from chicken or rabbit skeletal muscle correspond, presumably, to cytoplasmic proteins trapped inside the vesicular lumen during homogenization [7], as seems to be the case for rabbit serum albumin [6].

The total phospholipid and cholesterol contents of the frog T-tubules preparation are very similar to those of T-tubules isolated from rabbit muscle (Table I). Almost identical molar ratios of cholesterol to phospholipid were found for T-tubules isolated from either rabbit or frog muscle.

Integrity of the isolated vesicles

The proportion of sealed vesicles present in the preparation was determined by the difference in ouabain-sensitive $(Na^+ + K^+)$-ATPase activity and

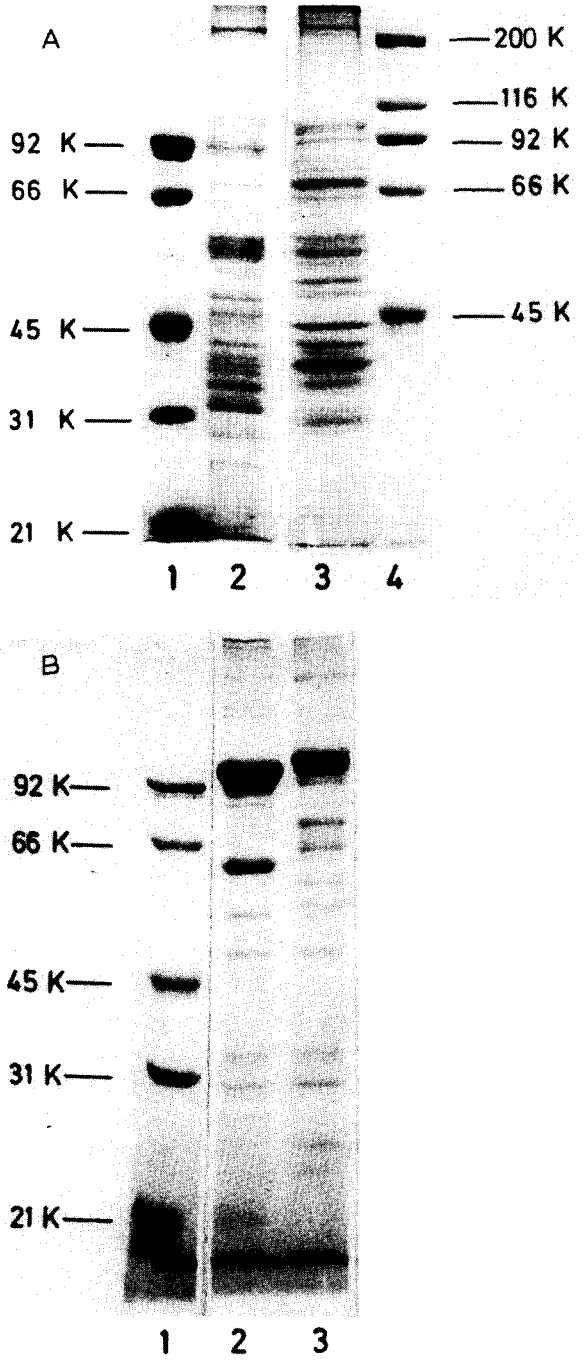

Fig. 1. SDS containing polyacrylamide gels of T-tubules and sarcoplasmic reticulum isolated from rabbit or frog skeletal muscle. (A) Lane 1, standards; lane 2, 40 μg of T-tubules isolated from frog muscle; lane 3, 40 μg of T-tubules isolated from rabbit muscle; lane 4, standards. (B) Lane 1, standards; lane 2, 25 μg of sarcoplasmic reticulum isolated from rabbit muscle; lane 3, 25 μg of sarcoplasmic reticulum isolated from frog muscle.

TABLE I

CHOLESTEROL AND PHOSPHOLIPID CONTENT OF ISOLATED T-TUBULE VESICLES

The molar ratio of cholesterol to phospholipid was calculated for each individual preparation. Numbers represent means ± S.D. The number of preparations used is in parenthesis. For experimental details, see Material and Methods. Values are expressed per mg of protein.

	Frog	Rabbit
Cholesterol (μmol·mg^{-1})	1.13 ± 0.17 (4)	1.01 ± 0.18 (2)
Phospholipid (P_i μmol·mg^{-1})	2.07 ± 0.21 (4)	1.88 ± 0.35 (2)
Cholesterol/phospholipid	0.55 ± 0.11 (4)	0.54 ± 0.01 (2)

ATP-dependent ouabain binding, measured with or without detergent in the assay solution. In the absence of detergents, only leaky vesicles should display ouabain sensitive ($Na^+ + K^+$)-ATPase or ATP-dependent ouabain binding, as expected from the fact that the binding sites for ATP and ouabain are located in opposite sides of the membrane, the ATP site being exposed to the cytoplasm and the ouabain site to the external medium. Several detergents have been used to unmask latent ATPase or ATP-dependent ouabain binding of muscle membranes [9,25–27]. We tested the effect of increasing concentrations of SDS, deoxycholate and saponin on both activities; in agreement with other studies in a different membrane system [28], we found that saponin gave consistently better results as unmasking agent. A narrow range of concentrations of SDS or deoxycholate is required to unmask activity, since higher concentrations inactivate the ($Na^+ + K^+$)-ATPase or reduce ATP-dependent ouabain binding. In contrast, saponin unmasked both activities without appreciable inactivation over a wider range of concentrations than deoxycholate or SDS. A comparison between the effectivity of deoxycholate and saponin as unmasking agents for ATP-dependent ouabain binding showed that only a fraction of the sites exposed by saponin have been unmasked by deoxycholate when binding starts to decrease following further increase in deoxycholate concentrations (Fig. 2).

A comparison between ($Na^+ + K^+$)-ATPase activity measured with or without saponin revealed that only a fraction of the total activity was

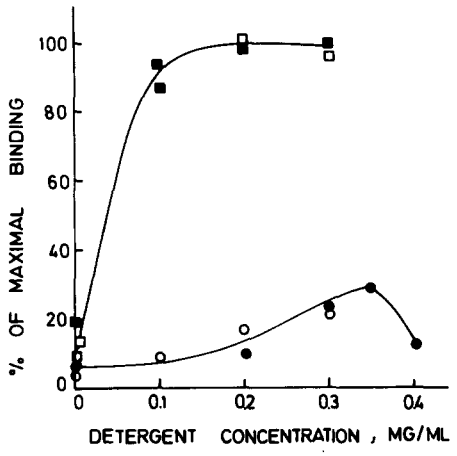

Fig. 2. Effect of saponin (□, ■) and deoxycholate (○, ●) as unmasking agents for ATP-dependent [^{3}H]ouabain binding. Values of 100% binding, measured in the presence of saponin, correspond to 228 pmol per mg of protein for T-tubules isolated from frog muscle, and to 170 pmol per mg for T-tubules isolated from rabbit muscle, Solid symbols, rabbit; open symbols, frog.

expressed in the absence of detergents (Table II). We calculate from the increase in activity observed in the presence of saponin that about 80% of the vesicles present in the preparation are sealed.

ATP-dependent ouabain binding was measured with or without saponin. Addition of saponin caused a marked stimulation in the ouabain binding sites (Table II), indicating that in agreement with the results obtained with the $(Na^+ + K^+)$-ATPase, 85% of the vesicles present in the preparation are sealed. ATP-dependent ouabain binding gave a single component, with a K_d of $11.1 \cdot 10^{-9}$ M [19] either in the absence or in the presence of detergents.

The $(Na^+ + K^+)$-ATPase activity and the ATP-dependent ouabain binding of T-tubules isolated from rabbit muscle, measured in the presence of saponin, give comparable values to those of T-tubules isolated from frog muscle (Table II). As judged by the increase in both activities caused by addition of saponin, the T-tubules isolated from rabbit muscle contain also mostly sealed vesicles (Table II).

Sidedness of the vesicles

To determine the sidedness of the sealed vesicles, we measured ATP-dependent binding of digoxin,

TABLE II

INTEGRITY AND SIDEDNESS OF ISOLATED T-TUBULES VESICLES

Integrity	Frog	Rabbit
ATPase activity (μmol·mg^{-1}·min^{-1})		
Total ATPase activity	0.53	3.15
Total ATPase activity + ouabain	0.40	2.98
$(Na^+ + K^+)$-ATPase	0.13	0.17
Total ATPase activity + saponin	1.17	2.93
Total ATPase activity + saponin + ouabain	0.55	1.96
$(Na^+ + K^+)$-ATPase + saponin	0.62	0.97
% sealed vesicles	79.0	82.5
Ouabain binding (pmol·mg^{-1})		
Control	35	28
+ Saponin	237	169
% sealed vesicles	85.2	83.4
Sidedness		
Digoxin binding (pmol·mg^{-1})		
Control	232	168
+ Saponin	220	155
% sealed outside-out [a]	none	none

[a] The % sealed outside-out (external side out) is calculated as the difference in digoxin binding with and without saponin.

a lipid-soluble derivative of ouabain. The same procedure was described elsewhere [27] to determine sidedness of T-tubules isolated from triads. In the absence of detergents, all vesicles with the ATP-binding site accessible (leaky and inside-out vesicles) will bind digoxin, and only vesicles sealed with the outside-out orientation will fail to do so. In the presence of detergents all vesicles will bind digoxin. Thus from the difference between digoxin binding with and without detergents the proportion of vesicles sealed with the outside-out orientation can be calculated. The same values for ATP-dependent digoxin binding were found with or without saponin (Table III), clearly indicating either that there are no vesicles sealed with the outside-out orientation, or that they represent a very small fraction of the total. A recent report, using T-tubules isolated from rabbit muscle by disruption of triadic junctions, also showed that 80–90% of the vesicles present in the preparation are sealed, and that all sealed vesicles have the inside-out orientation, as described by ATP-de-

pendent binding of ouabain and digoxin measured with and without SDS [27].

ATPase activities

The unmasked activity of the ouabain-sensitive ($Na^+ + K^+$)-ATPase of the T-tubules isolated from frog muscle (Table III) is higher than the values initially reported in T-tubule preparations isolated from chicken or rabbit muscle (see Discussion). We measured ($Na^+ + K^+$)-ATPase activity at different temperatures and in the presence of saponin for the frog preparation, to check whether assaying at 37°C caused enzyme inactivation, and we found steady increase in activity on raising the temperature from 25°C to 37°C (data not shown).

In agreement with other reports [7,8], we found negligible values of Ca^{2+}-ATPase activity and high values of a Mg^{2+}-ATPase that utilizes Mg-ATP or Ca-ATP as substrates (Table III). However, in contrast to the behavior or the enzyme present in T-tubules from rabbit, the Mg^{2+}-ATPase of the T-tubules from frog has a peculiar kinetic behavior. After addition of ATP to start the reaction, the amount of P_i liberated increased linearly only for a short period of time, after which the rate of P_i liberation decreased significantly (Fig. 3). Hence, we measured both the initial rate and the final rate and both activity values are given (Table IV). Addition of deoxycholate decreased somewhat the final rate, while addition of lysophosphatidylcholine or of saponin increased the final rates; none of these detergents abolished the biphasic pattern

TABLE III

ATPase ACTIVITIES OF ISOLATED T-TUBULES VESICLES

The Mg^{2+}-ATPase activity of the T-tubules isolated from frog muscle represents the initial rate of the reaction. For experimental details, see text. Numbers are given as means ± S.D.; in parenthesis is the number of preparations studied. The ($Na^+ + K^+$)-ATPase activity was measured with saponin.

	Frog	Rabbit
Mg^{2+}-ATPase ($\mu mol \cdot mg^{-1} \cdot min^{-1}$)	3.8 ± 0.2 (2)	3.4 ± 1.0 (2)
Ca^{2+}-ATPase ($\mu mol \cdot mg^{-1} \cdot min^{-1}$)	< 0.01 (5)	< 0.01 (2)
($Na^+ + K^+$)-ATPase ($\mu mol \cdot mg^{-1} \cdot h^{-1}$)	38.6 ± 1.5 (3)	50.4 ± 2.4 (2)

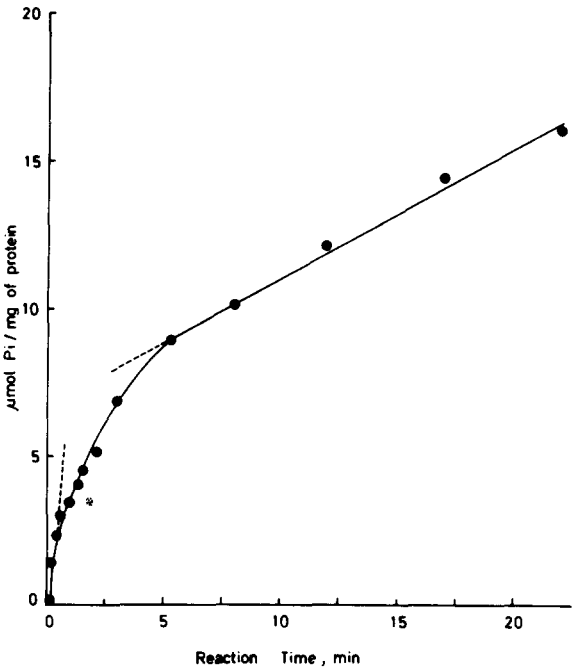

Fig. 3. Time-course of ATP hydrolysis by the Mg^{2+}-ATPase of the T-tubules isolated from frog muscle. ATP hydrolysis was measured at 25°C, as described in detail in the text.

TABLE IV

EFFECT OF DETERGENTS ON THE Mg^{2+}-ATPase ACTIVITY OF T-TUBULES ISOLATED FROM FROG MUSCLE

Rates are calculated as indicated in the experiment shown in Fig. 3. The rates are expressed in $\mu mol \cdot mg^{-1} \cdot min^{-1}$. The detergent concentration used ($mg \cdot ml^{-1}$) is given in parenthesis. lysoPC, lysophosphatidylcholine.

	Initial rate	Final rate
Control	3.72	0.44
+ deoxycholate (0.2)	3.78	0.26
+ saponin (0.2)	3.80	1.04
+ lysoPC (1.0)	6.71	1.20

(Table IV). Neither did changing the pH, the enzyme concentration or assaying the ATPase activity with Ca-ATP instead of Mg-ATP as substrate (data not shown).

Discussion

The results shown in this work indicate that T-tubules isolated from frog muscle share several

properties with T-tubules isolated from skeletal muscle of other vertebrates. In order to compare the properties of the different T-tubule preparations we have listed their cholesterol content, cholesterol to phospholipid molar ratios and ($Na^+ + K^+$)-ATPase activities in Table V, and their Mg^{2+}-ATPase and Ca^{2+}-ATPase activities in Table VI.

The T-tubules isolated from frog muscle have a high cholesterol content comparable to the highest values reported in the literature (Table V), and cholesterol to phospholipid molar ratios also comparable to other reported values. Thus, a high cholesterol content seems to be a general characteristic of all T-tubule preparations. It is likely that this high content of cholesterol is responsible for the marked lack of fluidity of the T-tubule membranes relative to other vertebrate membranes studied [39].

It was initially thought, based on ouabain binding measurements on whole and detubulated muscle fibers [40], that the density of ouabain receptors (i.e. of Na^+-pump molecules) was higher in the surface membrane than in the T-tubules. The first measurements of ($Na^+ + K^+$)-ATPase activity in isolated T-tubules (Table V) seemed to confirm this assumption, since much lower values than those reported in plasma membrane preparations [41] were found. However, more recent determinations of ($Na^+ + K^+$)-ATPase activity, including the present values, have revealed much higher activities (Table V), with values comparable to those reported for plasma membranes isolated from rabbit skeletal muscle [25]. It is interesting to note that the ratio of ($Na^+ + K^+$)-ATPase activity to cholesterol content for T-tubules still forming part of triadic junctions isolated from rabbit muscle [26] is around 58, very close to the ratios obtained in this work for the free T-tubules isolated from the same source (Table V). Hence, it is clear from

TABLE V

CHOLESTEROL CONTENT AND ($Na^+ + K^+$)-ATPase ACTIVITY OF T-TUBULES ISOLATED FROM DIFFERENT SOURCES

C/PL, cholesterol to phospholipid molar ratio. Values are given per mg of protein.

	($Na^+ + K^+$)-ATPase ($\mu mol \cdot mg^{-1} \cdot h^{-1}$)	Cholesterol ($\mu mol \cdot mg^{-1}$)	$\frac{(Na^+ + K^+)\text{-ATPase}}{\text{Cholesterol}}$	C/PL molar ratio
Rabbit				
Lau et al. [4,29]	6.2 [a]	0.64	9.7	0.40
Rosemblatt et al. [6]	12.0 [b]	0.90	13.3	0.55
Mitchell et al. [26]	39.0 [c]			
Mitchell et al. [26] [d]	4.61 [c]	0.08	57.6	0.13
Kirley and Schwartz [30]	48	0.94	51.1	
This work	50.4 [e]	1.01	49.9	0.54
Chicken				
Scales and Sabbadini [5]	3.2 [f]			
Sabbadini and Okamoto [7] / Sumnicht and Sabbadini [31]	26.9 [g]	0.94	27.9	0.86
Frog				
Narahara et al. [32]	8–21 [h]			
This work	38.6 [c]	1.13	34.2	0.55

[a] Measured in the presence of 2 M NaI.
[b] Extrapolated value determined at 30°C in the presence of Triton X-100.
[c] Measured at 37°C using SDS as unmasking agent.
[d] Values determined in T-tubules forming part of triadic junctions (pyrophosphate triads).
[e] Measured at 37°C using saponin as unmasking agent.
[f] Measured at 37°C.
[g] Measured at 37°C in the presence of valinomycin and monensin.
[h] Measured at 30°C.

TABLE VI

Mg^{2+}-ATPase AND Ca^{2+}-ATPase ACTIVITIES OF T-TUBULES ISOLATED FROM DIFFERENT SOURCES

Activities are presented in $\mu mol \cdot mg^{-1} \cdot min^{-1}$.

	T (°C)	Mg^{2+}-ATPase	Ca^{2+}-ATPase
Rabbit			
Lau et al. [4] [a]	37	0.431	0.922 [b]
Rosemblatt et al. [6] [c]	32	5.35	0.56
Hidalgo et al. [8] [d]	25	1.2–3.4	< 0.01 [b]
Kirley and Schwartz [31] [c]		12.7	2.33
Michalak et al. [32] [c]	37	0.052	0.024 [b]
This work [d]	25	3.4	< 0.01 [b]
Chicken			
Scales and Sabbadini [5] [c]	37	0.32	1.44 [b]
Malouf and Meissner [33] [d,e]	25	3.9	0.2 [b]
Malouf et al. [35] [d,e]	32	8	0 [b]
Sabbadini and Okamoto [7] [c]	25	3.9	0–0.15 [f]
Baskin and Kawamoto [36] [a]		0.43	0.12
Rat			
Beeler et al. [37] [d,e]	37	8.9 [g]	0
Mouse			
Mrack [38] [c]	24	3.1	
Frog			
Narahara et al. [32] [d]	30		< 0.13
This work [d]	25	3.8 [g]	< 0.01

[a] Isolated by disruption of triadic junctions by passage through a French press.

[b] Measured in the presence of ionophore A23187.

[c] Isolated by density and removing contamination sarcoplasmic reticulum by loading with insoluble salts of calcium (phosphate or oxalate).

[d] Isolated by density from contaminating sarcoplasmic reticulum.

[e] The authors did not specify whether the light membrane fraction used in these studies contained T-tubules and/or surface membranes.

[f] Measured in the presence of ionophore X537A.

[g] Values represent initial rates.

these results that the T-tubules isolated from rabbit muscle by density fractionation have the same proportion of cholesterol and $(Na^+ + K^+)$-ATPase as those described for T-tubules still forming part of triads (junctional T-tubules). This is an important observation, since comparisons of $(Na^+ + K^+)$-ATPase to cholesterol ratios might allow a distinction between surface membranes and T-tubules. Surface membranes have similarly $(Na^+ + K^+)$-ATPase activities but less cholesterol than T-tubules [41], hence lower ratios should be obtained for the latter. In agreement with this proposal we have found that preparations enriched in surface membranes (isolated from frog muscle as described elsewhere [19]) have the same $(Na^+ + K^+)$-ATPase activity as T-tubules but lower cholesterol content [41]. Thus, in frog muscle the ratio of $(Na^+ + K^+)$-ATPase to cholesterol is 60 or higher for the surface-membrane rich preparations [41], and around 39 for T-tubules (Table V). Furthermore, it is likely that highly purified surface membranes will have an even higher ratio of $(Na^+ + K^+)$-ATPase to cholesterol than those displayed by the partially purified preparations.

In addition to a high $(Na^+ + K^+)$-ATPase activity, T-tubules isolated from frog muscle display no detectable Ca^{2+}-ATPase activity and high initial values of Mg^{2+}-ATPase activity. It was initially reported that T-tubules isolated from chicken or rabbit muscle had significant Ca^{2+}-ATPase [4–6]. More recent reports, however, all agree in describing little or no activity (Table VI), making it likely that the Ca^{2+}-ATPase values initially described represented residual sarcoplasmic reticulum contamination.

The Mg^{2+}-ATPase activity is considerably lower for T-tubules isolated by mechanical disruption of triadic junctions than for T-tubules isolated directly by density (Table VI), with only two exceptions [5,33]. A low Mg^{2+}-ATPase activity was reported for T-tubules isolated by density from chicken [5] muscle. However, Sabbadini and Okamoto [7] using a similar preparation found much higher Mg^{2+}-ATPase activity than that initially described by Scales and Sabbadini [5]. Thus, it is apparent that in all cases, except one [33], a high Mg^{2+}-ATPase activity is a characteristic of T-tubules isolated by density. The fact that the Mg^{2+}-ATPase of T-tubules isolated by mechanical disruption of triadic junctions is about 10-times lower suggests that the activity of this enzyme depends on unknown factors that cause a decrease in activity after French press treatment. It is interesting to recall in this regard that the Mg^{2+}-ATPase activity of a light membrane fraction containing T-tubules and or surface membrane isolated from rat muscle [37], and the Mg^{2+}-ATPase of T-tubules isolated from frog are both inhibited during the course of the reaction. In the case of the

Mg^{2+}-ATPase of rat muscle [37], either ATP or a non-hydrolyzable ATP analog inhibit the enzyme; the inhibition can be prevented by addition of concanavalin A, wheat germ agglutinin or rabbit antiserum against the membrane. A large variety of ionic and non-ionic detergents increase the rate of ATP-dependent inactivation, and cross linking with glutaraldehyde prevents it and decreases the sensitivity of the enzyme to detergents [37]. It was proposed from these results that the inactivation of the Mg^{2+}-ATPase by ATP was related to the mobility of the enzyme in the membrane, increasing the mobility with detergents increased inactivation, and decreasing the mobility by cross-linking the membrane proteins with lectins, antiserum or glutaraldehyde, prevented inactivation [37]. The present results, however, show that the Mg^{2+}-ATPase activity of T-tubules isolated from frog muscle is affected differently by various detergents, all of which, presumably, increase the mobility of the enzyme in the membrane. Thus, we found that deoxycholate increased inactivation, while saponin and lysophosphatidylcholine exerted a partially protective effect. Accordingly, we cannot conclude that the enzyme found in T-tubules isolated from frog muscle is regulated by a similar mechanism to that proposed for the Mg^{2+}-ATPase present in the light membrane fraction isolated from rat muscle [39]. Clearly more information is needed regarding the regulation of the Mg^{2+}-ATPase of T-tubules, which, in turn, might shed some light on its so far unknown physiological role.

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