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Isolation of Terminal Cisternae of Frog Skeletal Muscle

CALCIUM STORAGE AND RELEASE PROPERTIES*

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Sarcoplasmic reticulum (SR) terminal cisternae (TC) of frog (*Rana esculenta*) fast-twitch skeletal muscle have been purified by isopycnic sucrose density gradient centrifugation. Biochemical characteristics and Ca^{2+} release properties have been investigated and compared to those of the homologous fraction of rabbit skeletal muscle TC. The frog SR fraction obtained at the 38/45% sucrose interface appears to be derived from the terminal cisternae region as judged by: (*a*) thin section electron microscopy showing vesicles containing electron opaque material and squarelike (feet) projections at the outer surface; (*b*) protein composition (Ca^{2+} -ATPase, calsequestrin, and high M_r proteins); (*c*) Ca^{2+} fluxes properties. The content of calsequestrin was higher in frog TC by 50% and the Ca^{2+} binding capacity (624 or 45 nmol of Ca^{2+}/mg of TC protein, depending upon experimental conditions) was 3–4 times that of rabbit TC. Species-specific antigenic differences were found between junctional SR proteins of frog and rabbit TC.

After active Ca^{2+} preloading in the presence of pyrophosphate (Palade, P. (1987) *J. Biol. Chem.* 262, 6135–6141), caffeine and doxorubicin elicited Ca^{2+} release from either TC fraction but with much faster rates in frog TC than in rabbit TC (14 *versus* 3 μmol of Ca^{2+}/min/mg of protein).

The present results provide new evidence for the existence of marked differences in Ca^{2+} release properties between TC of amphibian and mammalian fast-twitch muscle. Higher Ca^{2+} binding capacity and faster release rates in frog TC might compensate for the comparably greater diffusion distance being covered by the released Ca^{2+} from the Z-line to the actomyosin cross-bridges in the A-I overlap region.

The SR[1] is a specialized endomembrane system in skeletal muscle fibers which regulates the myoplasmic Ca^{2+} concentration, thereby controlling muscle contraction and relaxation (for review see Ref. 1). Uptake of Ca^{2+} into the lumen of SR by an ATP-dependent Ca^{2+} pump brings about relaxation. Ca^{2+} release from the SR terminal cisternae via Ca^{2+} channels (2) elicits contraction. The molecular mechanism of Ca^{2+} release from TC and the mechanism of E-C coupling, *i.e.* how depolarization of transverse tubule is linked to Ca^{2+} release from TC, are yet to be fully clarified (3).

Several experimental approaches have led to a few established facts. Ca^{2+} is stored into the TC lumen bound to calsequestrin (Ref. 4 and references therein) and is released via Ca^{2+} channels (2); transverse tubule-TC coupling takes place at the triad junction (5, 6). The supporting evidence has been obtained, and hypotheses concerning E-C coupling (3, 7–9) have been proposed using either intact, cut and skinned fibers, or isolated SR preparations of amphibian (3, 5–7, 9) or mammalian (2, 8) skeletal muscles. Frog muscles have been preferentially used in physiological studies whereas biochemical studies have been mostly done on rabbit skeletal muscle. General conclusions have been drawn irrespective of the muscle source and of the experimental approach. There are, however, well recognized morphological (10–12), pharmacological (13–16), and physiological (15, 16) differences between amphibian and mammalian muscles which might be relevant to the Ca^{2+} release process and E-C coupling. In this respect, different structural and electrical properties of transverse tubule (17–20) might also be significant.

In order to fill the gap in biochemical knowledge of TC from amphibian skeletal muscle, we purified a TC fraction from frog fast-twitch skeletal muscle and investigated its protein composition and Ca^{2+} binding capacity and some aspects of the Ca^{2+} release process.

EXPERIMENTAL PROCEDURES

Materials—Doxorubicin was kindly provided by Farmitalia Carlo Erba (Nerviano, Italy). Caffeine, antipyrylazo III, fast blue BB salt, and ruthenium red were obtained from Sigma, *p*-nitrophenylphosphate, α-naphthylphosphate, pyruvate kinase, lactate dehydrogenase, Na_2-phosphocreatine, and creatine kinase from Boehringer Mannheim, the cationic carbocyanine dye Stains All from Eastman, $^{45}CaCl_2$ from Amersham Corp., alkaline phosphatase-conjugated anti-(chicken) IgG and anti-(guinea pig) IgG from Kirkegaard & Perry Laboratories, Inc., A23187 from Behring Diagnostics, and nitrocellulose membranes from Bio-Rad.

Preparation of SR Fractions—SR was isolated from the predominantly fast-twitch skeletal muscles of New Zealand White rabbit and fractionated into longitudinal tubules of SR (R2 at the 27/32% sucrose interface) and TC (R4 at the 38/45% sucrose interface) as previously described by Saito *et al.* (21). Junctional SR was obtained from TC by Triton X-100 treatment followed by Tris-EDTA, pH 8.0, incubation to remove the bulk of calsequestrin (22). SR fractions were suspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4 (buffer A) and stored at −70 °C until used. The proteolytic inhibitor phenylmethylsulfonyl fluoride (100 μM) was present in all solutions.

Frog (*Rana esculenta*) SR was isolated from the fast-twitch skeletal muscles of the hind legs and fractionated as in the former case. However, frog SR preparation was scaled down since only about 50 g of muscle were used each time. The ground meat (50 g) was homogenized in 250 ml of buffer A and 100 μM phenylmethylsulfonyl fluoride

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; SDS, sodium dodecyl sulfate; TC, terminal cisternae of SR; E-C coupling, excitation-contraction coupling; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; ELISA, enzyme-linked immunosorbent assay.

using an Osterizer Cyclo-trol blender for 1 min in position "Blend." The homogenate was centrifuged in an SS 34 rotor for 10 min at 7,700 rpm. The pellet was rehomogenized as before, centrifuged at 7,700 rpm in an SS 34 rotor for 20 min, and the supernatant was filtered through 3–4 layers of cheesecloth and centrifuged in a Beckman 60 Ti rotor for 90 min at 40,000 rpm. The pellet or unfractionated SR (on average 65 mg/50 g of muscle) was resuspended in buffer A and layered onto a discontinuous 4-step sucrose gradient. The gradient steps, 2.5 ml each, consisted of 45, 38, 32, and 27% (w/w) sucrose buffered with 5 mM imidazole, pH 7.4. Gradient tubes were centrifuged in a Beckman SW 40 rotor at 24,000 rpm for 14–16 h. Frog SR fractions were either withdrawn from the gradient tubes and kept in high sucrose or centrifuged in a Beckman 60 Ti rotor for 60 min at 46,000 rpm, resuspended in 0.9 M sucrose, 5 mM imidazole, pH 7.4, and stored at 0–4 °C for 1–2 days. We never succeeded in recovering functional frog TC fractions after freezing at −70 °C, *i.e.* Ca^{2+} loading was abolished. Protein concentration was determined according to Lowry *et al.* (23) using bovine serum albumin as standard.

Slab Gel Electrophoresis—SDS-slab polyacrylamide gel electrophoresis was carried out as described by Laemmli (24) on 5–15% polyacrylamide linear gradient gels (gel width = 0.75 or 1.5 mm). Slab gels were stained either with Coomassie Brilliant Blue or Stains All (25). Densitometric scans of Coomassie Blue-stained slab gels were carried out as previously described (26). The relative amounts of Ca^{2+}-ATPase and calsequestrin were determined by direct planimetry.

Determination of Calsequestrin Content of TC—Calsequestrin was isolated from both rabbit and frog TC (27). Calsequestrin content was determined as described by Williams and Beeler (28) with reference to calibration curves obtained with 0.5–10 μg of purified calsequestrin. After SDS-gel electrophoresis and Stains All staining the absorbance of each calsequestrin band was measured in a Shimadzu chromatoscanner CS-380.

Biochemical Assays—(Ca^{2+},Mg^{2+})-ATPase activity was measured by a coupled-enzyme assay following NADH oxidation at 340 nm in a Perkin-Elmer 551S spectrophotometer. The assay was carried out at either 25 or 37 °C in a medium containing 20 mM histidine, pH 7.2, 0.1 M KCl, 5 mM $MgSO_4$, 2 mM ATP, 0.15 mM NADH, 0.5 mM phosphoenolpyruvate, 5 units of pyruvate kinase, 5 units of lactate dehydrogenase, and 2–5 μg of TC protein. Ca^{2+}-dependent ATPase activity is the difference between total ATPase activity, measured with 0.2 mM $CaCl_2$, and basal ATPase activity, measured with 0.2 mM EGTA. Maximal Ca^{2+}-dependent ATPase activity was measured after addition of A23187 (1.5 μg/ml), a Ca^{2+} ionophore.

Ca^{2+} loading was measured using the metallochromic indicator antipyrylazo III by following ΔA (710–790 nm) in a Hewlett-Packard 8451 A spectrophotometer. The assay was carried out at 25 or 37 °C in a medium containing 92 mM potassium phosphate, pH 7.0, 200 μM antipyrylazo III, 1 mM Na_2-ATP, 1 mM $MgSO_4$, and 30–50 μg of TC protein in the presence and absence of 20 μM ruthenium red. The reaction was started by adding 25–50 μM $CaCl_2$.

Ca^{2+} binding to purified calsequestrin from both rabbit and frog TC was measured by equilibrium dialysis. Ionic composition of the assay medium is detailed in the legend to Table II.

Phospholipid phosphorus was quantitated as described by Rouser and Fleischer (29). Succinate cytochrome *c* reductase activity was measured at 30 °C as previously described (30). Total cholesterol (both free and cholesterol esters) was measured as previously described (31).

Immunological Assays—One-step ELISA and immunoblot were carried out as described (26, 32). The anti-(rabbit junctional SR) serum was raised in hens by weekly intramuscular injections of about 300 μg of junctional SR in buffer A diluted 1:1 with 1 ml of incomplete Freund's adjuvant. The animal was bled after four injections, and the Ig fraction was extracted from the serum as described by Orlans *et al.* (33).

The anti-(rabbit 350-kDa protein) serum was raised in guinea pigs by weekly subcutaneous injections of a suspension containing, in a final volume of 3 ml, 0.5 ml of incomplete Freund's adjuvant and 18 acrylamide gel slices, retaining about 5 μg of 350-kDa protein each, homogenized in buffer A. The animal was bled after five injections, and polyclonal antibodies were affinity-purified as described by Bisson and Schiavo (34).

^{45}Ca-ligand Overlay—^{45}Ca-ligand overlay was carried out essentially as described by Maruyama *et al.* (35). After SDS-gel electrophoresis and transfer to nitrocellulose paper, blots were incubated for 20 min at room temperature in 5 mM imidazole, pH 7.4, 60 mM KCl, 5 mM $MgSO_4$, 10 μM $^{45}CaCl_2$ (specific activity, 1,500,000 cpm/nmol), rinsed in 100 ml of 30% ice-cold ethanol, dried, and exposed to Amersham β_{max} Hyperfilm for 6–7 days.

Ca^{2+} Release Assay—Net Ca^{2+} loading and release were observed by dual wavelength spectrophotometry using antipyrylazo III as a Ca^{2+} indicator. The assay was carried out, essentially as described by Palade (36), at 25 or 37 °C, and the 1-ml loading/release medium contained 60–80 μg of TC protein, 7.5 mM Na_2-pyrophosphate, pH 7.0, 0.1 M KCl, 1.5 mM $MgSO_4$, 1.5 mM Na_2-ATP, 200 μM antipyrylazo III, 5 mM Na_2-phosphocreatine, and 20 μg of creatine kinase. Ca^{2+} loading was performed by the addition of several consecutive pulses of $CaCl_2$ (10–25 nmol/pulse). Following Ca^{2+} preloading and when steady state was attained, caffeine or doxorubicin was added at specified concentrations.

Electron Microscopy—Samples (0.5 mg/ml) were prepared for thin-

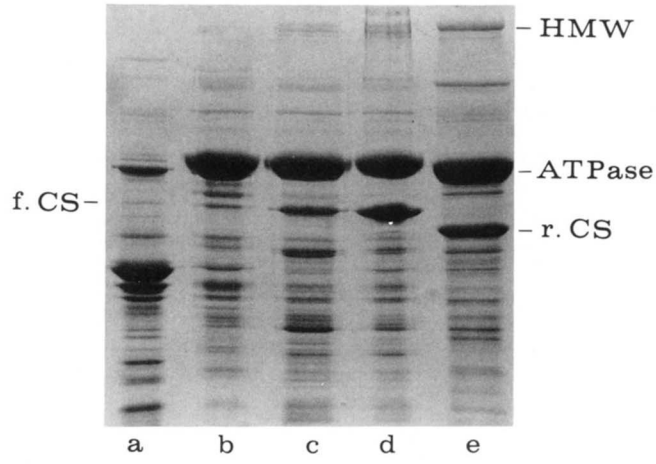

FIG. 1. **SDS-polyacrylamide gel electrophoresis of frog and rabbit skeletal muscle fractions.** Frog fractions (R1–R4, *a–d*) and rabbit R4 fraction were separated on a linear 5–15% polyacrylamide gradient gel (24) that was stained with Coomassie Blue. 30 μg of protein were applied per each lane: *a*, R1; *b*, R2; *c*, R3; *d*, R4; *e*, rabbit R4. Note that the high molecular weight protein is less represented in frog TC than in rabbit TC. Abbreviations used: *HMW*, high molecular weight protein; *ATPase*, Ca^{2+}-ATPase; *f.CS*, frog calsequestrin; *r.CS*, rabbit calsequestrin.

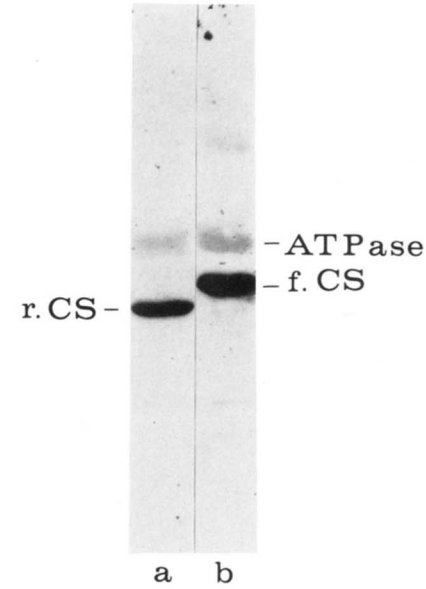

FIG. 2. **^{45}Ca ligand overlay of rabbit and frog TC fractions (autoradiogram).** Rabbit (*a*) and frog (*b*) TC fractions (50 μg of protein) were separated on a linear 5–15% polyacrylamide gel, transferred onto a nitrocellulose membrane, and processed for ^{45}Ca ligand overlay (35). The autoradiogram was obtained after 7 days of exposure. Note also that ATPases are faintly labeled by ^{45}Ca. Abbreviations as in Fig. 1

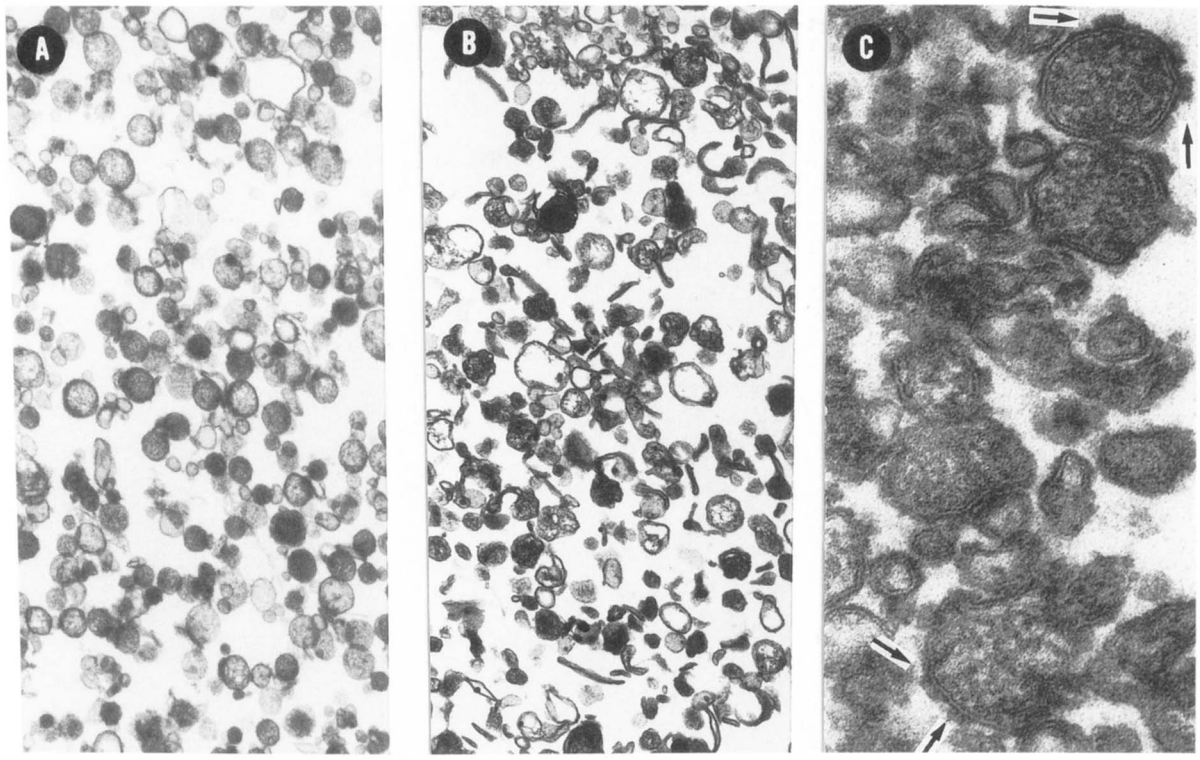

FIG. 3. **Thin-section electron microscopy of isolated TC fractions of frog and rabbit skeletal muscles.** Samples were prepared as described under "Experimental Procedures." *Panel A,* rabbit TC (×26,400) and *Panel B,* frog TC (×26,400) show many vesicles with electron-opaque material. *Panel C,* frog TC (×120,000) shows high magnification pictures of selected vesicles with typical squarelike projections or junctional feet structures (*arrows*). The sample was fixed in glutaraldehyde with 1% tannic acid that allows distinction between junctional face-containing feet and Ca^{2+} pump-containing membrane. Electron opaque content stains heavily after tannic acid enhancement (21).

section electron microscopy essentially as described in Mitchell *et al.* (37). After fixation in suspension with 0.25% glutaraldehyde, samples were centrifuged at 15,000 × *g* for 12 min, and the pellets were postfixed with 1% OsO_4, stained with 0.5% uranyl acetate, and then embedded in Durcupan. Sections were cut perpendicular to the axis of the centrifuge.

RESULTS

Isolation, Protein Composition, and Morphology of Frog TC—A procedure, originally developed by Saito *et al.* (21) in rabbit skeletal muscle, was adapted to frog skeletal muscle for purifying a TC fraction. The TC fraction was obtained by isopycnic centrifugation on a discontinuous sucrose gradient as the band collected at the 38/45% interface (R4). The longitudinal tubules of SR fraction banded at the 27/32% sucrose interface (R2). SDS-slab gel electrophoresis shows that frog R2 fraction is enriched in Ca^{2+}-ATPase protein (Fig. 1*b*) which is consistent with its predominant origin from the longitudinal tubules of SR. Frog R4 fraction, similar to the homologous fraction of rabbit SR, appears to be highly enriched in calsequestrin (Fig. 1*d*) and is, thus, referable to terminal cisternae (21). High M_r proteins (38), tentatively identified as junctional feet components in rabbit TC (Fig. 1*e*), appear to have a slightly different M_r in frog TC (Fig. 1*d*). Seiler *et al.* (39) already noted a heterogeneity between homologus sets of high M_r proteins of TC from skeletal and cardiac muscle. In the Laemmli gel system, frog calsequestrin has an apparent M_r of 72,000 (*lane d*) higher than that of rabbit calsequestrin (M_r 64,000; *lane e*) as previously shown (32). Identification of frog calsequestrin relies upon metachromatic staining with Stains-All (32) and specific Ca^{2+} binding as detected by ^{45}Ca overlay (Fig. 2*b*). As measured by densitometry of Coomassie Blue-stained gels, frog TC has a higher content of calsequestrin (see also Table II) resulting in a higher CS/Ca^{2+}-ATPase ratio when compared to rabbit TC (0.8 *versus* 0.6, $n = 3$).

Thin-section electron microscopy shows that the R4 fraction of both rabbit (Fig. 3*A*) and frog (Fig. 3*B*) consisted of vesicles containing electron opaque material, *i.e.* calsequestrin. Squarelike projections at the outer (myoplasmic) surface of rabbit TC vesicles have been identified as the "junctional feet structures" (21). After tannic acid enhancement (Fig. 3*C*), junctional feet could be detected in frog TC vesicles as discrete units separated from one another (*cf.* Ref. 21). In contrast with rabbit R4 fractions, frog R4 fractions also contained vesicles resembling transverse tubule and a few triads (*cf.* Ref. 40; data not shown). The presence of transverse tubule was also indicated by the higher content of cholesterol in frog as compared to rabbit R4 fractions (on average 29 and 14 nmol/mg of protein, respectively). The ultrastructure of the triad junction (10) and of the feet processes (41) is very much alike in both mammalian and amphibian skeletal muscles.

Biochemical Characteristics—Table I summarizes several biochemical properties of frog TC. The yield, the lipid-to-protein ratio, and the Ca^{2+}-dependent ATPase activity, in the presence and absence of A23187, were similar to those of rabbit TC. The activation energy (E_a) of Ca^{2+}-dependent ATPase and of Ca^{2+} loading was also comparable in rabbit and frog TC fractions. The stimulation of the Ca^{2+}-loading rate by ruthenium red, instead, was lower in frog as compared to rabbit TC. Frog TC contained no more than 0.5% mitochondria as indicated by the very low activity of diagnostic enzymes (*cf.* Ref. 40).

TABLE I

Biochemical characteristics of purified TC fractions of frog and rabbit skeletal muscle

All assays were carried as described under "Experimental Procedures." When activation energy (E_a) was determined, ATPase activity (−A23187) and Ca^{2+} loading (−ruthenium red) were measured as described under "Experimental Procedures" in the temperature range 13–40 °C. E_a was calculated from the slope (a) of Arrhenius plots, where $E_a = 2.3\ aR$. E_a values are similar to those previously reported. The values reported are means for the number of determinations on different TC preparations shown in parentheses.

	Frog	Rabbit	
1) Yield (mg/50 g muscle)	5.1 (5)	6.8 (6)	
2) Phospholipid content (μmol P_i/mg protein)	0.52 (3)	0.55 (3)	
3) Ca^{2+}-dependent ATPase			
Rate (μmol P_i/min/mg protein)			
−A23187	1.35 (3)	1.11 (3)	4.28[a]
+A23187	2.44 (3)	1.79 (3)	5.63[a]
E_a (−A23187) (kcal/mol)	12.4	11.4	
4) Ca^{2+} loading			
Rate (μmol Ca^{2+}/min/mg protein)			
−Ruthenium red	0.28 (3)	0.15 (3)	0.39[a]
+Ruthenium red	0.48 (3)	0.62 (3)	1.12[a]
E_a (−ruthenium red) (kcal/mol)	9.8	11.2	
5) Succinate cytochrome c reductase (nmol cytochrome c reductase/min/mg protein)	0–4 (3)	40.4 (3)	

[a] Data obtained at 37 °C.

TABLE II

Ca^{2+} storage capacity of frog and rabbit TC fractions

The amount of calsequestrin (CS) protein relative to the total TC protein was determined by densitometry of Stains-All-stained slab gels (see "Experimental Procedures" for details). The large difference in the Ca^{2+}-binding capacity of calsequestrin (column A *versus* column B) is likely due to a shift in K_D values from 4–40 μM to 0.8–1.3 mM in the presence of K^+ and other cations (64). Data are duplicate determinations carried out on two different preparations.

	Amount of CS	Ca^{2+} bound to CS		Estimated Ca^{2+} bound to TC[c]	
		A[a]	B[b]	A	B
	mg CS/mg TC protein	*nmol Ca^{2+}/mg CS*		*nmol Ca^{2+}/mg TC protein*	
Frog	0.48	1300	94.1	624	45.2
Rabbit	0.32	830	39.8	266	12.7

[a] Data derived from Damiani *et al.* (32). Ca^{2+} binding was measured by equilibrium dialysis in the presence of 5 mM $CaCl_2$ and 5 mM Tris-Cl, pH 7.5, *i.e.*, ionic conditions which allow determination of maximal Ca^{2+}-binding capacity.

[b] Ca^{2+} binding was measured by equilibrium dialysis on 0.70 mg of purified calsequestrin in the presence of (mmol/liter): 0.16 sodium phosphate, pH 7.0, 0.35 $^{45}CaSO_4$ (specific activity, 2500 cpm/nmol), 1.1 potassium phosphate, pH 7.0, 0.17 $MgSO_4$, 0.4 KCl, *i.e.* conditions devised to mimic intraluminal ion composition of TC (42); ion concentrations were chosen to avoid Ca^{2+}-phosphate precipitation.

[c] Estimated Ca^{2+} bound to TC was determined: amount of calsequestrin × Ca^{2+} bound to calsequestrin.

A direct determination (28) of the calsequestrin content of R4 fractions indicated that frog TC contain 50% more calsequestrin than rabbit TC (Table II). This result coupled to the higher Ca^{2+}-binding activity of frog calsequestrin (Ref. 32) implies that the maximal amount of Ca^{2+} bound to frog TC is 3 times higher than that bound to rabbit TC (fourth column in Table II). Frog calsequestrin also displayed a higher Ca^{2+} binding capacity than rabbit calsequestrin (94 *versus* 40 nmol of Ca^{2+}/mg of calsequestrin; Table II) when Ca^{2+} binding was measured under "physiological" conditions, *i.e.* ionic conditions derived from the elemental composition of frog TC (first column of Table II in Ref. 42).

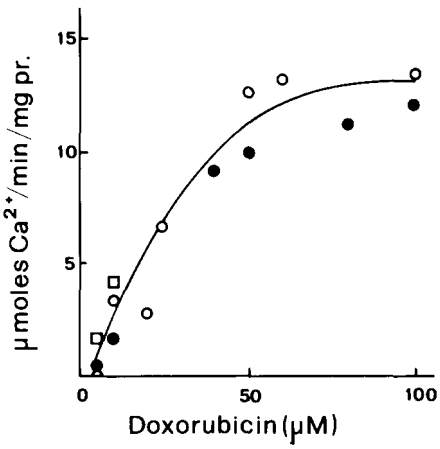

FIG. 4. **Doxorubicin-induced Ca^{2+} release from TC of frog skeletal muscle.** The Ca^{2+} release assay was carried out essentially as described by Palade (36) using the metallochromic dye antipyrylazo III as Ca^{2+} indicator. Frog TC were actively loaded with Ca^{2+} (2.3 μmol/mg of protein) at 25 °C. After completion of Ca^{2+} preloading, doxorubicin at specified concentrations was added to elicit Ca^{2+} release. Release rates were determined with respect to a Ca^{2+} pulse administered at the end of each experiment to recalibrate the dye. Different symbols refer to three different frog TC preparations. Under identical experimental conditions (at 37 °C), rabbit TC preloaded with Ca^{2+} (1.85 μmol/mg of protein) never attained release rates higher than 3 μmol of Ca^{2+}/min/mg of protein (not shown; see Ref. 55).

Drug-induced Ca^{2+} Release from TC—Ca^{2+} release from TC was elicited after active preloading of Ca^{2+} in the presence of 7.5 mM sodium pyrophosphate (36), an ATP-regenerating system, and low free Mg^{2+} (about 0.1 mM). Assays were carried out at either 25 or 37 °C for frog and rabbit TC, respectively, in order to mimic physiological temperature values. In preliminary experiments, the dependence upon the Ca^{2+} preloading was investigated using saturating concentrations of releasing agents, *i.e.* 10 mM caffeine or 100 μM doxorubicin (43). Increasing levels of internalized Ca^{2+} gave higher Ca^{2+} release rates, and maximal rates were attained at 2.30 and 1.85 μmol of Ca^{2+}/mg of protein for frog and rabbit TC, respectively. Using either caffeine or doxorubicin, frog TC displayed rates of Ca^{2+} release 3–4-fold higher than those of rabbit TC (not shown).

In a second set of experiments, the dependence upon drug concentration was investigated. After plateau levels of Ca^{2+} preloading were attained, TC fractions were challenged with increasing drug concentrations. With either caffeine (not shown) or doxorubicin (Fig. 4), frog TC displayed higher rates of Ca^{2+} release with maximal rates of 14 μmol of Ca^{2+}/min/mg of protein. Around 45% of the preloaded Ca^{2+} was released after 2 min. Half-maximal activation of Ca^{2+} release rates was obtained at 3–4 mM caffeine and 27–30 μM doxorubicin in rabbit and frog TC, respectively.

Species Specificity of Junctional SR Proteins—In addition to slight differences in M_r properties of feet proteins, we found heterogeneity in antigenic properties between rabbit and frog TC (Fig. 5) using anti-(rabbit junctional SR) antibodies, *i.e.* against junctional SR membrane proteins. Junctional proteins of frog TC showed a weak cross-reactivity with anti-(rabbit junctional SR) antibodies as judged by both ELISA

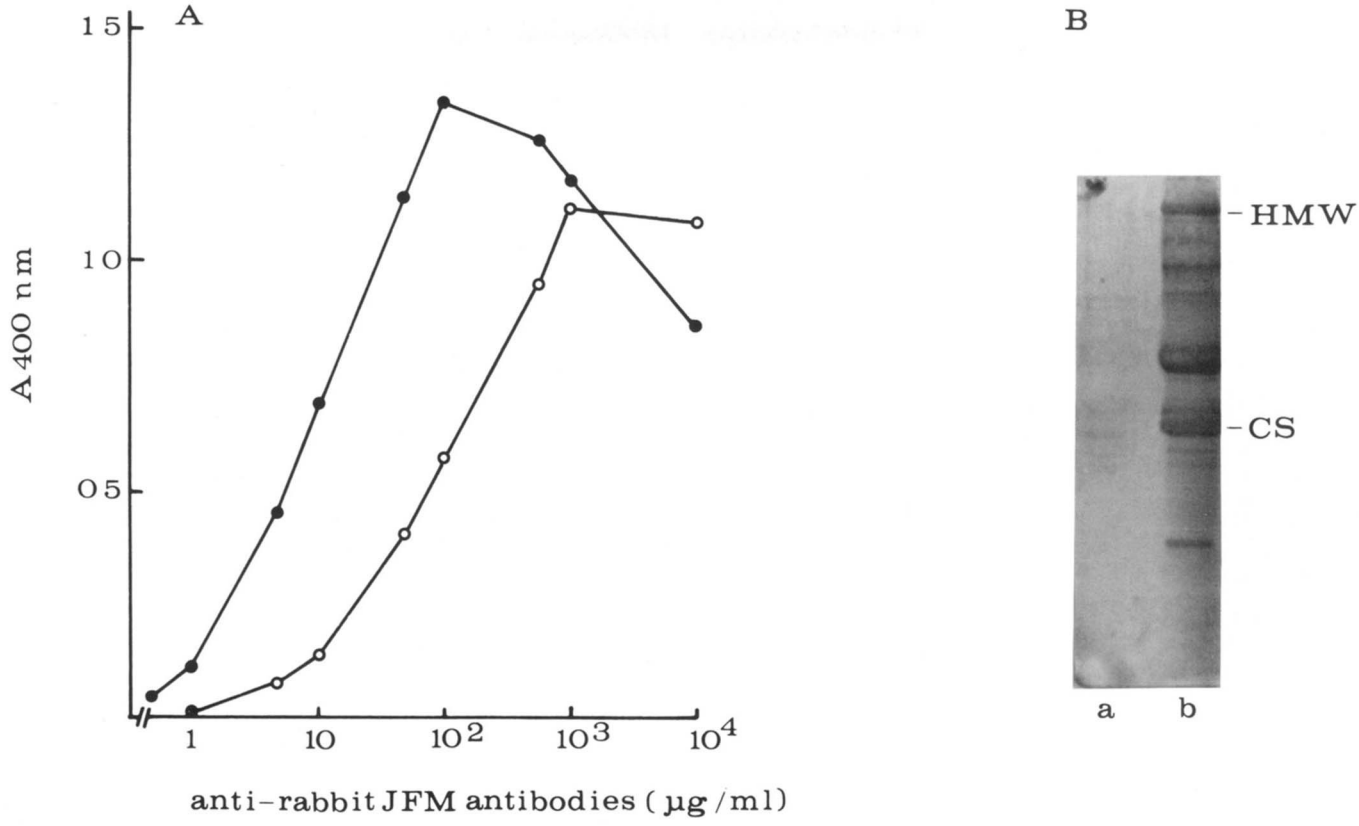

FIG. 5. **One-step ELISA and immunoblot of rabbit and frog TC fractions with anti-(rabbit junctional SR) antibodies.** *Panel A,* ELISA was performed as described in Ref. 26 with anti-(rabbit junctional SR) antibodies (at the concentrations indicated on the abscissa) in plate wells coated with antigen solution at a concentration of 5 µg/ml. The phosphatase activity bound to the wells was determined by incubations for 30 min at room temperature with *p*-nitrophenylphosphate and by measuring the A_{400} after stopping the reaction with alkali. ●, rabbit TC; ○, frog TC. *Panel B,* immunoenzymatic staining of rabbit (*b*) and frog (*a*) TC fractions (15 µg of protein) with anti-(rabbit junctional SR) antibodies. Immunoblot was revealed as described (32) by incubating with anti-(rabbit junctional SR) antibodies (10 µg/ml) and then with alkaline phosphatase-conjugated anti-(chicken) IgG. Immunoblot must be compared with ELISA data points at 10 µg/ml anti-(rabbit junctional SR). *HMW,* high molecular weight protein; *CS,* calsequestrin; *JFM,* junctional SR.

(Fig. 5*A*) and indirect immunoenzymatic staining of Western blots (Fig. 5*B*).

The 350-kDa feet component of junctional SR has been indicated in rabbit SR as a putative constituent of the Ca^{2+} channel by photolabeling studies with [^{14}C]doxorubicin (47) and binding studies with [^{3}H]ryanodine (48, 49). Fig. 6 shows that affinity-purified anti-(rabbit 350-kDa protein) antibodies did not cross-react at all with the homologous high molecular weight protein of frog TC (*lane d*). Antibody specificity is shown in *lane c*.

DISCUSSION

This paper reports the purification and functional characterization of an enriched TC preparation from frog fast-twitch skeletal muscles based on several established parameters (21, 50). In distinction from previous studies using separation by sedimentation alone (13, 44–46, 51), we provide here the first attempt to isolate a TC fraction from amphibian muscles by isopycnic sucrose density gradient centrifugation.

This paper contains two major findings, suggesting the existence of previously unnoticed differences in Ca^{2+} release and Ca^{2+} storage properties of frog TC, as compared to mammalian TC.

A. Unitary conductance of rabbit TC Ca^{2+} channels (2) has been reported to be around 100 picoSiemens, and similar values have been observed for frog Ca^{2+} channels (56). Frog TC release Ca^{2+} at rates 4-fold higher than rabbit TC, thus suggesting that Ca^{2+} release channels are present at a higher density in frog TC. Since net Ca^{2+} loading rates appear to be similar in both TC fractions (Table I), differences in net Ca^{2+} release rates are likely due to different unidirectional Ca^{2+} efflux rates. An alternative or complementary explanation for different Ca^{2+} release rates is that the Ca^{2+} concentration gradient across the SR membrane is different, *i.e.* the free Ca^{2+} concentration is higher in the lumen of frog TC as compared to that of rabbit TC; reliable estimates of intraluminal free Ca^{2+} concentrations are, however, not available in the literature.

The stimulation of the Ca^{2+}-loading rate by ruthenium red reflects the existence of Ca^{2+} channels in the "open state" which are blocked by the drug (50). The lower extent of ruthenium red stimulation in frog TC as compared to rabbit TC (Table I; see also Ref. 50) can be accounted for by a different Mg^{2+} and ruthenium red sensitivity of the channel, a smaller percentage of Ca^{2+} channels in the "open state," and/or the presence of junctionally associated transverse tubule (31, 50).

B. The maximal Ca^{2+} storage capacity of frog TC is 3-fold higher than that of rabbit TC. Experiments carried out under "physiological" ionic conditions also indicate that frog TC bind larger amounts of Ca^{2+}. Marked differences in Ca^{2+} storage capacity might be related to ultrastructural differences

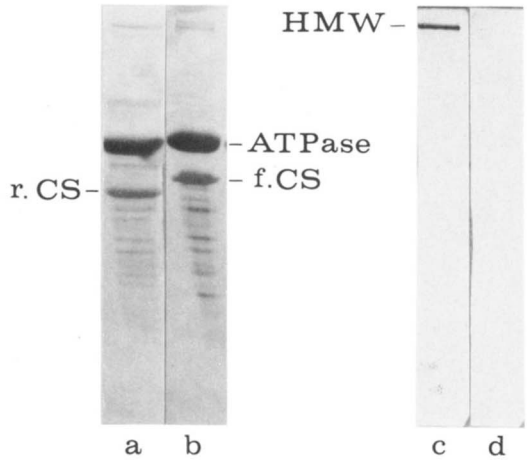

FIG. 6. **Immunoblot of rabbit and frog TC fractions with affinity-purified anti-(rabbit 350-kDa protein) antibodies.** SDS-polyacrylamide gel electrophoresis of rabbit and frog TC fractions (15 μg) was carried out as described under "Experimental Procedures." Electrophoretic transfer to nitrocellulose was carried out overnight at 0.1 A. Blots were stained with 0.2% Ponceau Red in 3% trichloroacetic acid (*a, b*). Immunoblot (*c, d*) was revealed as described (32) by incubating with anti-(rabbit 350-kDa protein) antibodies (5 μg/ml) and then with alkaline phosphatase-conjugated anti-(guinea pig) IgG. Rabbit TC, *lanes a* and *c*; frog TC, *lanes b* and *d*. Abbreviations as in Fig. 1.

between amphibian and mammalian skeletal muscles. In amphibians there is one triad/sarcomere, the triad is located at the Z-line, and the diffusion distance between the Ca^{2+} release sites and the actomyosin cross-bridges in the A-I overlap is relatively large, *i.e.* 0.5 μm plus the radial diffusion. On the other hand, in mammals there are two triads/sarcomere, the triad is located at the A-I boundary, and, hence, the released Ca^{2+} has to diffuse only radially. Thus, in frog muscles larger amounts of Ca^{2+} stored in TC and faster Ca^{2+} release rates might compensate for the greater diffusion distance of Ca^{2+}. Calculated Ca^{2+} release rates (1–40 μM/ms) are available for frog fibers only (52–54), and confirmation of our observations awaits kinetic measurements in mammalian muscles as well.

The time to peak tension (~20 ms) is almost identical in mammals (*e.g.* rabbit, cat) and frog (sartorius) fast-twitch muscles (Refs. 57 and 58 and references therein) at 36 and 22 °C, respectively. Since the structural and steady-state kinetic properties of actin and myosin from either muscle are similar (57, 59), specific E-C coupling steps must be different. The contraction time is dependent upon several distinct steps, and evidence has already accrued as to differences between frog and rabbit muscles. (i) The propagation of transverse tubule action potential is faster in amphibian muscles (20); (ii) the area of transverse tubule in junctional contact with SR is greater in mammals (20); (iii) the coupling lag, *i.e.* the time elapsed between the action potential upswing and Ca^{2+} release from TC, is temperature-dependent (60) and, thus, likely different between amphibian and mammalian muscles. Our findings indicate that Ca^{2+} release from frog TC compensates for other critical steps and ultrastructural restraints so that contraction time of fast-twitch muscles is unchanged. The physiological relevance of our findings relies upon the assumption that the channels being investigated here are the same which operate during E-C coupling.

This paper also shows that there is species-specific heterogeneity involving junctional SR proteins (Figs. 5 and 6). If Ca^{2+} release channels are localized in the junctional SR, a plausible speculation is that molecular component(s) of the Ca^{2+} channel of frog and rabbit TC are not identical. Species-specific heterogeneity, however, does not affect the drug-binding sites of the Ca^{2+} channel as judged by similar sensitivity of both frog and rabbit SR to doxorubicin and caffeine (present study), inositol 1,4,5-trisphosphate (8, 61), and ryanodine (62, 63).

The availability of a purified TC fraction from frog skeletal muscle shall allow further studies on the Ca^{2+} release mechanism and facilitate integration between biochemical and physiological investigations.

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