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Biochemical Characterization, Integrity, and Sidedness of Purified Skeletal Muscle Triads*

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The release of Ca^{2+} from the terminal cisternae of sarcoplasmic reticulum in muscle fiber triggers muscle contraction. The signal for Ca^{2+} release is mediated via the triad junction, *i.e.* the junctional association of terminal cisternae and transverse tubule. Recently, highly purified morphologically intact triads were isolated from rabbit skeletal muscle (Mitchell, R. D., Palade, P., and Fleischer, S. (1983) *J. Cell Biol.* 96, 1008–1016). In this study, biochemical characterization of two variants of purified triad preparations (Pyrophosphate and Standard) is provided.

Terminal cisternae of triads sequester Ca^{2+} at rates comparable to those of purified heavy sarcoplasmic reticulum which is referable to terminal cisternae (Meissner, G. (1975) *Biochim. Biophys. Acta* 389, 51–68). The permeability for calcium ions, as reflected by a 2–3-fold stimulation of (Ca^{2+},Mg^{2+})-ATPase activity in the presence of the Ca^{2+} ionophore A23187, and by the Ca^{2+} leak rate, is comparable in triads and heavy sarcoplasmic reticulum. Several transverse tubule characteristics are present in triads. Four of them, *i.e.* cholesterol content, ouabain binding, dihydroalprenolol binding (β-adrenergic receptor), and ouabain-sensitive (Na^+,K^+)-ATPase activity, are comparably enriched in the Pyrophosphate triads and therefore appear to be quantitative indices of the amount of transverse tubule. Adenylate cyclase and basal ATPase are unreliable in this regard.

Methodology for analyzing membrane integrity and sidedness was applied (adenylate cyclase activity) and modified (ouabain-sensitive (Na^+,K^+)-ATPase activity) to characterize the transverse tubule of the triad. In addition, a new method was developed making use of ouabain binding to study sidedness. These studies show that the transverse tubule is largely sealed and inside out in orientation, *i.e.* with the cytoplasmic face exposed.

This report indicates that the t-tubule and sarcoplasmic reticulum components of the triads possess transport capability and retain permeability barriers for ions. Therefore, the isolated triads appear to be suitable for studying the physiological Ca^{2+} release process *in vitro*.

In vertebrate skeletal muscle fibers, the release of Ca^{2+} from the terminal cisternae of the SR[1] triggers muscle contraction. Physiologically Ca^{2+} release occurs in response to t-tubule membrane depolarization by an action potential. The link between t-tubule depolarization and Ca^{2+} release is poorly understood (1) and the molecular mechanism of the Ca^{2+} release from SR remains obscure (1). The isolation and characterization of purified triads[2] are a direct approach to the study of the nature of this intracellular junction and the Ca^{2+} release process. The isolation of purified triads has recently been achieved (2). The morphology of the isolated triad was found to have close resemblance to the *in situ* structure (3).

Besides purity (2) and retention of morphology (3), isolated triads must display functional integrity to be of use in Ca^{2+} release studies. In this report, we have characterized isolated triads with regard to: 1) enzymic criteria of the t-tubule and SR, *i.e.* the component organelles of triad; 2) transport capability and permeability barriers for ions; and 3) membrane integrity and sidedness.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were reagent grade from Fisher (Pittsburgh, PA) unless otherwise stated. Density gradient grade sucrose was obtained from Schwarz/Mann (Orangeburg, NY) and from EM laboratories (Elmsford, NY). SDS was obtained from BDH Chemicals Ltd. (Poole, England). [α-^{32}P]ATP, [^{3}H]cAMP (30–50 Ci/mmol), [^{3}H]ouabain (19.3 Ci/mmol), and *levo*-[*propyl*-1,2,3-^{3}H]dihydroalprenolol hydrochloride (40.6 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Myokinase, GPP(NH)p, adenosine deaminase, arsenazo III (grade 1), Na_2-ATP (predominantly vanadate-free), ouabain, L-alprenolol-D-tartrate, valinomycin, ruthenium red, quercetin, and creatine phosphate were obtained from Sigma (St. Louis, MO). Creatine phosphokinase was obtained from both Sigma and Boehringer

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; t-tubule, transverse tubule; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; GPP(NH)p, guanyl-5'-yl imidodiphosphate; DHA, dihydroalprenolol.

[2] The term triad derives from electron microscopy of muscle in which the transverse tubule apposed between two terminal cisternae of sarcoplasmic reticulum, each in junctional association. The term "triad" will be used to denote a structure composed of one or more junctional associations of transverse tubule with terminal cisternae. In this context, triad is not intended to suggest that it necessarily consists of three components but rather refers to a structure consisting of two or more components in junctional association. Of the "probable" triad structures observed in thin section electron microscopy, there were on the average 1.45 and 1.53 terminal cisternae to t-tubules for Standard and Pyrophosphate triads, respectively (2).

Mannheim (Indianapolis, IN). Monensin and A23187 were obtained from Calbiochem (La Jolla, CA). Alamethicin was a gift of Dr. George B. Whitfield of the Upjohn Company (Kalamazoo, MI). Forskolin was isolated from the roots of *Coleus forskholii* and was a gift of Dr. David Garbers, Vanderbilt University, Nashville, TN.

Preparation of Triads

Female New Zealand White rabbits (2–3 kg) were obtained from Hilltop Rabbits (Columbia, TN) and maintained at the Vanderbilt University Animal Care Center. The predominantly fast twitch muscles of the hind-limbs were used, together with the back muscles.

Purified triads were isolated using either of two variants, as previously described (2). Briefly, the Standard variant involved isolation of microsomes by homogenization of ground muscle in 10% (w/w) sucrose, 0.5 mM EDTA, pH 7.2. Microsomes were then placed on a sucrose gradient to separate light SR from triad SR. A second discontinuous sucrose gradient, containing 20 mM $Na_4P_2O_7$, 20 mM NaH_2PO_4, and 1 mM $MgCl_2$ (pyrophosphate mix) served to disaggregate contractile proteins, and to further purify the triad preparation. A variation of the Standard method was also used (Pyrophosphate variant) which incorporated the pyrophosphate mix in the homogenization media as well as in all solutions in the subsequent steps. The major difference is that the Standard variant was exposed to the pyrophosphate mix for approximately 6 h, whereas the Pyrophosphate variant was exposed throughout the preparation, approximately 12 h.

All data reported in the text were obtained on purified triads (stage II, band IV, in Ref. 2) which, after isolation, were resuspended in 10% (w/w) sucrose, 5 mM K-HEPES, pH 7.2, unless otherwise stated. All assays were carried out on samples which were either never frozen or were frozen and thawed only one time.

Protein concentration was determined according to Lowry *et al.* (4) using bovine serum albumin as the standard. Phosphorus was measured by the method of Chen *et al.* (5) as described in Rouser and Fleischer (6).

Pretreatment of Triads with Alamethicin or SDS

In order to expose latent plasma membrane enzymic activities in triads containing sealed t-tubules, samples were preincubated with SDS or alamethicin. When pretreated with alamethicin, the triads (1–3 mg/ml) were preincubated for 20 min at room temperature (7) with varying concentrations of alamethicin added from a stock solution (30 mg/ml in absolute ethanol). The final concentration of alamethicin are expressed on a weight basis relative to protein. The amount of ethanol added ranged from 0.25 to 3.3% (v/v). When pretreated with SDS, the triads (1 mg/ml) were preincubated for 30 min at room temperature with varying concentrations of SDS in a medium containing 40 mM imidazole-HEPES and 2 mM Tris-EDTA, pH 7.5 (8).

It remains to be resolved whether alamethicin at the concentration used causes leakiness by forming channels in the membrane (9) or by a detergent-like action (10). Since SDS inactivates adenylate cyclase activity (7), in most of these studies, alamethicin was used to estimate sidedness and integrity of t-tubules. A concentration curve is required to optimize activity when alamethicin or SDS is used to study latency.

Adenylate Cyclase

Adenylate cyclase activity was determined according to a modification of the method of Jakobs *et al.* (11), as previously described (2). The assay was carried out for 5 min at 37 °C in a medium containing 25 mM Tris-Cl, pH 7.5, 5 mM $MgCl_2$, and 20–50 μg of protein in a final volume of 0.2 ml. Phosphodiesterase activity and production of competitive inhibitors were precluded as previously described (2).

Cholesterol Content

Total cholesterol (both free cholesterol and cholesterol esters) was measured as described by Allain *et al.* (12) using a Sigma kit (No. 350 B). These measurements were carried out until production of quinone-imine dye was complete (60 min, *i.e.* a much longer incubation than suggested by Sigma). The absorbance of the quinone-imine dye was recorded at 500 nm, using a Hewlett-Packard 8450A spectrophotometer, and found to be linear with respect to cholesterol in the range of concentrations used. Each determination was performed in duplicate, using 200–300 μg of protein.

[^{3}H]Dihydroalprenolol Binding

DHA binding was carried out as described by Caswell *et al.* (13), using about 200 μg of protein for each determination. [^{3}H]DHA concentration ranged from 0.25 to 16.66 nM. Specific binding was defined as the difference between total and nonspecific binding, which was measured in the presence of 10 μM L-alprenolol. The maximal number of binding sites was determined by means of a Scatchard plot. The assay was carried out in the absence of any detergent since alprenolol is membrane permeable.

(Na^+,K^+)-ATPase

Triads (1–3 mg of protein/ml) were pretreated with varying concentrations of detergent as described above. The assay was carried out at 37 °C as previously reported (2, 8). Phosphate production was measured using a modification (8) of the method of Ottolenghi (14). Ouabain-sensitive (Na^+,K^+)-ATPase activity is defined as the difference between total ATPase, and basal ATPase, *i.e.* the amount of phosphate released in the absence and presence of ouabain, respectively.

[^{3}H]Ouabain Binding

The quantitation of the ouabain-binding sites was carried out using either of two procedures which are described below.

A. [^{3}H]Ouabain Binding Promoted by Phosphate and Magnesium (Mg^{2+}-P_i-dependent Ouabain Binding)—Triads were equilibrated for 18–20 h, in the coldroom on ice, in a medium containing 3 mM Tris-PO_4, 3 mM $MgSO_4$, 10 mM Tris-Cl, pH 7.2, and 10% (w/w) sucrose (see "Results"). Triads were then pretreated, where applicable, with either alamethicin or SDS, as described above. The assay, in a final volume of 0.25 ml, was carried out at 37 °C in a medium containing 3 mM Tris-PO_4, 3 mM $MgSO_4$, 10 mM Tris-Cl, pH 7.2, 1 mM EGTA, 1 μM [^{3}H]ouabain, and 40–50 μg of protein, in the presence or absence of 0.1 mM unlabeled ouabain. After a 30-min incubation, 0.22 ml was filtered through a 0.22-μm Millipore filter, which was immediately washed with 15 ml of ice-cold buffer (without ouabain). The filters were dried and then dissolved in 0.5 ml of ethylene glycol monoethyl ether (Cellosolve) for 30–45 min before adding 10 ml of aqueous counting scintillant (ACS, The Radiochemical Centre, Amersham, U. K.) and determining radioactive content. Specific ouabain binding was defined as the difference between total and nonspecific binding, *i.e.* binding measured in the absence and in the presence of 0.1 mM unlabeled ouabain, respectively.

B. [^{3}H]Ouabain Binding Promoted by ATP and Sodium Ion (ATP-dependent Ouabain Binding)—The assay, in a final volume of 0.25 ml, was performed at 37 °C for 30 min in a medium which contained 40 mM Tris-Cl, pH 7.4, 120 mM NaCl, 1 mM EGTA, 10 mM $MgSO_4$, 1 μM [^{3}H]ouabain, and 40–50 μg of protein. Total binding was measured in the presence of 10 mM Na_2-ATP; nonspecific binding with 0.1 mM unlabeled ouabain, and without 10 mM Na_2-ATP. Filtration, washing of the filter, and counting were carried out as described in procedure A.

(Ca^{2+},Mg^{2+})-ATPase

(Ca^{2+},Mg^{2+})-ATPase activity was measured at 25 °C in a medium containing, in a final volume of 0.5 ml, 100 mM KCl, 5 mM $MgSO_4$, 5 mM Na azide, 0.16 mM Tris-EGTA, 0.2 mM $CaCl_2$, 10 mM K-HEPES, pH 7.0, and 10 μg of protein, in the absence and presence of 1.5 μM A23187. After 5-min preincubation at 25 °C, the reaction was started by adding 5 mM Tris-ATP and stopped after 20, 40, 60, and 80 s with 0.5 ml of 20% trichloroacetic acid, 12% ascorbic acid, 1% ammonium molybdate. Phosphate production was determined using a modification of the method of Ottolenghi (14). Rates were determined by least squares fit. Calcium-independent, Mg^{2+}-stimulated ATPase was measured in the presence of 1 mM Tris-EGTA, without addition of 0.2 mM $CaCl_2$, and accounted for 2–4% of the total (+A23187) ATPase activity.

Phosphate-facilitated Ca^{2+}-loading Rate

The Ca^{2+}-loading rate was determined using the metallochromic indicator arsenazo III (15), by following ΔA ($A_{660} - A_{740}$) in a Hewlett-Packard 8450A spectrophotometer. The assay was carried out at 25 °C in a medium containing, in a final volume of 1 ml, 125 mM potassium phosphate, pH 7.0, 1 mM $MgCl_2$, 1 mM Na_2-ATP, 10 μM arsenazo III, and 20–50 μg of protein. The reaction was initiated by adding 25 nmol of $CaCl_2$. In some of the assays, 20 μM ruthenium red was added just prior to the addition of Ca^{2+}.

Oxalate-facilitated Ca^{2+}-loading Rate

The assay was carried out as described for the phosphate-facilitated loading except that the medium contained 10 mM K-HEPES, pH 7.0,

100 mM KCl, 5 mM K oxalate, 1 mM $MgCl_2$, 1 mM Na_2-ATP, 10 μM arsenazo III, and 20–50 μg of protein.

Passive Calcium Diffusion (Ca^{2+} Leak)

Calcium uptake was carried out at 25 °C in a medium containing 100 mM KCl, 10 mM $MgSO_4$, 1 mM Na_2-ATP, 5 mM creatine phosphate, 20 μg/ml creatine phosphokinase, 10 μM arsenazo III, 10 mM K-HEPES, pH 7.0, and 300–360 μg of protein, in a final volume of 1 ml. The spectrophotometric assay was used to monitor uptake of 25 nmol of $CaCl_2$. At the completion of Ca^{2+} uptake, 300 μM quercetin (stock solution, 40 mM in absolute ethanol) was added to stop Ca^{2+} pump activity (16, 17). In some of the assays, 20 μM ruthenium red was added prior to quercetin addition. The Ca^{2+} leak was followed for 5–10 min. At the end of this time period, 25 nmol of $CaCl_2$ was added to recalibrate the arsenazo III. The assay conditions were designed to prevent pump reversal (absence of ADP, presence of quercetin (17, 18)), and spontaneous Ca^{2+} release (low calcium-preloading levels and high Mg^{2+} (17)).

SDS-Slab Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out according to Laemmli (19) using a stacking gel of 4.5% (w/v) acrylamide and a linear gradient separating gel of 7.5–15% acrylamide. The thickness of the slab gel was 0.75 mm. Electrophoresis was carried out with a current of 20 mA/slab and was stopped when the marker dye (0.01% bromphenol blue) had reached the bottom of the slab. The gel was stained in a solution containing 0.25% Coomassie brilliant blue G in 10% (v/v) methanol and 10% (v/v) acetic acid for 30 min at 50 °C and destained in 10% methanol, 10% acetic acid at room temperature. The protein standards used for estimation of molecular weights (see Fig. 1) were obtained from Bio-Rad (Richmond, CA). Densitometric scanning of slab gels was performed using a densitometer (E-C Apparatus Corp.) with a 557-nm band pass filter.

RESULTS

General Characteristics of Triads

Two types of purified triads were available for study (2). The methodology of isolation was similar for both and differed only in terms of the point of introduction of the pyrophosphate mixture which served to disaggregate contractile proteins enabling more effective separation. The Standard variant was exposed to this pyrophosphate mixture beginning with the second gradient centrifugation (~6 h), whereas the Pyrophosphate variant was exposed throughout the isolation procedure from the homogenization of the muscle onward (~12 h).

SDS-polyacrylamide gel electrophoresis of purified triads is shown in Fig. 1. Standard and Pyrophosphate triads exhibited similar protein composition. The predominant bands are referable to heavy SR (20), *i.e.* Ca^{2+} pump protein and calcium-binding protein or calsequestrin (20, 21). Several minor differences were noted between the two variants: 1) somewhat higher ratio of Ca^{2+} pump protein relative to calcium-binding protein in Standard triads (see also Table I); the calcium-binding protein is considered an indicator of intact heavy SR (20, 21); and 2) slight enrichment of minor protein bands of 60,000, 28,000, and 26,500 daltons in Pyrophosphate triads.

The phospholipid content as reflected by the bound phosphorus was 0.65 and 0.73 μmol/mg of protein for Pyrophosphate and Standard triads, respectively (Table I). The molar ratio of cholesterol to phospholipid was nearly 2-fold greater in Pyrophosphate triads (Table I), in the same proportion as other t-tubule characteristics (see below).

The triad component organelles are sarcoplasmic reticulum and transverse tubule. Characteristics referable to each of the component organelles are summarized in Table I. Ca^{2+}-loading rate and (Ca^{2+},Mg^{2+})-ATPase activity approximated those for purified heavy SR (20), the major component of the triads, and were consistently 20% higher for the Standard variant than for the Pyrophosphate variant. The Ca^{2+}-loading rate

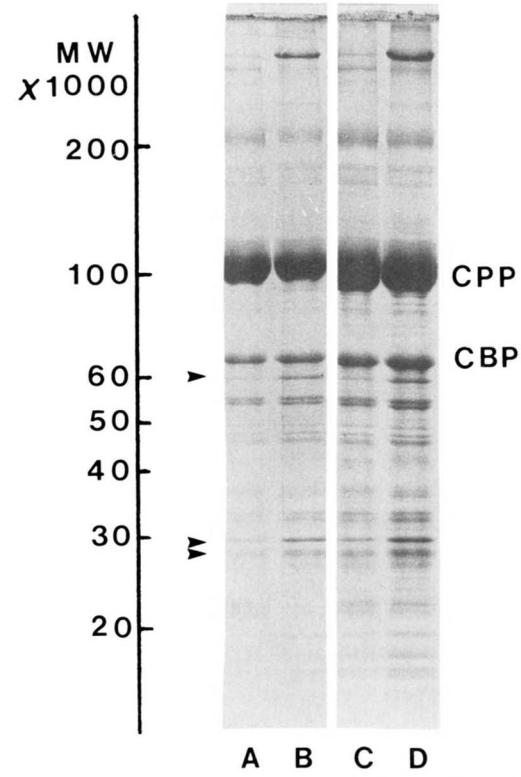

FIG. 1. **SDS-polyacrylamide slab gel electrophoresis of purified triads.** Standard (*A* and *C*) and Pyrophosphate (*B* and *D*) triads were isolated as described under "Experimental Procedures." Electrophoresis was carried out as described by Laemmli (19), using a linear polyacrylamide gel gradient (7.5–15%). About 8 μg (*A* and *B*) or 16 μg (*C* and *D*) of protein was applied per lane. *CPP*, Ca^{2+} pump protein; *CBP*, calcium-binding protein. Apparent molecular weight values were estimated from graphs of relative mobilities *versus* log of molecular weight using myosin heavy chain (200,000), galactosidase (116,500), phosphorylase (98,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), and lysozyme (14,300) as standards. There is a slight enrichment in the Pyrophosphate variant of three minor bands of 60,000, 28,000, and 26,500 Da which are denoted by *arrowheads*.

was enhanced by ruthenium red (22), 1.5-fold for the Standard variant and 2-fold for the Pyrophosphate variant. Thus, in the presence of ruthenium red, the Ca^{2+}-loading rates of both preparations were nearly the same.

Ouabain binding, DHA binding, and ouabain-sensitive (Na^+,K^+)-ATPase activity are characteristics of sarcolemma and t-tubule (7, 8, 13, 23, 24). DHA binding is a measure of the β-adrenergic receptor of the adenylate cyclase system (13). Scatchard plot analysis of DHA binding showed a single class of binding sites (13) with similar K_D for both Standard and Pyrophosphate triads (Table I). All three characteristics were present in significant amounts and were similarly enriched 1.5–1.9 fold in Pyrophosphate *versus* Standard triads. These findings suggest that there is more t-tubule in the Pyrophosphate as compared with Standard triads.

Basal adenylate cyclase (2, 13) and ouabain-insensitive basal ATPase (25, 26) are also considered to be diagnostic of sarcolemma and t-tubule. Therefore, it seemed puzzling at first that these two activities were found to be approximately half as high in Pyrophosphate triads as in Standard triads (Table I). Experiments were performed to evaluate whether the relatively lower activity of adenylate cyclase in the Pyrophosphate triads could be reactivated with modulators. The catecholamine-sensitive adenylate cyclase system is composed of at least three proteins (27): 1) the hormone receptor; 2) the

TABLE I

Characteristics of purified triads

Triads were isolated and all measurements were carried out as described under "Experimental Procedures." Ca^{2+}-loading rates measured in the presence of 5 mM K oxalate were found to be slightly lower than those measured in 125 mM K phosphate, the differences between Pyrophosphate and Standard being conserved (data not shown). (Ca^{2+},Mg^{2+})-ATPase activity of heavy SR, isolated according to Meissner (20), was stimulated 2-fold by A23187. The percentage of total protein represented by the Ca^{2+} pump protein (CPP) and calcium-binding protein (CBP) was determined by densitometry of SDS-polyacrylamide slab gels (Fig. 1), after staining with Coomassie blue. The molar ratio of CPP/CBP was determined by assuming M_r = 105,000 and 65,000 (20) for the CPP and CBP, respectively. A molar ratio of approximately 3 was reported for purified heavy SR (20). Ouabain-sensitive (Na^+,K^+)-ATPase activity was the difference between the activity measured in the absence (total) and in the presence (basal) of 1 mM ouabain, after preincubation with optimized detergent concentrations (0.14–0.18 mg of SDS/mg of protein or 0.4–0.6 mg of alamethicin/mg of protein). Maximal ouabain binding was determined after preincubation with optimized concentrations of alamethicin (0.4–0.6 mg/mg of protein). Similar values were obtained when SDS was used as detergent. Basal adenylate cyclase activity was measured in the presence of 5 mM $MgCl_2$. The maximal number of DHA-binding sites and K_D were determined by Scatchard plot analysis. The values reported are means ± S.D. for the number of determinations on different triad preparations shown in parentheses.

Characteristic	Standard triads	Pyrophosphate triads	Ratio pyrophosphate/Standard
Bound phosphorus (μmol/mg protein)	0.73 ± 0.14 (4)	0.65 ± 0.11 (5)	0.89
Cholesterol (μmol/mg protein)	0.051 ± 0.009 (3)	0.083 ± 0.006 (5)	1.62
Chol/PL (molar ratio)[a]	0.07	0.13	1.86
Sarcoplasmic reticulum			
Phosphate-facilitated Ca^{2+}-loading rate (μmol/min/mg protein)			
−Ruthenium red	1.38 ± 0.14 (3)	1.15 ± 0.18 (3)	0.86
+Ruthenium red (20 μM)	2.10 ± 0.12 (3)	2.31 ± 0.42 (3)	1.10
(Ca^{2+},Mg^{2+})-ATPase activity (μmol P_i/min/mg protein)			
−A23187	1.50 ± 0.21 (3)	1.26 ± 0.19 (3)	0.84
+A23187 (1.5 μM)	3.75 ± 0.47 (3)	2.65 ± 0.44 (3)	0.71
CPP/CBP (molar ratio)	4.14 ± 0.39 (3)	3.13 ± 0.19 (3)	0.76
T-tubule			
Ouabain-sensitive (Na^+,K^+)-ATPase (μmol P_i/h/mg protein)	2.86 ± 0.62 (6)	4.61 ± 0.90 (6)	1.61
Basal (ouabain-insensitive) ATPase (μmol P_i/h/mg protein)	7.98 ± 1.82 (6)	3.79 ± 0.43 (6)	0.47
Maximal ouabain binding (pmol/mg protein)	5.07 ± 0.67 (5)	9.62 ± 1.39 (5)	1.90
Basal adenylate cyclase (pmol/min/mg protein)	57.5 ± 8.5 (4)	27.1 ± 7.0 (4)	0.47
DHA binding			
Maximal number of binding sites (pmol/mg protein)	0.258 ± 0.027 (5)	0.394 ± 0.038 (4)	1.53
K_D (nM)	1.70 ± 0.23 (5)	1.84 ± 0.19 (4)	

[a] The cholesterol to phospholipid (Chol/PL) molar ratio was calculated assuming the bound phosphorus to be entirely referable to phospholipid.

regulatory subunit; and 3) the catalytic subunit responsible for the conversion of ATP to cAMP. Hormone activation can be bypassed using agents acting on the regulatory subunit (GPP(NH)p (28), fluoride (28), or high Mg^{2+} (20 mM) (29)), or on the catalytic subunit, *e.g.* Mn^{2+} (29, 30). Additionally, adenylate cyclase activity is greatly enhanced by forskolin, a diterpene effector. At present, the site of action of forskolin is not well defined, but is either on the regulatory subunit (31), on the catalytic subunit (32–35), or on both (36, 37).

Results obtained with Standard triads are shown in Fig. 2*A*. Although Standard and Pyrophosphate triads each displayed activation using 10 mM NaF, 200 μM GPP(NH)p, 20 mM Mg^{2+}, 3 mM Mn^{2+}, or 200 μM forskolin, data normalized against DHA binding (Fig. 2*B*) showed no preferential increase in Pyrophosphate triads. These experiments indicate that the catalytic subunit of adenylate cyclase was partially inactivated or extracted in the Pyrophosphate triads.

Transport Capability and Permeability Barrier for Ions

The calcium-pumping characteristics of the terminal cisternae of the triads are presented in Table I and have already been discussed above. The addition of the Ca^{2+} ionophore A23187 stimulated the (Ca^{2+},Mg^{2+})-ATPase activity 2- to 3-fold in both preparations (Table I). This enhancement of ATPase activity is indicative of a sealed SR compartment, largely impermeant to calcium ions.

The passive Ca^{2+} leak rate was measured more directly by actively preloading 60–70 nmol of Ca^{2+}/mg of protein in the absence of a precipitating anion and then inhibiting the Ca^{2+} pump with quercetin (16). The Ca^{2+} leak appeared to be at least biphasic as determined by a semilog plot of Ca^{2+} remaining in SR *versus* time (not shown). The faster phase had a half-time of about 25–28 s and a first order rate constant of approximately 0.025 s^{-1} (Table II). These values are in close agreement with those already reported for sarcoplasmic reticulum (38, 39). The first order rate constant of the fast phase was decreased approximately 2-fold by ruthenium red (Table II). The Ca^{2+} leak characteristics were comparable for Pyrophosphate and Standard triads as well as for heavy SR (Table II). These studies demonstrate that the SR of the triads behaves similarly to heavy SR with regard to energized Ca^{2+} accumulation (20) and Ca^{2+} leak rate.

The t-tubules of the triads, which contain (Na^+,K^+)-ATPase, are capable of energized Na^+ uptake (Ref. 40 and Table V). Later in the results, the t-tubules are documented to be sealed and inside out.

Analysis of Membrane Sidedness and Integrity of t-Tubule

t-Tubules can exist in three configurations with regard to membrane sidedness: 1) sealed right-side out, oriented with their extracellular face exposed; 2) sealed inside out, oriented with their cytoplasmic face exposed; or 3) leaky, with both

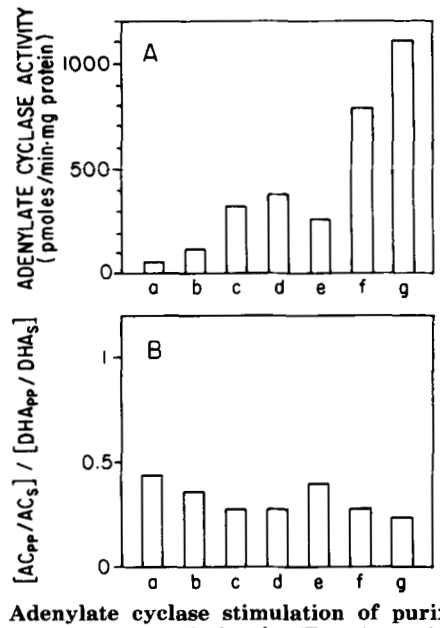

FIG. 2. **Adenylate cyclase stimulation of purified triads.** Triads were isolated as described under "Experimental Procedures." Adenylate cyclase activity was measured in the presence of: *a*, 5 mM $MgCl_2$ (basal); *b*, 5 mM $MgCl_2$, 3 mM $MnCl_2$; *c*, 20 mM $MgCl_2$; *d*, 20 mM $MgCl_2$, 3 mM $MnCl_2$; *e*, 5 mM $MgCl_2$, 3 mM $MnCl_2$, 200 μM GPP(NH)p; *f*, 5 mM $MgCl_2$, 3 mM $MnCl_2$, 10 mM NaF; *g*, 5 mM $MgCl_2$, 3 mM $MnCl_2$, 200 μM forskolin. In *A*, the results obtained for Standard triads are shown. In *B*, the equivalent data obtained for Pyrophosphate triads are expressed as the ratio of adenylate cyclase activity of Pyrophosphate/Standard variants after normalization by DHA binding (0.434 and 0.264 pmol/mg of protein, for Pyrophosphate and Standard triads, respectively). By this normalization procedure, restoration of adenylate cyclase activity in Pyrophosphate triads would be reflected by an activity ratio of 1.0. *AC*, adenylate cyclase; *DHA*, dihydroalprenolol binding; *PP*, Pyrophosphate triads; *S*, Standard triads.

TABLE II

Effect of ruthenium red on Ca^{2+} leak

Triads were isolated as described under "Experimental Procedures" and heavy SR according to Meissner (20). Ca^{2+} leak was monitored, using arsenazo III as the calcium indicator. Triads and heavy SR (300–360 μg of protein) were preloaded in the absence of precipitating anions with 25 nmol of $CaCl_2$, *i.e.* below their maximal capacity. At the completion of Ca^{2+} uptake, 300 μM quercetin was added to stop Ca^{2+} pumping. Ruthenium red (20 μM) was added, where applicable, just prior to the addition of quercetin. At the end of each experiment a 25-nmol $CaCl_2$ pulse was added to recalibrate the arsenazo III. Ca^{2+} leak was biphasic as determined by a semilog plot of Ca^{2+} remaining in SR *versus* time. In the table, the first order rate constant (k) and the $t_{1/2}$ of the fast phase are given. The $t_{1/2}$ for Ca^{2+} uptake of triads was approximately 6 s.

Fraction	k	$t_{1/2}$
	s^{-1}	*s*
Pyrophosphate triads		
−Ruthenium red	0.0245 ± 0.0021 (3)	28.4 ± 2.5 (3)
+Ruthenium red	0.0116 ± 0.0018	60.7 ± 9.3
Standard triads		
−Ruthenium red	0.0269 ± 0.0027 (3)	25.9 ± 2.6 (3)
+Ruthenium red	0.0150 ± 0.0015	46.6 ± 4.6
Heavy SR		
−Ruthenium red	0.0307	22.6
+Ruthenium red	0.0150	46.2

faces exposed. Estimates of both membrane integrity and sidedness are generally made by determining the degree of increase in the binding of a ligand or enzymic activities before and after the addition of detergents (7, 8, 41–44) or alamethicin (7), which make latent binding sites accessible. Latency was measured by ouabain binding, adenylate cyclase, and (Na^+,K^+)-ATPase activities. With regard to the (Na^+,K^+)-ATPase, the ouabain-binding site is not accessible in inside-out tubules, whereas the ATP (or phosphate)-binding site is not accessible in right-side out t-tubules (45). With respect to adenylate cyclase, the ATP-binding site is not accessible in right-side out t-tubules (7).

Ouabain Binding

Ouabain binds preferentially to the phosphoenzyme form of the catalytic subunit of the (Na^+,K^+)-ATPase which can be phosphorylated by either ATP or inorganic phosphate (46). Previous studies examining ouabain binding to t-tubule were performed in the presence of ATP (23). Since ATP and ouabain bind to opposite faces of the (Na^+,K^+)-ATPase (45), membrane integrity could be assessed but not sidedness (23).

A new method for ouabain binding was developed to determine membrane sidedness in which phosphate is used instead of ATP to form phosphoenzyme. In this procedure, triads are preincubated in the presence of 3 mM $MgCl_2$, 3 mM Tris-phosphate (see "Experimental Procedures" for details), in the cold for 18–20 h. This incubation time was found to be sufficient to permit both Mg^{2+} and phosphate to permeate across the membranes (Fig. 3), enabling the (Na^+,K^+)-ATPase molecules to be phosphorylated, irrespective of vesicle orientation or membrane integrity. Under these conditions, ouabain binding is limited only to leaky and sealed right-side out t-tubules (last time point in Fig. 3 and *b* in Fig. 4). The total number of ouabain-binding sites (*c* in Fig. 4) can be measured by exposing latent sites and, by difference, the inside out sealed t-tubules are obtained. Titration of either Pyrophosphate or Standard triads with alamethicin (Fig. 4) revealed that most of the ouabain-binding sites were latent; thus, the t-tubules were predominantly sealed and inside-out in orientation. The percentage of leaky vesicles was estimated by measuring the ouabain binding promoted by ATP and Na^+ (rather than by Mg^{2+} and P_i) in the absence of alamethicin (*a* in Fig. 4), divided by the total ouabain binding (*c* in Fig. 4).

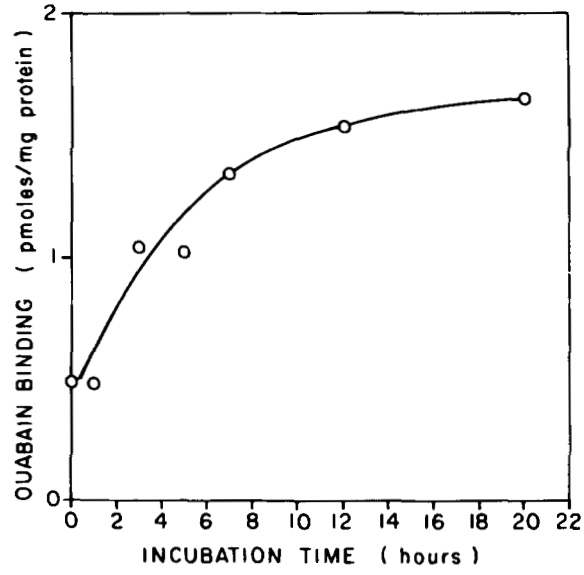

FIG. 3. **Preincubation time for Mg^{2+}-P_i-dependent ouabain binding.** Triads were isolated according to the Pyrophosphate variant. 1.1 mg of protein was incubated on ice in a medium containing 3 mM $MgSO_4$, 3 mM Tris-PO_4, 10% sucrose, and 10 mM Tris-Cl, pH 7.2, in a final volume of 0.5 ml. Aliquots (25 μl) were withdrawn at the indicated times and assayed for ouabain binding as described under "Experimental Procedures," "[^{3}H]Ouabain Binding," procedure A. No detergent was present.

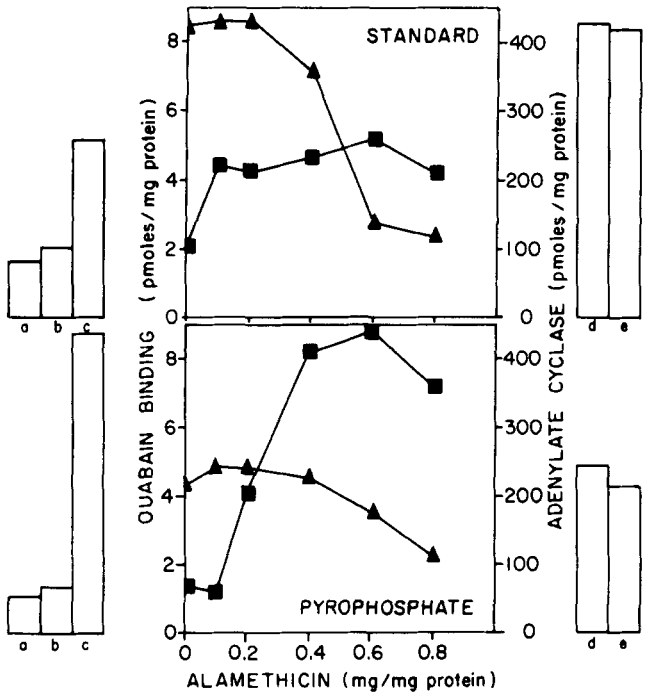

FIG. 4. **Effect of alamethicin on Mg^{2+}-P_i-dependent ouabain binding and fluoride-stimulated adenylate cyclase activity of purified triads.** Triads were isolated according to Standard and Pyrophosphate variants, and preincubated with alamethicin, where applicable, as described under "Experimental Procedures." Adenylate cyclase activity (▲) in the presence of 10 mM NaF and ouabain binding (■) were measured as described in the text. The scales for ouabain binding and adenylate cyclase activity in the *histograms* to the *left* and *right*, respectively, are obtained directly from the *ordinates* of the graph. The histograms to the *left* describe the quantitation of sidedness and integrity of the t-tubules by means of ouabain binding. *a*, ATP-dependent ouabain binding, without alamethicin, measures leaky t-tubules. *b*, Mg^{2+}-P_i-dependent ouabain binding, without alamethicin, measures leaky vesicles plus sealed right-side out t-tubules. *c*, Mg^{2+}-P_i-dependent ouabain binding, with alamethicin (0.6 mg/mg of protein), measures all the t-tubules, *i.e.* leaky plus sealed right-side out and inside out. Thus, the percentage of leaky t-tubules (a/c) for Pyrophosphate and Standard variants is 12.1 and 31.1. The percentage of sealed right-side out t-tubules $[(b - a)/c]$ is 3.8 and 8.5 and the percentage of sealed inside out t-tubules $[(c - b)/c]$ is 84.1 and 60.4, for Pyrophosphate and Standard triads, respectively. The average for five determinations is given in Table III. The shape of the curve for ouabain binding with increasing concentrations of alamethicin by Pyrophosphate triads is consistently different from that by Standard triads. However, for purpose of quantitation, the maximal ouabain binding is always obtained for both preparations between 0.4 and 0.6 mg of alamethicin/mg of protein (*cf.* Table I). The histograms to the *right* are of adenylate cyclase activity which quantitates the percentage of sealed right-side out t-tubules. *d*, adenylate cyclase activity, optimized with alamethicin, (0.1–0.2 mg/mg of protein) measures all the t-tubules. *e*, adenylate cyclase activity, without alamethicin, measures leaky plus sealed inside out t-tubules. Thus, the percentage of sealed right-side out t-tubules $[(d - e)/d]$ is 12 and 2.3 for Pyrophosphate and Standard triads, respectively.

The percentage of sealed right-side out t-tubules was, thus, calculated by difference $[(b - a)/c$ in Fig. 4]. Examples of such determinations are given for Standard and Pyrophosphate triads in Fig. 4 and are summarized in Table IV. By these criteria, the t-tubule of the Pyrophosphate variant is 90% sealed and 87% inside out, and is superior to the Standard variant in this respect.

Adenylate Cyclase Activity

Quantitation of right-side out t-tubules is made in a complementary manner by examining the increase in adenylate cyclase activity using alamethicin (7). Exposure of either Standard or Pyrophosphate triads to increasing concentrations of alamethicin up to 0.2 mg/mg of protein (Fig. 4) resulted in only a small increase in adenylate cyclase activity, indicating that 12% or fewer of the t-tubules were sealed and right-side out. Higher concentrations of alamethicin inhibited adenylate cyclase activity (Fig. 4). This procedure does not discriminate between sealed inside out and leaky t-tubules.

(Na^+,K^+)-ATPase Activity

The latency of (Na^+,K^+)-ATPase activity in the presence and absence of ouabain represents one of the more established methods to determine plasma membrane integrity and sidedness (7, 8, 41–44). The method was therefore applied to the t-tubule of triads.

Problems with the application of (Na^+,K^+)-ATPase Assay to Estimate Membrane Integrity of t-Tubule in Triads—Two assumptions are implicit in the use of (Na^+,K^+)-ATPase activity for determining membrane integrity in the absence of detergent. The first premise is that the enzyme turnover is not limited by build-up of ionic gradients (*i.e.* Na^+ and K^+ are freely permeable). Otherwise, rapid changes of $[Na^+]_i$ and $[K^+]_i$ resulting from pump activity (47, 48) may alter the normal mode ($3Na^+$ for $2K^+$) of the pump (45, 49, 50), with decrease or cessation of ATP hydrolysis (47, 48, 51). The second premise is that ouabain (52) is excluded from the interior of sealed vesicles, at the levels (*i.e.* 0.1 to 1 mM) which are routinely used for such assay (7, 8, 41–44, 53).

These assumptions do not seem to be valid for triads. 2 μM valinomycin,[3] a K^+-selective ionophore (9), was found to enhance ouabain-sensitive (Na^+,K^+)-ATPase (Fig. 5*A*). The increase was concentration dependent with respect to ouabain, ranging from 2–3-fold at 1 μM to 8-fold at 1 mM. The stimulation was greater in the Pyrophosphate than in the Standard triads. Monensin, a $(Na^+,K^+)/H^+$ exchanger (9), gave similar results, although higher levels of ionophore (10–20 μM) were necessary to elicit the effect (data not shown).

It was expected that the dissipation of ionic gradients with ionophores, in the absence of detergent, would result in a higher total ATPase activity (8) rather than an increase in ouabain sensitivity. These data suggested that some ouabain permeated the membrane even in the absence of detergent, perhaps enhanced by valinomycin. Fig. 5*B* shows that ouabain binding (on the same triad fractions used in the experiment shown in Fig. 5*A*) did not change significantly in the presence of valinomycin, indicating that the membrane permeability to ouabain was not altered by the mere addition of valinomycin. It would appear, therefore, that the high levels (1 mM) of ouabain ordinarily used in the (Na^+,K^+)-ATPase assay result in sufficient permeation to inhibit an appreciable percentage of the activity, even in the absence of detergent, making estimates of integrity unreliable. For Pyrophosphate and Standard triads in Fig. 5*A*, maximal ouabain-sensitive (Na^+,K^+)-ATPase activity, with alamethicin and 1 mM ouabain, was 5.14 and 3.45 μmol of P_i/h/mg, respectively. By the usual estimate for leaky vesicles, *i.e.* the ouabain-sensitive (Na^+,K^+)-ATPase in the absence of detergent divided by this activity in the presence of detergent, the percentage of leaky t-tubules would be 80 and 76 for the two triad preparations. This analysis is clearly not valid.

Therefore, to assess integrity of t-tubule by means of ouabain-sensitive (Na^+,K^+)-ATPase activity, an *ad hoc* assay was carried out with 2 μM valinomycin and 1 μM ouabain to

[3] 2 μM valinomycin (stock solution, 1 mM in absolute ethanol) was found to be an optimal concentration to give maximal total (Na^+,K^+)-ATPase activity (data not shown).

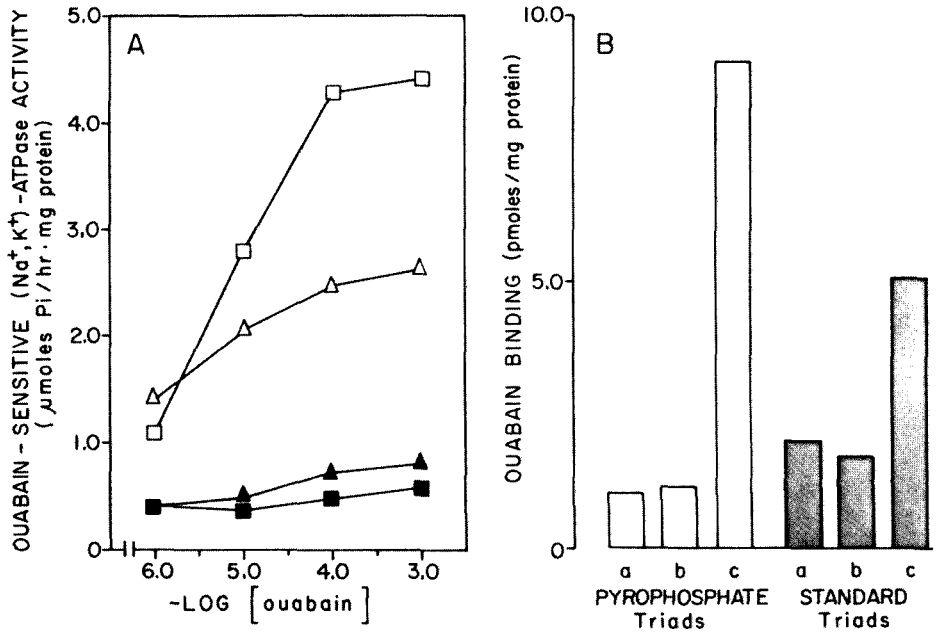

FIG. 5. **Influence of valinomycin on ouabain-sensitive (Na^+,K^+)-ATPase activity and ouabain binding of triads.** *A*, ouabain-sensitive (Na^+,K^+)-ATPase activity as a function of ouabain concentration assayed in the presence (□, △) and absence (■, ▲) of 2 μM valinomycin. Standard (*triangles*) and Pyrophosphate (*squares*) triads were isolated as described under "Experimental Procedures." (Na^+,K^+)-ATPase activity was measured on 40 μg of protein. Ouabain-sensitive (Na^+,K^+)-ATPase was the difference between the activity measured in the absence and presence of ouabain, respectively. No detergent was added. *B*, Mg^{2+}-P_i-dependent ouabain binding of triads. Triads (40 μg of protein) were preincubated in the presence of Mg^{2+} and phosphate for 20 h as described in the text and then assayed: *a*, without further addition; *b*, with 2 μM valinomycin; *c*, after further preincubation with 0.6 mg of alamethicin/mg of protein.

TABLE III

(Na^+,K^+)-ATPase activity of triads

(Na^+,K^+)-ATPase activity was determined as described under "Experimental Procedures." Ouabain-sensitive (Na^+,K^+)-ATPase activity is the difference between the activity measured in the absence (total) and in the presence (basal) of 1 μM ouabain. Values reported in lines c and f were obtained after preincubation with optimized concentrations of alamethicin (0.4–0.6 mg/mg of protein). Estimates of sidedness and integrity could then be made. Total ATPase activity in the presence of valinomycin (b) estimates leaky vesicles plus sealed inside out t-tubules. Total ATPase activity with both valinomycin and alamethicin (c) estimates all the t-tubules. Thus, the percentage of sealed right-side out t-tubules $[(c - b)/c]$ is 12 and 8 for Standard and Pyrophosphate variants. Ouabain-sensitive (Na^+,K^+)-ATPase activity in the presence of valinomycin (e) estimates leaky t-tubules. Ouabain-sensitive (Na^+,K^+)-ATPase activity with alamethicin (f) estimates all the t-tubules. In this study, the percentage of sealed, both right-side out and inside out, t-tubules $[(f - e)/f]$ is 70 and 85 for Standard and Pyrophosphate triads. If the added ouabain sensitivity with valinomycin (line e *versus* line d) derives from sealed inside out rather than leaky t-tubules, the calculated percentage of sealed t-tubules would be somewhat underestimated. The values reported are means ± S.D., for six different triad preparations. The ouabain-sensitive (Na^+,K^+)-ATPase activity assayed side-by-side in the presence of much higher ouabain concentration (1 mM), after preincubation with optimized alamethicin concentration, averaged 2.95 and 4.56 μmol of P_i/h·mg of protein for the Standard and Pyrophosphate triads, respectively. The finding that 1 μM ouabain is not as effective as 1 mM ouabain in inhibiting (Na^+,K^+)-ATPase is likely due to the well known effect of K^+ on the K_D for ouabain. K_D is ~50 nM in the absence of K^+ (23). 20 mM KCl, present in the (Na^+,K^+)-ATPase assay, strongly increases the K_D for ouabain (60, 61) so that 1 μM ouabain may not be near saturating concentration.

	Standard triads	Pyrophosphate triads
	μmol P_i/h·mg protein	
1. Total (Na^+,K^+)-ATPase		
a. No addition	7.8 ± 2.3	4.7 ± 1.4
b. +2 μM valinomycin	8.6 ± 2.3	6.6 ± 1.5
c. +2 μM valinomycin and alamethicin	9.8 ± 2.1	7.2 ± 1.8
2. Ouabain-sensitive (Na^+,K^+)-ATPase (+1 μM ouabain)		
d. No addition	0.32 ± 0.14	0.40 ± 0.19
e. +2 μM valinomycin	0.74 ± 0.29	0.57 ± 0.10
f. +2 μM valinomycin and alamethicin	2.46 ± 0.33	3.80 ± 0.40

TABLE IV

Analysis of sidedness and integrity of transverse tubule of purified triads

Ouabain binding, carried out as described in Fig. 4, gives complete sidedness information. Ouabain-sensitive and total (Na^+,K^+)-ATPase activity, carried out as described in Table III, gives similar information. Adenylate cyclase activity was measured in the presence of 10 mM NaF (Fig. 4). The quantitation of leaky, sealed, right-side out, or inside out t-tubules was performed as described in Fig. 4 and Table III. The values reported are the mean ± S.D., for the number of determinations on different triad preparations shown in parentheses.

	Ouabain binding	Ouabain-sensitive (Na^+,K^+)-ATPase	Total (Na^+,K^+)-ATPase	Adenylate cyclase
	% t-tubule in each orientation			
Standard Triads				
Sealed t-tubules	62.2 ± 4.9 (5)	69.9 ± 9.4 (6)		
Right-side out	2.5 ± 2.6		12.3 ± 3.6 (6)	2.3
Inside out	59.7 ± 5.0		[57.7][a]	
Leaky t-tubules	37.8 ± 4.9	30.1 ± 9.4		
Pyrophosphate Triads				
Sealed t-tubules	89.5 ± 7.7 (5)	85.0 ± 8.6 (6)		
Right-side out	3.9 ± 2.5		8.3 ± 2.1 (6)	12
Inside out	86.5 ± 5.6		[76.7][a]	
Leaky t-tubules	10.5 ± 7.7	15.0 ± 8.6		

[a] The percentage of inside out t-tubules, values in brackets, was calculated by subtracting the percentage of right-side out t-tubules (from the total (Na^+,K^+)-ATPase analysis) from the percentage of sealed t-tubules (from the ouabain-sensitive (Na^+,K^+)-ATPase analysis).

minimize build-up of ion gradients and to limit the levels of intratubular ouabain (see below and Tables III and IV).

Estimates of Membrane Integrity and Sidedness—The percentage of sealed t-tubules is estimated by measuring the percentage of latent ouabain-sensitive (Na^+,K^+)-ATPase activity (8). The large increase of ouabain-sensitive (Na^+,K^+)-ATPase activity after preincubation with alamethicin (Table III) revealed that t-tubules were largely sealed in both variants (Table IV). This analysis gives estimates of sealed and leaky t-tubules which are comparable to those obtained using ouabain binding (Table IV).

The percentage of sealed right-side out t-tubules was estimated by measuring total (Na^+,K^+)-ATPase activity (Table III). Total (Na^+,K^+)-ATPase activity in the presence of either valinomycin or monensin estimates both leaky and sealed inside out t-tubules (8). Total (Na^+,K^+)-ATPase activity with optimized alamethicin concentration reflects the activity of all the t-tubules. Therefore, the percentage of sealed right-side out t-tubules can be calculated by difference (8). As shown in Table III, the increase in total (Na^+,K^+)-ATPase activity measured with alamethicin and 2 μM valinomycin was small, indicating that approximately 10% of the t-tubules was right-side out. The percentage of inside out t-tubules can be obtained by subtracting this value from the percentage of sealed t-tubules, obtained from the latency of the ouabain-sensitive (Na^+,K^+)-ATPase activity.

A summary of sidedness and integrity for both Standard and Pyrophosphate triads using the three approaches is given in Table IV. The t-tubules in both preparations are largely sealed as measured by ouabain binding and ouabain-sensitive (Na^+,K^+)-ATPase activity. Pyrophosphate triads exhibit greater membrane integrity (85–89% *versus* 62–70%), and the sealed t-tubules are oriented largely inside out (>90%). The percentage of right-side out t-tubules is small, 12% or less, as estimated by ouabain binding, total (Na^+,K^+)-ATPase, or adenylate cyclase activities.

DISCUSSION

This study provides the first detailed biochemical and functional characterization of purified triads. The purity of the triads was documented previously by particle counting[4] at the level of the electron microscope (2) and was supported by diagnostic enzyme analysis. The purified triads consist predominantly of terminal cisternae of sarcoplasmic reticulum and transverse tubule, in junctional association. Some lateral cisternae are also observed on occasion, still associated with terminal cisternae (3). The ultrastructural detail of the junction has been preserved in the isolated triads (3). Most of the triadic protein is referable to SR terminal cisternae and has been estimated[5] to be 1 order of magnitude greater than t-tubule, even though the ratio of terminal cisternae to t-tubule in the isolated fraction is only about 1.5 to 1 (2).

Other purified rabbit skeletal muscle membrane fractions are now available and provide a context for interpreting the characteristics of the triads (Table V). These include highly purified SR (54) and light and heavy SR referable to lateral and terminal cisternae of sarcoplasmic reticulum, respectively (20), t-tubule (24, 26), and sarcolemma (8). t-Tubule can be nonjunctional or junctionally associated with terminal cisternae (55, 56). The t-tubule preparation by Caswell and collaborators (13, 23, 24, 57, 58) is especially interesting in that it is of documented junctional origin.

The lipid content of the purified triads is somewhat greater than that of heavy SR (Ref. 20 and Table I). The latter has considerably less lipid than light SR (Table V). The lipid content of t-tubule (26, 57) and sarcolemma (8) is higher yet (*cf.* Table V). The lower lipid content of Pyrophosphate *versus* Standard triads (Table I) is consistent with a higher proportion of sealed terminal cisternae, which is also reflected in the higher ratio of the calcium pump protein/calcium-binding protein in the Pyrophosphate triads.

The cholesterol content is nearly 2-fold greater for Pyrophosphate *versus* Standard triads and would appear to reflect a higher content of t-tubule. The greater proportion of t-tubule in the Pyrophosphate variant is supported by a comparable (1.6–1.9-fold) enrichment in the number of ouabain- and DHA-binding sites, as well as ouabain-sensitive (Na^+,K^+)-ATPase activity; each is diagnostic of t-tubule and not of SR.

The comparable enrichment of the four characteristics suggests: 1) there is more t-tubule membrane in Pyrophosphate *versus* Standard triads; 2) the higher amount of t-tubule membrane possibly indicates longer t-tubules since particle counting[4] gave similar ratios of t-tubule to terminal cisternae for the two variants of triads (2); and 3) the amount of t-tubule membrane is similarly reflected by these four characteristics so that each is a reliable index of the amount of t-tubule membrane in the triads.

Adenylate cyclase (7, 8, 13, 44) and basal (ouabain-insensitive) ATPase (25, 26) activities have been reported to be characteristic of sarcolemma and t-tubule. We find both to have lower specific activity in Pyrophosphate *versus* Standard triads by a factor of 2.

Since agents which act directly on the catalytic subunit (Mn^{2+} and seemingly forskolin) do not restore the deficient adenylate cyclase activity in Pyrophosphate triads, we conclude that the catalytic subunit was inactivated and/or extracted in the isolation of the triads. Longer exposure to pyrophosphate mixture, which was used to solubilize contractile elements, is likely responsible for such damage.

High basal (ouabain-insensitive) ATPase activity has been reported to be localized in t-tubule and sarcolemma, both in rabbit (26) and chicken (25) skeletal muscle. The role of this activity is obscure. Yet, highly purified sarcolemma from skeletal muscle prepared in our laboratory (8) as well as t-tubule of junctional origin prepared in Caswell's laboratory (24, 58) have low levels of this activity. Such divergent results between the different laboratories suggest that basal ATPase is inactivatable and/or extractable from the membrane and therefore is not a reliable index of the amount of sarcolemma and/or t-tubule in a membrane fraction.

It is as yet unresolved whether the extensions of the surface membrane, *i.e.* sarcolemma, junctional, and nonjunctional t-tubule, are similar. Purified membrane fractions are becoming available to address this problem. The present studies indicate that ouabain and DHA binding and ouabain-sensitive (Na^+,K^+)-ATPase activity are reliable indices of the amount of t-tubule. The lower specific activities of these characteristics of triads by 1 order of magnitude as compared with purified sarcolemma (Ref. 8 and Table V), reflect the low percentage of protein in triads which is referable to t-tubule.[5] Thus, t-tubule and sarcolemma seem to have similar specific activities for these three characteristics and for Na^+ uptake as well (Table V). This contention is further supported by preliminary studies in which partially purified t-tubule prepared from Standard triads (Table III in Ref. 2) had ouabain-sensitive (Na^+,K^+)-ATPase and adenylate cyclase activities which were similar to those of sarcolemma (Ref. 8 and Table V). The t-tubule fraction prepared in Caswell's laboratory, however, appears to have lower specific activities than our extrapolated values (Table V).

The ability of triads to function in Ca^{2+} release *in vitro* would have to depend on their permeability characteristics and transport capability. SR terminal cisternae of the triads

[4] Particle counting at the level of the electron microscope was carried out on thin sections (2). In this context, each membrane vesicle or compartment was considered a particle.

[5] This estimate is based on the following: 1) morphometric analysis (56) shows that terminal cisternae have a surface area three times larger than that of t-tubules; 2) the protein to phospholipid ratio is 3-fold higher in terminal cisternae than in t-tubules (20, 57); 3) (Ca^{2+},Mg^{2+})-ATPase activity of triads is close to that of purified heavy SR (Table V); 4) ouabain-sensitive (Na^+,K^+)-ATPase and basal adenylate cyclase activities in a partially purified t-tubule fraction isolated from Standard triads, according to Lau *et al.* (24) are 8–10 times higher than in purified triads (Table V).

TABLE V

Characteristics of rabbit skeletal muscle membrane fractions

	μmol P/mg protein	Cholesterol		(Ca^{2+},Mg^{2+})-ATPase	Ca^{2+}-loading rate	Adenylate cyclase		Binding		Na^+ uptake	Ouabain-sensitive (Na^+,K^+)-ATPase	Basal ATPase
		μmol/mg protein	Molar ratio Chol/PL[a]			Basal	+NaF	DHA	Ouabain			
				μmol/min·mg protein		*pmol/min·mg protein*		*pmol/mg protein*		*nmol/mg protein*	*μmol/h·mg protein*	
Fleischer laboratory												
Temperature of assay (°C)				25	25	37	37	25	37	37	37	37
SR (54)	0.76	0.03	0.04	4.24[b,c]	2.9							
LSR (20)	1.10			4.96[b,c]	3–6							
HSR (20)	0.52			3.17[b,c]	1–2							
SL (8)	2.37	0.92	0.39	<0.03		313		1.69[d]	118[d,e]	270	56.6[e]	18.6[e]
t-Tubule[f]						375					39[e]	
Standard triads (this study)	0.73	0.05	0.07	3.75[c]	1.38	57.5	420	0.26	5.07[e]	10–20[g]	2.86[e]	7.98[e]
Pyrophosphate triads (this study)	0.65	0.08	0.13	2.65[c]	1.15	27.1	240	0.39	9.62[e]		4.61[e]	3.79[e]
Caswell laboratory												
Temperature of assay (°C)				37				25	37	37	37	37
LSR (57, 58)	0.87	0.08	0.09	3.95[c]								
HSR (13, 23, 24, 57, 58)	0.53–0.75	0.05–0.08	0.008–0.1	2.1–2.42[c]						1.7	0.48–0.78[h]	2.76[h]
t-Tubule (13, 23, 24, 57, 58)	1.57	0.64	0.4	0.9–0.70[c]			2300	0.40–0.61	37[e]	198	4.32–6.18[h]	25.9[h]
TC-triads (13, 57)	0.64					13.0	111	0.15				
Rosemblatt *et al.* (26)												
Temperature of assay (°C)				37							37	37
t-Tubule	1.65	0.9	0.55	0.56							12[e,i]	329

[a] Chol/PL, cholesterol/phospholipid ratio; LSR and HSR, light and heavy SR; SL, sarcolemma; TC, terminal cisternae.
[b] L. Hymel, P. Volpe, B. Costello, and S. Fleischer, unpublished observations.
[c] Activity measured in the presence of A23187.
[d] P. Volpe, unpublished observations.
[e] Activity measured after preincubation with detergent.
[f] t-Tubule was partially purified according to Lau *et al.* (24) using standard triads (see also Table III in Ref. 2).
[g] Data derived from Mitchell (40). Na^+ uptake capacity measurements were carried out as described by Seiler and Fleischer (8), for 10 min at 37 °C using approximately 300 μg of protein for each determination.
[h] Activity measured in the presence of NaI.
[i] Extrapolated value.

are comparable to heavy SR, with respect to: 1) Ca^{2+}-loading rate; 2) enhancement of Ca^{2+}-loading rate with ruthenium red[6]; 3) Ca^{2+}-loading efficiency[6]; 4) stimulation of (Ca^{2+},Mg^{+})-ATPase activity with A23187; and 5) Ca^{2+} leak rate. Ruthenium red, an inhibitor which has been reported to block Ca^{2+}-induced Ca^{2+} release in heavy SR (59), was found to enhance the net Ca^{2+}-pumping rate, increase Ca^{2+}-pumping efficiency[6] and decrease the Ca^{2+} leak rate in triads, as well as in heavy SR. The Ca^{2+} leak of the triads was found to be comparable to that of heavy SR in that it displays first order kinetics and is at least biphasic.

The t-tubules of the triads are capable of energized accumulation of sodium ions (Ref. 40 and Table V). Valinomycin stimulation of (Na^{+},K^{+})-ATPase activity (Table III) indicates that K^{+} is transported and that t-tubule is poorly permeable to K^{+}. Therefore, both components of the triad, *i.e.* t-tubule, as reflected by (Na^{+},K^{+})-pump, and terminal cisternae, as reflected by the calcium pump, are able to transport ions, and display membrane integrity.

A major portion of this study deals with membrane integrity and sidedness of t-tubule of the triads. In order for the t-tubules to function in t-tubule depolarization-induced Ca^{2+} release *in vitro*, they should be sealed and inside out. This sidedness is a consequence of the anatomy of the muscle fiber, in which t-tubule invaginates transversely from the sarcolemma to the interior of the muscle fiber (55).

Studies of membrane integrity and sidedness in muscle were first carried out for heart sarcolemma (7, 41–44). Membrane integrity was estimated by means of ouabain-sensitive (Na^{+},K^{+})-ATPase activity since both faces of the membrane must be accessible to measure this activity. Later, procedures were developed to estimate membrane sidedness. Such procedures included formation of phosphoenzyme from ATP (7) or adenylate cyclase activity (7, 44), which measure accessibility of the cytoplasmic face. A more complete approach to estimate sidedness was cardiac glycoside-sensitive (Na^{+},K^{+})-ATPase activity (42, 43) using ouabain (considered impermeant) and digitoxigenin (considered membrane permeable).

More recently, membrane integrity and sidedness studies were carried out on t-tubule (23) and sarcolemma (8) from skeletal muscle. ATP-dependent ouabain binding was used to estimate membrane integrity (23). In the present study, we have introduced a new variant of this procedure which enables sidedness to be measured as well. In the new variant, phosphoenzyme is formed by prolonged preincubation with Mg^{2+} and P_i instead of ATP and Na^{+}. Thus, ouabain binding, in the absence of detergent, measures both leaky and sealed right-side out t-tubules. When the latency is removed, any increase in ouabain binding is referable to inside out t-tubules. This procedure now appears to be one of the methods of choice for determining membrane integrity and sidedness in muscle plasma membrane vesicle fractions (see below).

In a previously described procedure to measure membrane integrity and sidedness of purified skeletal muscle sarcolemma (8), (Na^{+},K^{+})-ATPase activity was assayed in the presence of ionophore to prevent build-up of ionic gradients. The difference in ouabain sensitivity in the absence and presence of detergent gives the percentage of both leaky and sealed vesicles. This procedure, applied to triads, gave estimates of leakiness which were much greater than those obtained by ouabain binding. Surprisingly, the ouabain sensitivity in the absence of detergent was enhanced by 2 μM valinomycin and this increase was dependent on the concentration of ouabain (Fig. 5*A*). At 1 mM ouabain, in the presence of valinomycin, approximately 80% of the optimized ouabain-sensitive (Na^{+},K^{+})-ATPase activity was obtained, erroneously indicating that most of the t-tubules of the triads were leaky. Valinomycin did not influence ouabain permeability (Fig. 5*B*), but, by preventing K^{+} depletion from the tubular lumen, increased phosphorylation of the (Na^{+},K^{+})-ATPase molecules and thereby ouabain inhibition. Thus, the data suggested that some ouabain crossed the membrane even in the absence of detergent and that the added ouabain sensitivity with valinomycin probably derived from sealed inside out rather than leaky t-tubules.

It must be stressed that the ouabain permeability phenomenon is particularly pronounced in t-tubule. In purified skeletal muscle sarcolemma (8), under identical experimental conditions, using 1 mM ouabain, either 2 μM valinomycin[7] or 2 μM monensin (Fig. 6 in Ref. 8) caused a 1.5–2-fold stimulation of the ouabain-sensitive (Na^{+},K^{+})-ATPase activity. Even so, the stimulated activity was about 20% of the maximal ouabain-sensitive (Na^{+},K^{+})-ATPase in this sarcolemma fraction (8) so that the overestimate of leaky vesicles[8] was modest compared with t-tubule of triads. A miniscule permeation of ouabain appears to be very effective in t-tubule where the internal volume is very small (57) and is much less effective in sarcolemma which has a larger internal volume (8). In order to use ouabain sensitivity to measure integrity of t-tubule of triad, we reduced the ouabain concentration to much lower concentration, *i.e.* 1 μM (Table III). This still gives some overestimate of leaky t-tubules (Fig. 5*A* and Table III, line *e versus* line d).

Caswell and co-workers have described a fraction from rabbit skeletal muscle designated "terminal cisternae-triads." It contains mainly terminal cisternae and some triads (24). The degree of triad enrichment has not been evaluated. The fraction has been used mainly for the preparation of purified t-tubules (13, 23, 24, 57, 58) and received only limited characterization (Table V). The adenylate cyclase activity (13) is less than one-half to one-fourth of this activity of the Pyrophosphate and Standard triads, respectively. DHA binding is 40% (13) of that shown by the Pyrophosphate triads. The t-tubule was reported to be 90% sealed by use of ATP-dependent ouabain binding (23). Sidedness information was not obtained. The integrity of the SR compartment in our triad preparation stands in contrast to a recent report by Brandt *et al.* (58) that the terminal cisternae in this fraction "have an intrinsic high permeability to Ca^{2+}."

The Pyrophosphate triads appear to have more t-tubule and a higher degree of t-tubule integrity than Standard triads. The sealed t-tubules are nearly all (>90%) inside out. These characteristics indicate that the Pyrophosphate triads might be somewhat preferable for study of t-tubule depolarization-induced Ca^{2+} release, provided that adenylate cyclase is not directly involved.

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[6] Ca^{2+}-loading efficiency is approximately 0.7 for triads and heavy SR and is increased to 1.2 by 20 μM ruthenium red. Ruthenium red increases the Ca^{2+}-loading rate and does not affect (Ca^{2+},Mg^{2+})-ATPase activity (L. Hymel, P. Volpe, B. Costello, and S. Fleischer, unpublished observations).

[7] P. Volpe, B. Costello, S. Seiler, and S. Fleischer, unpublished observations.

[8] Thus, the skeletal muscle sarcolemma fraction is more sealed than previously estimated (8). The estimate for leaky vesicles should be obtained from the difference in (Na^{+},K^{+})-ATPase activity ± ouabain ($(A - B)/E = 12\%$; Fig. 6 in Ref. 8) rather than as previously estimated using this measurement in the presence of ionophore ($(C - D)/E = 22\%$; Fig. 6 in Ref. 8). It should be noted that 1 mM ouabain was used in this study (8). When 1 μM ouabain was used, the difference ± valinomycin was reduced to 3% (not shown).

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