Nonmammalian vertebrate skeletal muscles express two triad junctional foot protein isoforms

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ABSTRACT Mammalian skeletal muscles express a single triad junctional foot protein, whereas avian muscles have two isoforms of this protein. We investigated whether either case is representative of muscles from other vertebrate classes. We identified two foot proteins in bullfrog and toadfish muscles on the basis of (*a*) copurification with [^{3}H]epiryanodine binding; (*b*) similarity to avian muscle foot proteins in native and subunit molecular weights; (*c*) recognition by anti-foot protein antibodies. The bullfrog and toadfish proteins exist as homooligomers. The subunits of the bullfrog muscle foot protein isoforms are shown to be unique by peptide mapping. In addition, immunocytochemical localization established that the bullfrog muscle isoforms coexist in the same muscle cells. The isoforms in either bullfrog and chicken muscles have comparable [^{3}H]epiryanodine binding capacities, whereas in toadfish muscle the isoforms differ in their levels of ligand binding. Additionally, chicken thigh and breast muscles differ in the relative amounts of the two isoforms they contain, the amounts being similar in breast muscle and markedly different in thigh muscle. In conclusion, in contrast to mammalian skeletal muscle, two foot protein isoforms are present in amphibian, avian, and piscine skeletal muscles. This may represent a general difference in the architecture and/or a functional specialization of the triad junction in mammalian and nonmammalian vertebrate muscles.

INTRODUCTION

The triad junctional foot protein is thought to play a key role in the events coupling surface membrane depolarization and contraction in vertebrate fast twitch skeletal muscle (1, 2). Recently, we obtained evidence for the coexistence of two isoforms of this protein in avian pectoral muscle (3). In contrast, only a single foot protein isoform appears to be expressed in mammalian fast twitch skeletal muscle (4–8). In view of this biochemical difference and the frequent use of nonmammalian vertebrate skeletal muscles for physiological and morphological studies, it was of interest to determine whether muscles from other vertebrates contain a single foot protein isoform like mammalian muscle, or resemble avian muscle and express two isoforms. Both mammals and birds have evolved from reptiles (9); consequently, there is no a priori reason to assume either possibility.

We have found that two foot protein isoforms also coexist in amphibian skeletal muscle fibers. Additionally, we present evidence for two foot protein isoforms in toadfish skeletal muscle. These data suggest that mammalian and nonmammalian vertebrate skeletal muscles differ in the number of foot protein isoforms they contain.

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Abbreviations used in this paper: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio] 1-propranesulfonate; CHES, 2[*N*-cyclohexylamino] ethanesulfonic acid; DFP, diisopropyl fluorophosphate; Mab, monoclonal antibody; PBS, phosphate-buffered saline; PEI, polyethylenimine; PMSF, phenylmethylsulfonylfluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, tris-[hydroxymethyl]aminomethane.

EXPERIMENTAL PROCEDURES

Materials

Fertilized white Leghorn chicken eggs were purchased from Weber Egg Co. (Rio Linda, CA), mature bullfrogs (*Rana catesbiana*) from W.A. Lemberger Co. (Oshkosh, WI), and toadfish from the Marine Biological Laboratories (Woods Hole, MA). Leupeptin, PMSF[1], CHAPS, L-α-phosphatidylcholine, agarose-linked goat anti-mouse IgG (whole molecule) antibodies, PEI, *n*-propyl gallate, DFP, and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO); alkaline phosphatase–conjugated goat anti-mouse IgG from Tago (Burlingame, CA); SDS-PAGE molecular weight standards from Bio-Rad Laboratories (Richmond, CA); paraformaldehyde from Polysciences, Inc. (Warrington, PA); OCT compound from Miles Laboratories, Inc. (Elkhart, IN); FITC-conjugated goat anti-mouse antibodies, normal goat, and normal mouse sera were obtained from Organon Technika (Durham, NC).

Membrane preparation

Microsomal membranes were prepared from chicken pectoral and thigh muscles, bullfrog hindlimb fast twitch muscles, and toadfish swim bladder and dorsal body wall muscles, using the method of Saito et al. (10) with minor modifications (3). The excised muscles were rinsed in ice-cold isotonic saline, minced, cleaned of all obvious nonmuscle tissue, suspended in 5 ml/g wet weight of a solution containing 0.3 M sucrose, 10 mM imidazole, pH 7.4, 0.23 mM PMSF, 1.1 μM leupeptin

(solution A), and homogenized three times for 1 min each at high speed in a Waring blender. After each homogenization, the pH was checked and, if necessary, adjusted to 7.4 with 1 M NaOH. The homogenate was centrifuged at 12,000 *g* for 15 min and the supernatant (S_1) was filtered through four layers of cheesecloth and saved. The pellet was resuspended in the initial volume of solution A, rehomogenized as above, and centrifuged at 12,000 *g* for 15 min. The resulting supernatant (S_2) was filtered through cheesecloth and the S_1 and S_2 supernatants were centrifuged separately at 135,000 *g* for 90 min. The microsomal membrane pellets termed P_1 and P_2, respectively, were resuspended in solution A at a protein concentration of 10–20 mg/ml, rapidly frozen in liquid N_2 and stored at −90°C. The microsomal fraction P_2 from chicken pectoral and P_1 from bullfrog hindlimb muscles, respectively, were found to contain the greatest levels of the foot proteins and were used in the present studies. The fractional distribution of the toadfish and chicken leg muscle foot proteins were not determined. For the preliminary characterizations of these muscles described in this report, the P_1 and P_2 fractions were combined. Microsomal membrane protein was determined by the method of Lowry et al. (11) using BSA as a standard.

Solubilization and partial purification of the foot proteins

The foot proteins present in the different muscles were solubilized with CHAPS in the presence of phosphatidylcholine as described previously (3). Membrane protein (10 mg/ml) was solubilized with 2% Triton X-100 in solution A containing 10 mM $CaCl_2$ to extract proteins other than the foot proteins. The nonsolubilized material was collected by centrifugation at 10,000 *g* for 30 min and resuspended at 5 mg/ml (assuming recovery of 25% of the starting membrane protein) in 0.5 M KCl, 20 mM Tris, pH 7.4, 5 mM DTT, 0.23 mM PMSF, 1.1 μM leupeptin (solution B). The foot proteins were then solubilized with 1.0% CHAPS/0.5% phosphatidylcholine in this same solution at a final protein concentration of 3 mg/ml. Solubilized and nonsolubilized material were separated by centrifugation at 100,000 *g* for 30 min. For some experiments, the Triton X-100 extraction step was omitted and membrane protein (5 mg/ml) was suspended in solution B and solubilized with CHAPS/phospholipid as described above.

The foot proteins were partially purified by sedimentation through continuous sucrose gradients (6). Solubilized protein was loaded onto 32-ml continuous 10–30% sucrose gradients and centrifuged at 113,000 *g* for 14–16 h. Gradient fractions (3 ml) were analyzed for the presence of foot protein subunit polypeptides by SDS-PAGE and for [^{3}H]epiryanodine binding as described below. The gradient fractions were either used immediately or rapidly frozen in liquid N_2 and stored at −90°C. Solubilized protein was quantitated with the method of Kaplan and Pedersen (12) using BSA as a standard.

PAGE and Western blot analyses

Membrane samples were prepared for SDS-PAGE (13) in a load buffer at final protein and SDS concentrations of 2.5 mg/ml and 2%, respectively. Sucrose gradient fractions were prepared in 4× concentrated load buffer. Aliquots (40–50 μl) were loaded onto continuous 4–20% linear polyacrylamide gradient SDS resolving minigels with 3% stacking gels and subjected to a constant voltage of 130 V at 0–4°C. The gels were stained for protein with 0.2% Coomassie brillant blue.

Western blotting onto nitrocellulose was accomplished in 10 mM CHES, pH 9.6, 10% ethanol (14) using a Transphor TE 50 (Hoefer Scientific Instruments, San Francisco, CA) at an initial setting of 100 V for the first 2 h and a setting of 40 V overnight. The proteins transferred to the nitrocellulose were visualized by brief staining with 1% Ponceau S in 5% acetic acid followed by destaining in water. Immunostaining of the blotted proteins was visualized using goat anti-mouse IgG conjugated to alkaline phosphatase using 5-bromo-4-chloro-3-indolyl-phosphate (*p*-toluidine salt) and nitroblue tetrazolium as substrates.

Immunoprecipitation of foot proteins

Murine, anti-chick skeletal muscle foot protein monoclonal (IgG_1) antibodies were used as primary antibodies in immunoprecipitation experiments. These antibodies were precipitated using goat anti-mouse IgG antibodies linked to Sepharose as described previously (3). Briefly, antibody beads were incubated with solubilized protein for 3 h at room temperature. The beads were collected by brief centrifugation and washed three times with a solution containing 0.5 M NaCl, 10 mM $NaPO_4$, pH 7.4. The original supernatants and the washed pellets were analyzed for the presence of foot protein subunit polypeptides by SDS-PAGE. As described below, two foot protein isoforms have been identified in the nonmammalian vertebrate skeletal muscles studied. These have been termed lower (L) and upper (U), based on differences observed in the apparent molecular masses of the polypeptide subunits associated with each isoform (cf. Fig. 1). The following monoclonal antibodies were used in these studies. For bullfrog skeletal muscle, Mab 26G = anti-L; Mab 29F = anti-U; Mab 34C = anti L/U. For toadfish skeletal muscle, Mab 59E = anti-L; Mab 34C = anti-U; Mab 76F = anti L/U. For chicken pectoral and thigh muscles, Mab 110E = anti-L; Mab 110F = anti-U; Mab 34C = antiL/U. Note that Mab 34C recognizes both foot protein isoforms in bullfrog and chicken skeletal muscles, but only one of the isoforms in toadfish muscle. The foot protein isoform specificity of the antibodies used in these studies is demonstrated in Results.

[^{3}H]Epiryanodine binding experiments

In the present experiments, [^{3}H]epiryanodine (15) was used with the knowledge that it binds three to four times less well to the high-affinity binding sites of the skeletal muscle foot proteins than does [^{3}H]ryanodine (3). The binding of [^{3}H]epiryanodine to solubilized foot proteins was measured by incubating 50 μl of solubilized protein for 1 h at 23°C in a final volume of 0.1 ml of 0.5 M KCl, 20 mM Tris, pH 7.4, 0.2 mM $CaCl_2$, 1% CHAPS, 0.5% phosphatidylcholine, 5 mM DTT 0.23 mM PMSF, 1.1 μM leupeptin containing 0.540 μM [^{3}H]epiryanodine (45.8 Ci/mmol sp act). Nonspecific binding was measured in the presence of 60 μM unlabeled ryanodine. Ligand binding was terminated by filtration through GF/B filters (Whatman Instruments, Clifton, NJ) that had been pretreated with 1% PEI (16) and washing of the filter seven times with 4-ml aliquots of an ice-cold solution containing 1.0 M NaCl, 20 mM Tris, pH 7.4, 200 μM $CaCl_2$. The radioactivity remaining on the filter was measured by liquid scintillation counting.

When the amount of [^{3}H]epiryanodine bound by solubilized foot proteins was measured after immunoprecipitation with anti-foot protein antibodies, 100 μl of solubilized protein was incubated in a final volume of 0.123 ml of the solution described in the preceding paragraph for 1 h at room temperature and then immunoprecipitated as described above. The [^{3}H]ligand remaining in the supernatant was measured after filtration through PEI-treated GF/B filters, while the radioactivity precipitated was determined by addition of washed precipitated material directly to vials for counting. The levels of nonspecific binding obtained with these binding protocols were different, and have been indicated in the appropriate figures.

Limited proteolysis of bullfrog muscle ryanodine-binding proteins

Bullfrog microsomal membrane proteins were solubilized with CHAPS in the presence of phosphatidylcholine as described above. The two

ryanodine-binding proteins were precipitated individually with polypeptide-specific antibodies and proteolyzed at room temperature with trypsin (0.5 μg/ml). The resulting peptide fragments were separated by SDS-PAGE and visualized by either staining the gel with Coomassie brilliant blue, as densitometric scans of the stained gel using a model GS 300 Scanning Densitometer (Hoefer Scientific Instruments) and by Western blotting using an antibody, 34C, which recognizes an epitope common to both high molecular mass polypeptide subunits (see Results).

Immunofluorescent labeling and confocal immunofluorescence microscopy

Adult bullfrogs were fixed by vascular perfusion with 4% paraformaldehyde in 0.1 M PBS and the sartorius muscle was removed and fixed further in the same solution for 2 h at 4°C. Tissue was cryoprotected in 0.5 M sucrose in PBS for 30 min and then in 1.0 M sucrose in PBS for 1 h. Tissue was frozen in OCT compound in either dry ice or liquid N_2.

Immunofluorescence labeling was conducted as described previously (17). Briefly, 10-μm-thick cryostat sections were mounted on gelatin-coated slides and rinsed with 0.1 M PBS containing 0.05 M glycine (PBS-Gly). Nonspecific binding was blocked by incubating the sections for 20 min in 1% normal goat serum, 0.5% BSA, and 0.5% gelatin in PBS. Sections were incubated with either monoclonal anti-foot protein antibodies (3), or with normal mouse serum for 1 h at 22°C. The sections were washed 10 times for 2 min each in PBS and incubated in goat anti–mouse IgG-FITC conjugate for 1 h at 22°C. The sections were again washed 10 times for 2 min each in PBS and covered with an anti-fade media consisting of 4% *n*-propyl gallate, 90% glycerol in PBS.

Immunofluorescent images were obtained using a Bio-Rad MRC-600 confocal laser scanning microscope fitted to a Zeiss Axiovert 35M inverted microscope. Images were stored on an AGA Optical Disk Recorder and photographed with a Lasergraphics LFR Plus camera using Kodak Ektachrome 100 film.

RESULTS

High molecular mass polypeptides are present in chicken, frog, fish, and rabbit skeletal muscle membranes

As a first step in investigating the nature of the foot protein isoforms in nonmammalian vertebrate muscle, we visualized the polypeptides having molecular masses of ~500 kD, in microsomal membranes from chicken pectoral and thigh, toadfish dorsal body wall, bullfrog hindlimb, and rabbit back muscles in a SDS gel stained for protein (Fig. 1). In contrast to rabbit muscle microsomes, which have been demonstrated previously to express a single foot protein isotype, bullfrog and toadfish muscle membranes were similar to those from chicken pectoral muscle in that they contained two prominent high molecular mass polypeptides. This suggests that two foot protein isoforms are expressed in both bullfrog and toadfish muscles. The polypeptides in the different muscles, particularly in bullfrog muscle, appear to differ in their apparent molecular masses. The extent of these differences is difficult to assess, since the relative mobilities of such large polypeptides can be variable. Chicken thigh muscle microsomal membranes also contain two high molecular mass polypeptides,

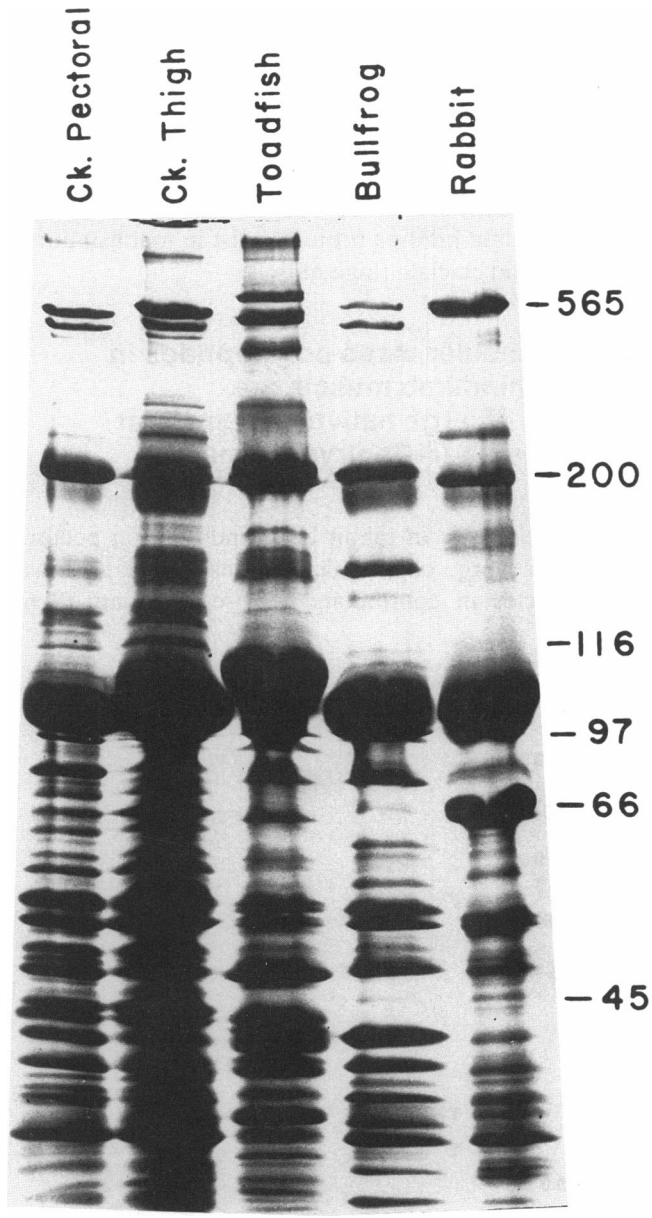

FIGURE 1 Multiple high molecular mass polypeptides are present in sarcoplasmic reticulum from nonmammalian vertebrate skeletal muscles. The proteins in microsomal membranes isolated from chicken pectoral (80 μg) and thigh (70 μg), toadfish back (80 μg), bullfrog hindlimb (50 μg), and rabbit back (37.5 μg) muscles were separated in a continuous 4–20% polyacrylamide gradient SDS gel and visualized by staining with Coomassie brilliant blue. The rabbit skeletal muscle foot protein (565 kD), myosin heavy chain (200 kD), beta-galactosidase (116 kD), phosphorylase b (98 kD), BSA (66 kD) and ovalbumin (45kD) were used as molecular mass standards.

indicating that multiple foot proteins exist in different avian muscles and are not unique to pectoral muscle. In the experiments described below, we have characterized the foot proteins present in bullfrog skeletal muscle and demonstrate that two unique isoforms coexist in the same muscle fibers. In addition, we present results, which indicate that two homooligomeric, high molecular mass ryanodine-binding proteins exist in toadfish dorsal body wall and chicken thigh muscles.

High molecular mass polypeptides in bullfrog hindlimb muscle are subunits of large native proteins that copurify with [^{3}H]epiryanodine binding

The foot proteins in rabbit back and chicken pectoral muscles are large oligomeric proteins that sediment as 30S particles in continuous sucrose gradients (3, 6). Therefore, we investigated whether the polypeptides identified as putative foot protein subunits in bullfrog skeletal muscle are associated with native proteins having a similar size. In these studies, bullfrog hindlimb and chicken pectoral microsomal membrane proteins were solubilized with CHAPS in the presence of phosphatidylcholine and sedimented through continuous 10–30% sucrose gradients. The gradients were fractionated and analyzed by SDS-PAGE for the presence of the high molecular mass polypeptides (Fig. 2, *A* and *B*). Although there were minor differences in the fractions containing these polypeptides, in both cases their distributions peaked in the same gradient fractions. An identical sucrose gradient distribution was obtained for the rabbit back muscle foot protein (data not shown). These results indicate that the polypeptides in avian, amphibian, and mammalian skeletal muscles are associated with, and presumably subunits of, proteins having a similar native oligomeric size. As shown in Fig. 3,

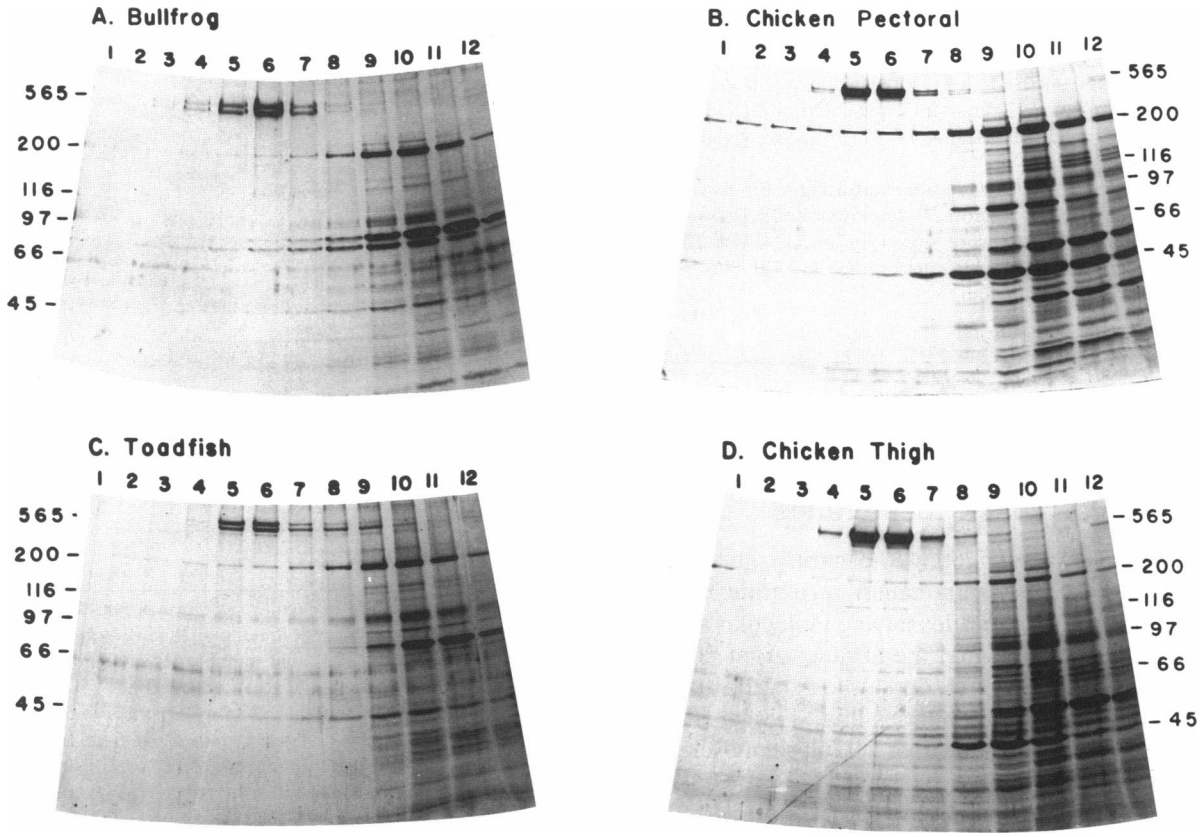

FIGURE 2 The sarcoplasmic reticulum in nonmammalian vertebrate muscle contains large proteins with comparable native and polypeptide subunit sizes. Proteins in microsomal membranes obtained from bullfrog hindlimb (*A*), chicken pectoral (*B*), toadfish back (*C*), and chicken thigh (*D*) were solubilized with CHAPS and sedimented through continuous 10–30% sucrose gradients as described in Experimental Procedures. The gradients were fractionated and the protein profiles of each fraction (numbered from the bottom of the gradient) determined using SDS-PAGE and staining with Coomassie brilliant blue. Comparable amounts of protein were loaded onto each gradient. The molecular mass standards used are those described for Fig. 1.

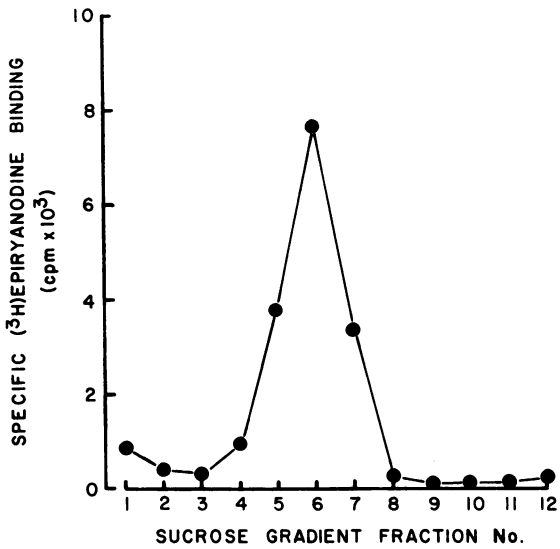

FIGURE 3 Specific [^{3}H]epiryanodine binding copurifies with the high molecular mass proteins solubilized from bullfrog hindlimb muscle sarcoplasmic reticulum membranes. Equal volumes of the sucrose gradient fractions shown in Fig. 2 *A*, were tested for the ability to bind [^{3}H]epiryanodine as described in Experimental Procedures.

specific [^{3}H]epiryanodine binding copurifies with the bullfrog muscle proteins.

Large native proteins in bullfrog muscle containing the high molecular mass polypeptides are homooligomeric ryanodine-binding proteins

To determine whether the triad junctional foot proteins in bullfrog skeletal muscle are hetero- or homooligomeric proteins, we identified anti-chicken skeletal muscle monoclonal antibodies that specifically recognize the individual polypeptide subunits of these proteins. These antibodies were used in immunoprecipitation experiments to determine first, whether the frog muscle high molecular mass polypeptides are subunits of hetero- or homooligomeric proteins. The rationale for this experiment is that if both polypeptides are subunits of a heterooligomeric protein, then antibodies specific for either polypeptide will precipitate proteins containing both polypeptides. Conversely, if the polypeptides are subunits of two homooligomeric proteins, then polypeptide-specific antibodies will precipitate proteins containing only the polypeptide recognized by the antibody being used. Second, these antibodies were used in a similar protocol to establish whether both polypeptides are subunits of ryanodine-binding proteins by assessing whether specifically bound [^{3}H]epiryanodine coimmunoprecipitates with each polypeptide. In these experiments, muscle microsomal proteins are solubilized with CHAPS and permitted to bind [^{3}H]epiryandine before immunoprecipitation with polypeptide-specific antibodies. The results of these experiments are shown in Fig. 4 and can be compared to those obtained with chicken pectoral muscle (Fig. 8).

Antibodies specific for each high molecular mass polypeptide in bullfrog skeletal muscle selectively immunoprecipitate proteins that contain only the polypeptide recognized by the antibody depleting that polypeptide

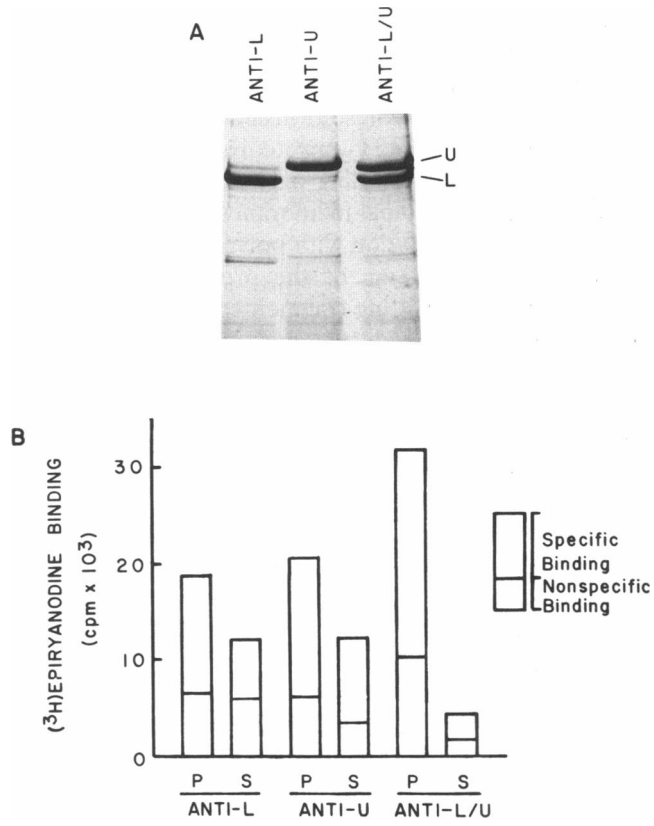

FIGURE 4 Bullfrog hindlimb muscle sarcoplasmic reticulum contains two homooligomeric, high molecular mass proteins that bind [^{3}H]epiryanodine. The large molecular mass proteins were solubilized from microsomal membranes with CHAPS and precipitated using monoclonal antibodies that recognize either only one (Mab 26G = anti-L; Mab 29F = anti-U) or both of the high molecular mass polypeptides (Mab 34C = antiL/U). (*A*) Immunoprecipitated proteins were resolved in continuous 4–20% polyacrylamide gradient SDS gels and visualized after staining with Coomassie brilliant blue. *U* and *L* refer to the higher and lower molecular mass polypeptides, respectively. The molecular mass standards were the same as those described for Fig. 1. (*B*) The solubilized proteins were incubated with [^{3}H]epiryanodine before immunoprecipitation. The level of binding associated with the precipitates (*P*) and remaining in the supernatants (*S*) after precipitation with either polypeptide specific, or an antibody that recognizes both polypeptides were measured.

from the supernatant (Fig. 4 *a*). These results indicate that in this tissue, both polypeptides are subunits of distinct homooligomeric proteins. Approximately equivalent amounts of specifically bound [^{3}H]epiryanodine immunoprecipitates with each of the proteins (Fig. 4 *B*). Together, these results demonstrate the existence of two homooligomeric ryanodine-binding proteins in bullfrog skeletal muscle.

Two ryanodine-binding proteins in bullfrog skeletal muscle are not related in a simple manner by proteolysis

A limited proteolysis/immunoprecipitation protocol was used to demonstrate that the polypeptide subunits of the two putative foot protein isoforms in bullfrog muscle are unique and not related by proteolysis. In this experiment, the ryanodine binding proteins were solubilized from bullfrog microsomal membranes with CHAPS and separated by precipitation with polypeptide-specific antibodies. After digestion of the precipitated proteins with trypsin, the resulting fragments were separated by SDS-PAGE, Western blotted, and probed with an antibody that recognizes an epitope common to both polypeptides. The rationale for this experiment is, if these polypeptides are related by proteolysis, then an epitope present in both polypeptides will segregate with similar proteolytic fragments from either polypeptide.

The results of this experiment are illustrated in Fig. 5. The solubilized protein mixture and the separate protein precipitates are shown in lanes 1–3 of *A*. The proteolytic fragments produced from each protein after 5- and 10-min exposures to trypsin (0.5 μg/ml) can be seen in lanes 4–7. A Western blot of a gel identical to that in *A* probed with an antibody recognizing an epitope common to both polypeptides can be seen *B* and densitometric scans of lanes *2* and *3* and *4* and *5* of the gel shown in *A* are presented in *C*. The results indicate that distinctive peptide maps are obtained for the two ryanodine-binding proteins. In addition, the protein consisting of the lower polypeptide is more resistant to trypsin (compare Fig. 5, lanes *4* and *5*). A similar difference in sensitivity to trypsin was observed for the chicken skeletal foot protein isoforms (3). Thus, two unique, large ryanodine-binding proteins exist in both frog and chicken skeletal muscles.

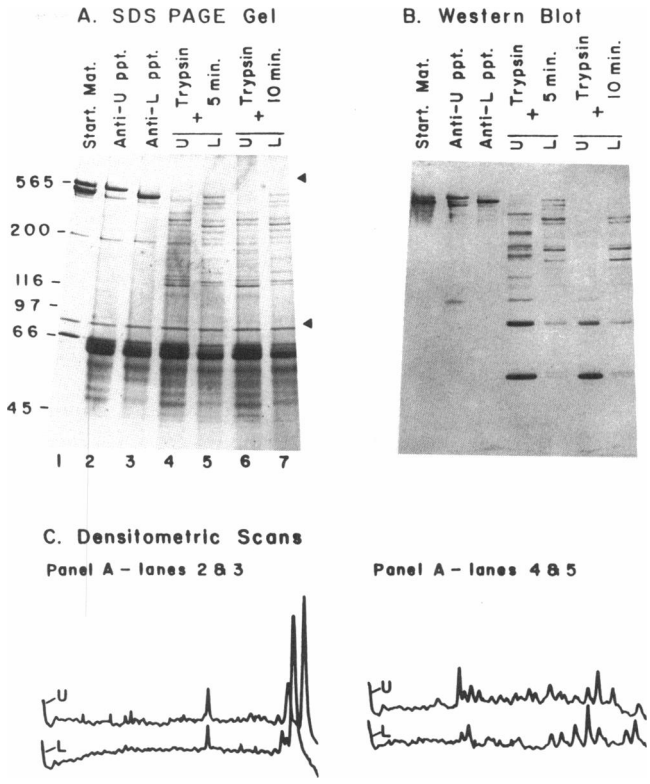

FIGURE 5 The two ryanodine-binding proteins in bullfrog skeletal muscle sarcoplasmic reticulum are unique and not related in a simple way by proteolysis. (*A*) Microsomal membrane protein was solubilized with CHAPS and sedimented through a continuous 10–30% sucrose gradient. Monoclonal antibodies specific for each polypeptide (Mab 26G = anti-L; Mab 29F = anti-U) were used to selectively precipitate each protein from an appropriate sucrose gradient fraction (lanes 1–3). The ratios of antibody and solubilized protein were adjusted to result in the precipitation of approximately equivalent quantities of each protein. The precipitates were incubated with trypsin (0.5 μg/ml) for either 5 or 10 min, at which time proteolysis was stopped by the addition of DFP (2 mM) and 4× SDS gel load buffer. The proteolytic fragments generated in each reaction were resolved in continuous 4–20% polyacrylamide gradient SDS gels (lanes *4–7*) and visualized by staining with Coomassie brilliant blue. The molecular mass standards used are the same as those described for Fig. 1. (*B*) The proteins present in an SDS gel identical to that shown in *A* were electrophoretically transferred onto nitrocellulose and probed with a monoclonal antibody (Mab 34C) that recognizes both the larger (*U*) and smaller (*L*) polypeptide subunits. (*C*) The proteins present before (*left scan*) and after (*right scan*) proteolysis of precipitates containing either the larger (*U*) and smaller (*L*) polypeptide subunits were also visualized as densitometric scans of the region of the gel shown in *A* indicated by the arrowheads on the right side of the figure. The scan proceeded from left to right, starting at the lower arrowhead.

Two ryanodine-binding proteins in bullfrog skeletal muscle coexist in the same muscle fibers.

The two foot protein isoforms identified in bullfrog muscle could be present in different muscle fiber types and represent phenotype-specific isoforms. To determine whether this is the case, polypeptide-specific antibodies were used in conjunction with indirect immunocytochemical techniques to localize these proteins in consecutive serial sections from the same frog muscle

fibers. As can be seen in Fig. 6, both proteins coexist in the same fibers. Moreover, the highly banded, punctate distribution of the fluorescence observed for both proteins is consistent with the Z line localization of the triad junction in bullfrog skeletal muscle (18) and is identical to that observed previously for chicken pectoral muscle (3).

Two homooligomeric ryanodine binding proteins also exist in toadfish skeletal muscle

To establish further whether the presence of two foot protein isoforms is a general characteristic of nonmammalian vertebrate skeletal muscle, we assessed whether two ryanodine-binding proteins exist in piscine muscle. The toadfish was selected for these experiments because previous work by Block et al. (19) suggests that two foot protein isotypes may be present in swim bladder muscle from this fish. In the present studies similar results were obtained for swim bladder and dorsal body wall muscles. Due to its greater abundance, the latter muscle was used most extensively. As shown in Figs. 1 and 2 *C*, two polypeptides having a molecular mass of ~500 kD are solubilized by CHAPS from toadfish muscle microsomal membranes and are associated with native proteins that sediment in a continuous sucrose gradient in a manner similar to that observed for the rabbit, chicken pectoral, and bullfrog muscle proteins.

Anti-chicken skeletal muscle foot protein antibodies that recognize each of the toadfish muscle polypeptides were identified and used to establish whether the polypeptides are subunits of homo- or heterooligomeric proteins and if these proteins bind [^{3}H]epiryanodine. As shown in Fig. 7, polypeptide-specific antibodies precipitated proteins containing only the polypeptide recognized by the antibody and specifically bound [^{3}H]epiryanodine coprecipitated with both proteins. Therefore, like chicken pectoral and bullfrog hindlimb muscles, the high molecular mass polypeptides in toadfish muscle are subunits of two homooligomeric ryanodine-binding proteins that are present in approximately equivalent

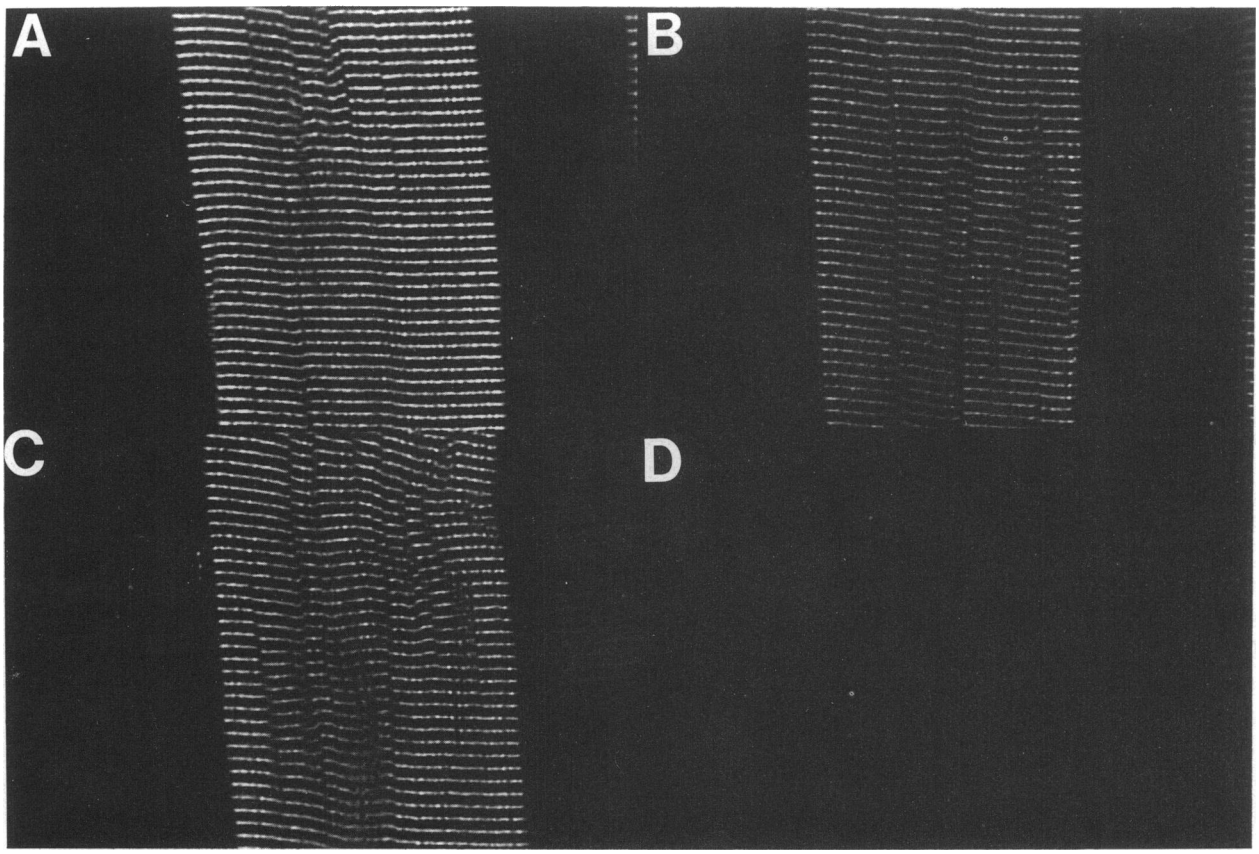

FIGURE 6 The two ryanodine-binding proteins in bullfrog skeletal muscle coexist in the same muscle fibers. Consecutive, serial longitudinal sections of the muscle were obtained and stained with two monoclonal antibodies (Mabs 32E and 29F) that recognize only the smaller polypeptide subunit (*A* and *B*), with an antibody (Mab 26G) specific for the larger polypeptide subunit (*C*), or with normal mouse serum (*D*). The antibodies used in *B* and *C* were the same as those used in the experiment shown in Fig. 4.

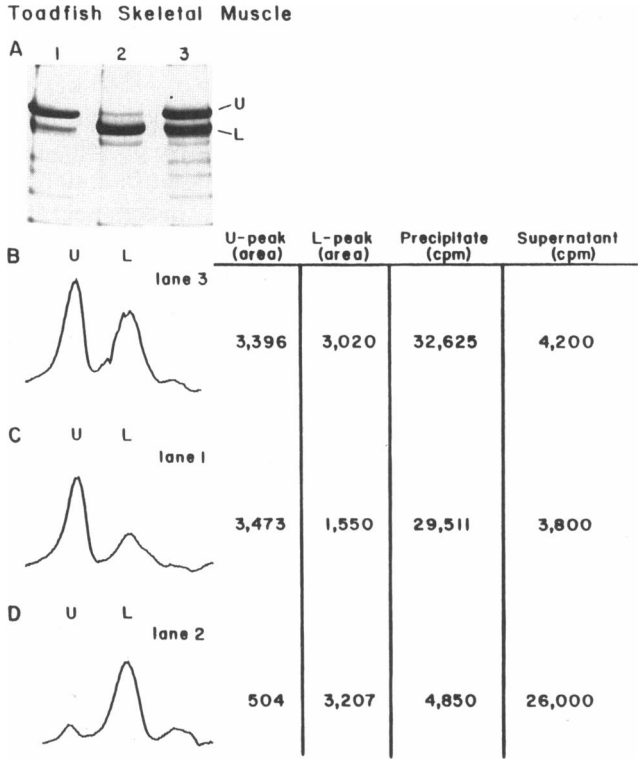

FIGURE 7 Toadfish skeletal muscle sarcoplasmic reticulum contains two homooligomeric proteins which both bind [^{3}H]epiryanodine. (*A*) Toadfish muscle microsomal membrane protein was solubilized with CHAPS and immunoprecipitated with monoclonal antibodies specific for either the larger (Mab 34C = anti-*U*, lane *1*), or smaller (*L*, Mab 59E = anti-L, lane *2*) polypeptide subunit, or with an antibody that recognizes both polypeptides (Mab 76F = anti-L/U, lane *3*). The precipitated proteins were resolved in a continuous 4–20% polyacrylamide gradient gel and visualized after staining with Coomassie brilliant blue. (*B–D*) The high molecular mass proteins present in each lane in *A* were quantitated by densitometric scanning and the areas under each peak given in relative units. In a parallel experiment, the solubilized proteins were incubated with [^{3}H]epiryanodine before immunoprecipitation. The levels of specifically bound ligand in both the precipitates and remaining in the supernatant after precipitation were determined.

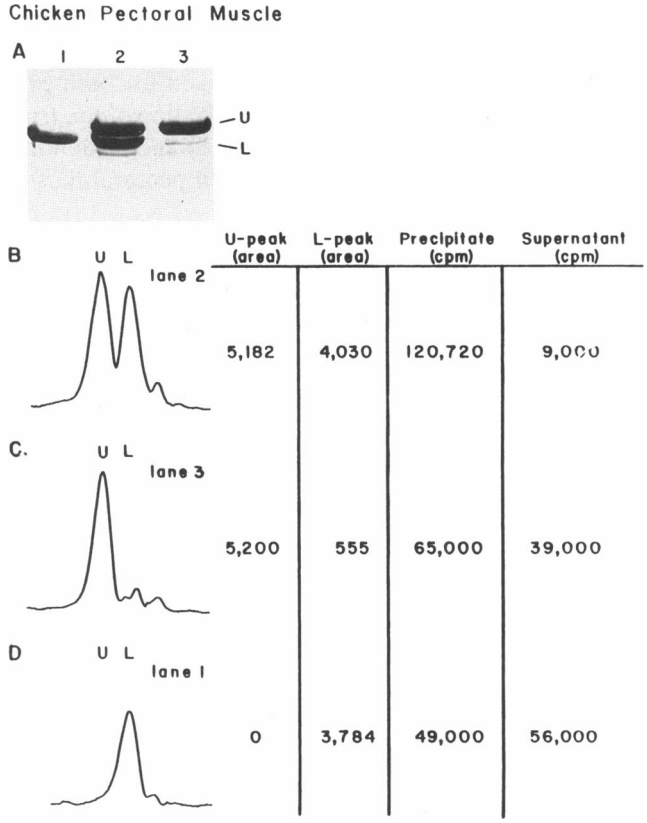

FIGURE 8 The two foot protein isoforms in chicken pectoral muscle bind approximately equivalent levels of [^{3}H]epiryanodine. An experiment identical to that described in Fig. 7 was conducted using chicken pectoral muscle microsomal membranes. In this study, Mab 110F = anti-U; Mab 110E = anti-L; and Mab 34C = anti-L/U.

amounts. In contrast to the chicken pectoral (Fig. 8) and bullfrog (Fig. 4) muscle proteins, the two toadfish proteins appear to differ, at least under the conditions used in the present experiments, in their ability to bind [^{3}H]epiryanodine (Fig. 7). Essentially equivalent levels of specifically bound ligand are precipitated when antibodies recognizing either, both polypeptides, or only the higher molecular mass polypeptide are used, and only a low level of specifically bound ligand remained in the supernatant. In contrast, a relatively low level of specifically bound [^{3}H]epiryanodine coprecipitated with the protein containing the lower molecular mass polypeptide, with most of the bound ligand remaining in the supernatant. This difference in binding remains when the concentration of [^{3}H]epiryanodine is increased fourfold, suggesting that a difference in binding capacity and not affinity may be involved (data not shown). The ryanodine binding proteins present in toadfish muscle also differed immunologically from those in chicken or bullfrog muscles. The epitope recognized by the monoclonal antibody 34C is present in both isoforms in the latter two muscles, whereas, only the toadfish muscle isoform containing the larger polypeptide subunit is immunoprecipitated by this antibody.

Chicken thigh muscle contains two homooligomeric ryanodine-binding proteins

In a final set of studies, we compared the ryanodine-binding proteins in chicken breast and thigh muscles, which contain different predominant fiber types. The two foot protein isoforms characterized in pectoral

muscle (Fig. 8), were also observed in thigh muscle, and shown to be [^{3}H]epiryanodine binding homooligomeric proteins (Fig. 9). The breast and thigh muscle differ in the relative amounts of the two polypeptides each contains. Both polypeptides are present in equivalent amounts in pectoral muscle, whereas the higher molecular mass polypeptide is more abundant in the thigh muscle (compare lanes *1* and *2* of Figs. 1 and 2 *D*, see Fig. 5 *A*). The differences in the relative abundances of the two high molecular mass polypeptides can be appreciated from both the quantity of each polypeptide and the level of specifically bound [^{3}H]epiryanodine immunoprecipitated by polypeptide-specific antibodies. The extent to which the difference in the levels of the two isoforms reflects phenotypic differences between fiber types is currently being investigated.

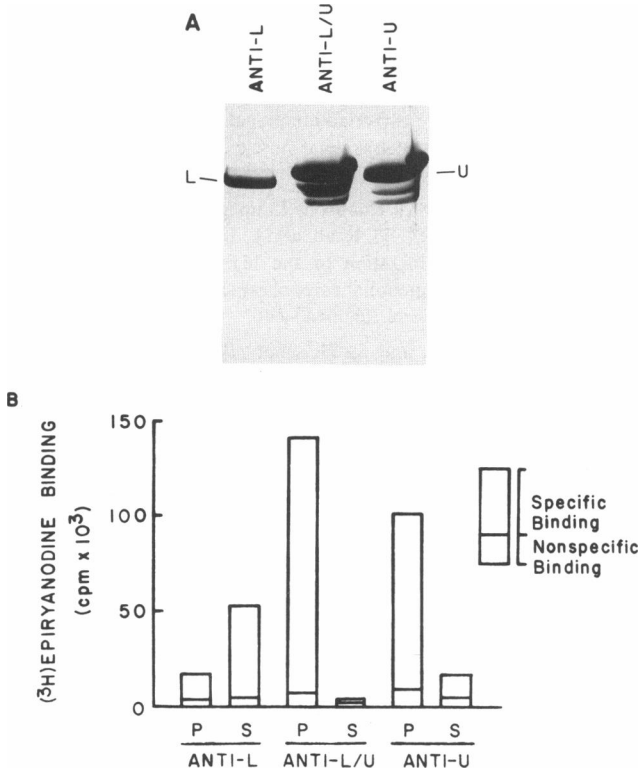

FIGURE 9 Chicken thigh muscle also contains two homooligomeric ryanodine binding proteins. (*A*) Microsomal membrane proteins were solubilized with CHAPS and precipitated with monoclonal antibodies specific for the larger polypeptide subunit (Mab 110F = anti-U), the smaller subunit (Mab 110E = anti-L), or both polypeptides (Mab 34C = anti-L/U). The precipitated polypeptides were separated in a continuous 4–20% polyacrylamide gradient SDS gel and visualized after staining with Coomassie brilliant blue. (*B*) In a parallel experiment, the solubilized proteins were incubated with [^{3}H]epiryanodine before immunoprecipitation and the levels of bound ligand in the precipitates (*P*) and remaining in the supernatants (*S*) were determined in each case.

DISCUSSION

The purpose of the present studies was to determine whether the expression of multiple foot protein isoforms is unique to chicken pectoral breast muscle, or a trait shared with other nonmammalian vertebrate skeletal muscles. The results obtained demonstrate that two large ryanodine-binding proteins coexist in the same frog skeletal muscle fibers. In addition, two biochemically and immunologically distinguishable ryanodine binding proteins are also identified in fish skeletal muscle and chicken thigh muscle. Based on the biochemical, immunological, and pharmacological similarities of the ryanodine-binding proteins in chicken, fish, and frog muscles, we suggest that in each case these proteins represent triad junctional foot proteins. On this basis, these data suggest that mammalian and nonmammalian skeletal muscles differ in the number of foot protein isoforms expressed.

Whether mammalian and nonmammalian vertebrate skeletal muscle have functional and/or structural differences due to the expression of different foot protein isoforms and whether such differences have exerted any selective pressure during evolution are not known. The two isoforms identified in mature chicken pectoral muscle are expressed at markedly different times during embryonic chick development (22). The differential expression of these proteins in developing muscle, and their continued expression in postmaturation muscle suggests that each foot protein isoform may make unique contributions to muscle cell function. The expression of different relative amounts of the two proteins in chicken thigh muscles, which differ from pectoral muscle in predominant muscle fiber type (18) may also be consistent with this possibility. Whether the foot protein isoforms are expressed differentially in chicken skeletal muscle fiber types and how this correlates with the functional characteristics of these proteins are currently being investigated. As discussed below, a single ryanodine-binding protein isoform appears to be expressed in chicken ventricular myocardium and in certain regions of the avian brain. The possibility that the foot protein isoforms differ in function is also suggested by the toadfish muscle proteins, which exhibit different capacities for binding [^{3}H]epiryanodine. We are currently characterizing the ligand binding and ion channel characteristics of the foot protein isoforms in piscine skeletal muscle to determine the molecular basis and functional significance of this difference.

Recently, Block et al. (19) reported morphological evidence for different foot protein subtypes. Analysis of freeze-fracture images of toadfish swim bladder muscle revealed that the foot proteins present in a triad junction

are alternately apposed by a tetrad of intramembrane particles present in transverse tubular membranes. The transverse tubule particles were suggested to represent the dihydropyridine receptor. These results provide evidence that triad junctional foot proteins in toadfish skeletal muscle may not be equivalent. It is tempting to speculate that our findings with toadfish muscle represent the biochemical counterpart of the structural data of Block et al. (19). Direct immunoelectronmicroscopic techniques are currently being used to determine the cellular distribution of each foot protein isoform in nonmammalian vertebrate muscle to test this possibility.

A final question concerns whether the foot protein isoforms are the products of a single or multiple genes. Cardiac and skeletal muscle foot protein genes have been identified in mammalian tissues (7, 8, 20). Therefore, a likely possibility is that both genes are expressed in nonmammalian vertebrate skeletal muscle, whereas only one foot protein gene is expressed in a tissue-dependent manner in mammalian muscle. Another possibility is that the two foot protein isoforms in nonmammalian vertebrate skeletal muscle result from alternative splicing of the transcript derived from a single gene. The determination of cDNA sequences unique to mammalian skeletal and cardiac foot protein genes (7, 8, 20) will permit questions concerning the regulation of the expression of these proteins to be addressed. It is of interest that expression of the two foot protein isoforms, at least in different chicken tissues, appears to be tissue specific. For example, studies conducted to date indicate that chick cardiac muscle contains only single foot protein isoform (Airey, J. A., and J. L. Sutko, unpublished observations, Dutro, S., and W. R. Trumble, personal communication) although it is not clear whether this isoform is one of those found in skeletal muscle. In addition, we have obtained evidence for two ryanodine-binding protein isoforms in chicken cerebellum, whereas, only a single isoform appears to exist in other brain regions (21, 23). Further characterizations of the functional properties of the ryanodine-binding protein isoforms and their roles in muscle, as well as nonmuscle cell function are necessary to establish why one or both isoforms are present in a given tissue, and why the expression of two isoforms appears to occur more commonly, if not exclusively in nonmammalian vertebrates.

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REFERENCES

1. Franzini-Armstrong, C. 1970. Studies of the triad. I. Structure of the junction in frog muscle fibers. *J. Cell Biol.* 47:488–499.
2. Caswell, A. H., and J.-P. Brunschwig. 1984. Identification and extraction of proteins that compose the triad junction of skeletal muscle. *J. Cell Biol.* 99:929–939.
3. Airey, J. A., C. F. Beck, K. Murakami, S. J. Tanksley, T. J. Deerinck, M. H. Ellisman, and J. L. Sutko. 1990. Identification and localization of two triad junctional foot protein isoforms in mature avian fast twitch skeletal muscle. *J. Biol. Chem.* 265:14187–14195.
4. Campbell, K. P., C. M. Knudson, T. Imagawa, A. T. Leung, J. L. Sutko, S. D. Kahl, C. D. Raab, and L. Madson. 1987. Identification and characterization of the high affinity [^{3}H]ryanodine receptor of the junctional sarcoplasmic reticulum Ca^{2+} release channel. *J. Biol. Chem.* 262:6460–6463.
5. Inui, M., A. Saito, and S. Fleischer. 1987. Purification of the ryanodine receptor and identity with feet structures of junctional terminal cisternae of sarcoplasmic reticulum from fast skeletal muscle. *J. Biol. Chem.* 262:1740–1747.
6. Lai, F. A., H. P. Erickson, E. Rousseau, Q.-Y. Liu, and G. Meissner. 1988. Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature* (*Lond.*). 331:315–319.
7. Takeshima, H., S. Nishimura, T. Matsumoto, H. Ishida, K. Kangawa, N. Minamino, H. Matsuo, M. Udea, M. Hanaoka, T. Hirose, and S. Numa. 1989. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature* (*Lond.*). 339:439–445.
8. Zorzato, F., J. Jujii, K. Otsu, M. Phillips, N. M. Green, F. A. Lai, G. Meissner, and D. H. MacLennan. 1990. Molecular cloning of cDNA encoding human and rabbit forms of the Ca^{2+} release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 265:2244–2256.
9. Romer, A. S. 1967. The Vertebrate Body. W. B. Saunders., Philadelphia, PA. 627 p.
10. Saito, A., S. Seiler, A. Chu, and S. Fleischer. 1984. Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. *J. Cell Biol.* 99:875–885.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
12. Kaplan, R. S., and P. L. Pedersen. 1985. Determination of microgram quantities of protein in the presence of milligram levels of lipid with amido black 10B. *Anal. Biochem.* 150:97–104.

13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (*Lond.*). 227:680–685.

14. Zorzato, F., and P. Volpe. 1988. Calcium binding proteins of junctional sarcoplasmic reticulum: detection by 45Ca ligand overlay. *Arch. Biochem. Biophys.* 261:324–329.

15. Sutko, J. L., L. J. Thompson, R. G. Schlatterer, F. A. Lattanzio Jr., A. S. Fairhurst, C. Campbell, S. Martin, L. Ruest, and P. Deslongchamps. 1985. Separation and formation of ryanodine from dehydroryanodine. Preparation of [^{3}H]ryanodine. *J. Label. Compd. Radiopharmaceut.* 23:215–222.

16. Bruns, R. F., K. Lawson-Wendling, and T. A. Pugsley. 1983. A rapid filtration assay for soluble receptors using polyethylenimine-treated filters. *Anal. Biochem.* 132:74–81.

17. Abramson, S. N., M. Ellisman, T. Deerinck, and P. Taylor. 1989. Differences in structure and distribution of the molecular forms of acetylcholinesterase. *J. Cell Biol.* 108:2301–2311.

18. Ogata, T. 1988. Morphological and cytochemical features of fiber types in vertebrate skeletal muscle. *CRC Crit. Rev. Anat. Cell Biol.* 1:229–275.

19. Block, B. A., T. Imagawa, K. P. Campbell, and C. Franzini-Armstrong. 1988. Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. *J. Cell Biol.* 107:2587–2600.

20. Otsu, K., H. F. Willard, V. K. Khanna, F. Zorzato, N. M. Green, and D. H. MacLennan. 1990. Molecular cloning of cDNA encoding the Ca^{2+} release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. *J. Biol. Chem.* 265:1372–13483.

21. Ellisman, M. H., T. J. Derrinck, Y. Ouyang, C. F. Beck, S. J. Tanksley, P. D. Walton, J. A. Airey, and J. L. Sutko. 1990. Identification and localization of ryanodine binding proteins in the avian central nervous system. *Neuron.* 5:135–146.

22. Sutko, J. L., J. A. Airey, K. Murakami, M. Takeda, C. Beck, T. Deerinck, and M. H. Ellisman. 1991. Foot protein isoforms are expressed at different times during embryonic chick skeletal muscle development. *J. Cell Biol.* 113:In press.

23. Walton, P. D., J. A. Airey, J. L. Sutko, C. F. Beck, G. A. Mignery, T. C. Südhof, T. J. Deerinck, and M. H. Ellisman. 1991. Ryanodine and inositol trisphosphate receptors coexist in avian cerebellar Purkinje neurons. *J. Cell Biol.* 113:In press.