Three Ryanodine Receptor Isoforms Exist in Avian Striated Muscles†

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ABSTRACT: Two isoforms of the ryanodine receptor (termed α and β) are coexpressed in avian fast twitch skeletal muscle, whereas a single isoform is expressed in avian cardiac muscle. We have investigated the relationship between these three proteins, comparing several different properties. First, the three receptor isoform subunits have different mobilities on SDS–polyacrylamide gels. Second, monoclonal antibodies against the chicken skeletal muscle receptor isoforms recognize shared and unique epitopes in each receptor protein, indicating there is not a simple antigenic relationship between the isoforms. Third, the three receptor isoforms exhibit different susceptibilities to proteolysis by trypsin, and limited tryptic digestion yields a different peptide map for each isoform. Fourth, in native sarcoplasmic reticulum membranes, the chicken muscle receptor isoforms are phosphorylated to different extents by the multifunctional calcium/calmodulin-dependent protein kinase II (β > cardiac > α). Fifth, the sites phosphorylated by the calcium/calmodulin-dependent protein kinase in the chicken cardiac and skeletal receptor isoforms are not equivalent. A polyclonal serum, produced against a synthetic peptide containing the site phosphorylated by this kinase in the mammalian cardiac muscle receptor, by immunoprecipitation showed markedly different avidities for the receptor isoforms, and recognized only the cardiac receptor isoform on Western blots. Sixth, the chicken ryanodine receptor isoforms differ in the extent to which they bind azido[^{125}I]calmodulin (α > β > cardiac). These results indicate that three distinct ryanodine receptor proteins are expressed in chicken striated muscles.

Ryanodine receptors are a family of proteins that serve as intracellular calcium release channels in both muscle and nonmuscle tissues (Campbell et al., 1987; Inui et al., 1987; Lai et al., 1988; Ellisman et al., 1990; McPherson et al., 1990; Walton et al., 1991). Members of this family have been identified and purified on the basis of their ability to bind the plant alkaloid ryanodine (Campbell et al., 1987; Inui et al., 1987; Lai et al., 1988). In addition to being a high-specificity receptor ligand, ryanodine alters the gating and conductance properties of the channel. Nanomolar to micromolar concentrations of ryanodine activate the channel and stabilize it in a long-lived subconductance state, whereas greater concentrations block the channel (Rousseau et al., 1987; Lattanzio et al., 1987; Smith et al., 1988).

Two ryanodine receptor isoforms, α and β, are localized to the triad junctions within the same chicken fast twitch skeletal muscle fibers (Airey et al., 1990). The coexpression of two receptor isotypes is not unique to avian muscle, also occurring in frog and fish skeletal muscles (Olivares et al., 1991). This is in contrast to mammalian skeletal muscle fibers, and to both mammalian and avian cardiac muscle, where only a single ryanodine receptor isotype appears to be expressed in each tissue (Campbell et al., 1987; Inui et al., 1987; Lai et al., 1988; Anderson et al., 1989; Airey et al., 1990; Dutro et al., 1993). The expression of two ryanodine receptor isoforms is not restricted to skeletal muscle, as a similar phenomenon is observed in avian (Ellisman et al., 1990; Walton et al., 1991) and piscine central nervous systems (Zupanc et al., 1992).

The coexpression of two isoforms in nonmammalian vertebrate skeletal muscle raises two questions. First, what is the origin and relationship between these proteins? Previous studies of the ryanodine receptors expressed in mammalian cardiac and skeletal muscle have demonstrated that these proteins, which are 66% homologous at the amino acid level, are the products of two genes located on different chromosomes (Otsu et al., 1990). Moreover, the ryanodine receptor isotype expressed in mammalian cardiac muscle is also expressed in the mammalian central nervous system (Otsu et al., 1990; Nakai et al., 1990; Witcher et al., 1992) and perhaps in mammalian smooth muscle (Nakai et al., 1990). The presence of the cardiac isoform in tissues other than cardiac muscle raised the possibility that the existence of two ryanodine receptor isoforms in nonmammalian skeletal tissue could result from the coexpression of the skeletal and cardiac muscle receptor gene products in this tissue (Airey et al., 1990). Consistent with this idea, Lai et al. (1992) recently compared the recognition patterns of two anti-receptor antibodies in frog fast twitch skeletal muscle and suggested that the two receptor isoforms in this tissue are counterparts of the mammalian cardiac and skeletal muscle receptor isoforms. To address this question, we have compared the biochemical properties of the ryanodine receptor isoforms expressed in avian and mammalian skeletal and cardiac muscles.

A second question concerns the functional significance of the coexistence two ryanodine receptor isoforms in nonmammalian vertebrate skeletal muscle, when a single receptor isotype appears to be adequate in mammalian skeletal, as well as mammalian and avian cardiac muscles. This is particularly interesting since excitation–contraction coupling events in mammalian and nonmammalian vertebrate skeletal muscles

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appear to be similar. The two receptor isoforms in avian skeletal muscles are likely to have distinct functions, as the expression of each isoform is initiated at a different time during embryonic development (Sutko et al., 1991) and both proteins are found in mature muscle (Airey et al., 1990). One possibility is that neither ryanodine receptor isoform coexpressed in nonmammalian skeletal muscle possesses all of the functional attributes exhibited by the single mammalian skeletal isoform. Alternatively, fundamental differences may exist between the processes linking muscle cell excitation and SR calcium release in mammalian and nonmammalian vertebrate muscles. Resolution of this issue will require dissection of the relationships and functional properties of the ryanodine receptor isoforms. As a first step in these studies, we have compared the effects of potential regulators of channel function on the receptor isoforms found in the different muscle types.

On the basis of differences in mobility in a gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] system, immunological differences, unique tryptic peptide maps, different levels of phosphorylation, and different ability to bind calmodulin, we suggest that three unique ryanodine receptor isoforms exist in avian striated muscle. Two receptor subtypes are coexpressed in skeletal muscle, while a third isoform is found singly in the myocardium. Moreover, these isoforms have the potential to be regulated in a differential manner. A preliminary report of these results has been presented (Sutko et al., 1992).

EXPERIMENTAL PROCEDURES

Materials. T-61 euthanasia solution was purchased from American Hoechst Corp. (Somerville, NJ); leupeptin, PMSF,[1] DFP, calmodulin, ovomucoid trypsin inhibitor, CHAPS, L-α-phosphatidylcholine, agarose-linked goat anti-mouse IgG (whole molecule) antibodies, and trypsin were from Sigma (St. Louis, MO); BCA reagents were from Pierce Chemical Co. (Rockville, IL); [γ-^{32}P]ATP was from NEN Dupont (Wilmington, DE); and alkaline phosphatase conjugated goat anti-mouse IgG was purchased from Fisher Scientific (Santa Clara, CA). Rat brain CaM kinase was generously supplied by Dr. H. Schulman (Stanford University).

Preparation of Microsomal Membranes and Solubilized Ryanodine Receptor Proteins. These methods have been described previously in detail (Airey et al., 1990). Briefly, crude microsomal membranes were prepared from pectoral and cardiac muscles excised from White Leghorn chickens (1–2 months old). Muscle was homogenized 3 times for 1 min at high speed in a Waring blender in 5 mL/g wet tissue weight in a solution containing 0.3 M sucrose, 10 mM imidazole, pH 7.4, 1.1 μM leupeptin, and 230 μM PMSF. The homogenate was centrifuged at 8000*g* for 14 min and the pellet rehomogenized and centrifuged as above. Supernatants from both centrifugations were combined. Microsomal membranes collected by centrifugation at 130000*g* for 90 min were resuspended in the above solution, rapidly frozen in liquid N_2, and stored at −90 °C. Membrane protein was measured using BCA with bovine serum albumin as a standard.

Skeletal and cardiac muscle microsomal membrane proteins were solubilized in a solution containing 0.5 M KCl, 20 mM Tris, pH 7.4, 5 mM DTT, 2% CHAPS, and 1% L-α-phosphatidylcholine for 30 min on ice at final membrane protein concentrations of 3 mg/mL (skeletal) and 6 mg/mL (cardiac). Solubilized and nonsolubilized materials were separated by centrifugation at 100000*g* for 30 min. Solubilized protein was either used immediately or rapidly frozen in liquid N_2 and stored at −90 °C.

SDS–PAGE and Western Blot Analyses. Samples were prepared for SDS–PAGE (Laemmli, 1970) in a 4×-concentrated sample buffer so that the final SDS concentration was 2% (Airey et al., 1990). Aliquots (40–60 μL) were loaded onto either 4–10% or 4–20% gradient minigels with 3% stacking gels, and a constant voltage of 130 V was applied at 0–4 °C. The separated proteins were visualized by staining with 0.2% Coomassie Brilliant Blue. Densiometric scans of stained SDS gels were obtained using a GS300 scanning densitometer (Hoeffer Scientific Instruments, San Francisco, CA) or a JAVA video analysis system (Jandel Scientific, San Rafael, CA).

Proteins were transferred onto nitrocellulose using a Transphor TE 50 (Hoeffer Scientific Instruments) at an initial setting of 100 V for the first 2 h and then at 35–40 V overnight in 10 mM TES, pH 8.2, and 10% ethanol. Blotted protein was visualized by brief staining with 0.5% Ponceau S (Salinovich & Montelaro, 1986) followed by destaining in distilled water. The proteins identified by the antibodies used in these studies were visualized using alkaline phosphatase conjugated to goat anti-mouse antibodies.

Antibodies and Immunoprecipitation of Ryanodine Receptor Proteins. Monoclonal antibodies produced in mice against the chicken skeletal muscle ryanodine receptor proteins were utilized as primary antibodies in these studies (Airey et al., 1990; Dutro et al., 1993). These antibodies and their specificities are identified in the legends to Table I and Figure 2. A polyclonal serum raised against a synthetic peptide containing the amino acid sequence comprising the CAM kinase phosphorylation site in the mammalian cardiac ryanodine receptor isoform was also used (Witcher et al., 1991).

Immunoprecipitations were carried out as described previously (Airey et al., 1990; Olivares et al., 1991; Dutro et al., 1993). Primary antibodies were bound to goat anti-mouse IgG, or goat anti-rabbit IgG antibodies linked to agarose. The antibody–agarose beads were washed 3 times in 0.5 M NaCl/10 mM NaH_2PO_4, pH 7.4, and resuspended at the original concentration obtained from the supplier. Aliquots of 0.1 mL of the beads were incubated with solubilized protein for either 3 h at room temperature or overnight at 0–4 °C. Antibody beads which had been incubated with hybridoma culture medium instead of primary antibody were used as negative controls. The antibody beads were washed 3 times with the above solution containing 0.5% CHAPS and 0.25% phosphatidylcholine and prepared as gel samples by removing all excess fluid and adding 50 μL of 4X sample buffer and 6 μL of 1 M DTT. In preliminary experiments, we determined the concentrations of monoclonal antibodies necessary to precipitate equivalent levels of each ryanodine receptor isoform for the proteolysis experiments. In all other experiments using immunoprecipitation, antibodies were added in sufficient excess so as to precipitate all of the antigen.

Tryptic Digestion of Ryanodine Receptor Isoforms. Tryptic maps of the ryanodine receptor isoforms were obtained in two ways. First, each receptor isoform was solubilized in CHAPS/phosphatidylcholine and precipitated with an appropriate

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropyl fluorophosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylaminonio]-1-propanesulfonate; IgG, immunoglobulin G; BCA, bicinchoninic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CaM kinase, calcium- and calmodulin-dependent protein kinase.

isoform-specific monoclonal antibody. The antibody 110F was used for the α-skeletal isoform, while the antibody 110E was used for the β-skeletal and cardiac isoforms. The immunoprecipitated complex was then digested with trypsin. Although proteins other than the ryanodine receptor are present in the precipitated complex, none are comparable in size to the ryanodine receptor subunits. Thus, large fragments originating from the latter polypeptides could easily be distinguished and compared using 4–20% SDS gels. This approach had the advantage of producing a much stronger signal, as the ryanodine receptor subunits were concentrated by the immunoprecipitation step. A second method was used to verify that the high molecular mass fragments generated by proteolysis were originating from the ryanodine receptor subunits. In these studies, the ryanodine receptors were purified by sucrose gradient sedimentation to ~90% homogeneity [cf. Lai et al. (1988)]. The skeletal muscle isoforms were again separated by immunoprecipitation, but in this case, the isoform utilized for tryptic digestion was not the one precipitated, but rather the one remaining in the supernatant. As described in detail under Results, the same results were obtained with both methods. Tryptic digestion was initiated by the addition of trypsin at a final concentration of 0.9 μg/mL to the immunoprecipitated complex (method 1), or 1.0 μg/mL to the supernatant isoforms (method 2), and permitted to proceed for 5–30 min at room temperature. Proteolysis was terminated by the addition of ovomucoid trypsin inhibitor and DFP at final concentrations of 20 μg/mL and 0.25 mM. The samples were then prepared for SDS–PAGE as described above.

Phosphorylation of Ryanodine Receptor Proteins. Phosphorylation of skeletal and cardiac microsomal membranes by CaM kinase was determined in an assay system containing 25 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 0.5 mM EGTA, 0.75 mM $CaCl_2$, and 50 μM [γ-^{32}P]ATP in the presence or absence of 1 μg of calmodulin and 0.02 μg of rat brain CaM kinase in a final volume of 30 μL (Witcher et al., 1991). The reactions were started by the addition of [γ-^{32}P]ATP and permitted to proceed for 2 min at 30 °C. The phosphorylation reaction was terminated by the addition of SDS gel sample buffer, and the protein species were resolved in 4–10% polyacrylamide gradient SDS gels. The following procedure was utilized to compare the extent of phosphorylation of each isoform in as quantitative and identical a manner as possible. The relative quantities of each of the isoform subunits present in each gel were determined densitometrically, using a high-gain video scanning system. The SDS gels were then dried and subjected to autoradiography to obtain a visual record of the radioactivity associated with each polypeptide band. Following this, the extent of ^{32}P incorporation into each of the ryanodine receptor subunit bands was quantitated by excising and counting the appropriate bands in a liquid scintillation counter (LKB Wallac, Bromma, Sweden). The cpm obtained for each band were then normalized for the quantity of protein present in that band, and this ratio obtained for each receptor isoform was compared to determine whether the isoforms were phosphorylated to different extents.

Cross-Linking of the Ryanodine Receptor Proteins with Azidocalmodulin. Azido[^{125}I]calmodulin was prepared as described previously (Seiler et al., 1984). Microsomal membranes were incubated in the dark in 70 mM MOPS (pH 7.1)/15 mM $MgCl_2$ with either 0.4 mM $CaCl_2$ or 1.5 mM EGTA and 60 μg/mL azido[^{125}I]calmodulin at either 0 or 30 °C for 20 min. The samples were then irradiated with UV light at 312 nm for 5 min. SDS gel sample buffer was then added, and the samples were run on 4–10% gels. After being stained with Coomassie Brilliant Blue, the gels were imaged and subjected to autoradiography, and the excised bands were counted in a γ counter (Packard, Downers Grove, IL). Quantitative methods identical to those used to compare phosphorylation of the different receptor isoforms, and described in detail in the preceding paragraph, were used to determine and compare the extent of calmodulin binding to the isoforms.

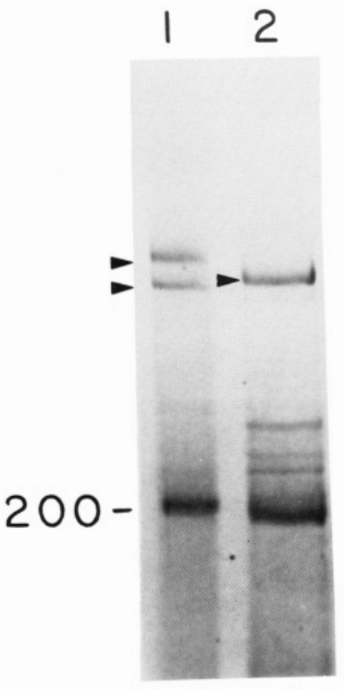

FIGURE 1: Avian skeletal and cardiac ryanodine receptors exhibit different mobilities in SDS–PAGE. A portion of a 4–10% gradient gel. Lane 1 is chicken pectoral muscle microsomes (25 μg of protein); lane 2 is chicken cardiac microsomes (90 μg of protein). Arrowheads indicate ryanodine receptor isoforms.

RESULTS

In the present studies, we have investigated the interrelationships between the ryanodine receptor isoforms expressed in chicken skeletal and cardiac muscles. If either the α- or the β-skeletal muscle isoform results from the expression of the cardiac ryanodine receptor protein in skeletal muscle, then one of the isoforms should have biochemical properties similar to those of the cardiac muscle receptor. Therefore, we compared these proteins for their subunit mobility in SDS–PAGE gels, the existence of shared epitopes, peptide maps produced by limited proteolysis, the extent of phosphorylation by CaM kinase, and the ability to bind calmodulin.

Skeletal and Cardiac Muscle Ryanodine Receptors Exhibit Different Mobilities on SDS Gels. The first indication that three distinct ryanodine receptor isoforms are expressed in chicken striated muscle was obtained when it was observed that these proteins exhibit slight, but consistent, differences in mobility in SDS gels. As shown in Figure 1, the cardiac muscle isoform has a mobility that is intermediate between the α- and β- skeletal muscle receptor isoforms. Using a number of gel systems in which the receptor isoforms are well resolved, we consistently found that the cardiac isoform has a different mobility from either skeletal isoform.

Avian Skeletal and Cardiac Muscle Receptor Isoforms Have both Shared and Unique Immunoreactivities. A panel of 31 monoclonal antibodies produced against the chicken skeletal muscle receptor isoforms was screened to determine

Table I: Specificities of Some Anti-Ryanodine Receptor Monoclonal Antibodies[a]

	ryanodine receptor isoforms			
Mab skel	chick skel α	chick skel β	chick card	rabbit
1C4	+			
3F5		+		
2G6	+	+		
2F9	+		+	+
110E		+	+	
3G5		+		+
34C	+	+	+	+

[a] (+) indicates that the antibody will precipitate this receptor isoform. Skeletal is abbreviated as skel and cardiac as card.

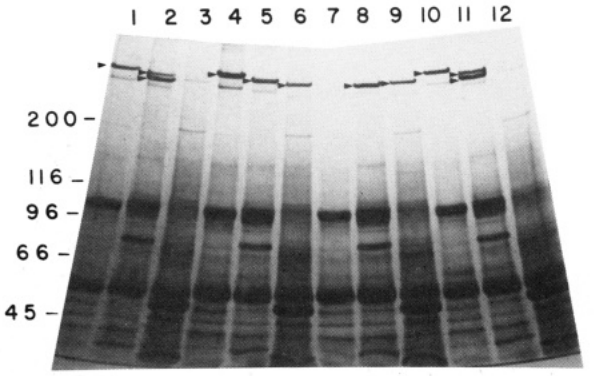

FIGURE 2: Three ryanodine receptor isoforms in chicken striated muscles exhibit both unique and shared epitopes. CHAPS-solubilized chicken and rabbit skeletal (150 μg of protein) and cardiac microsomes (300 μg of protein) were immunoprecipitated by monoclonal antibodies, 1E4 (lanes 1–3), 2F9 (lanes 4–6), 110E (lanes 7–9), and 3E2 (lanes 10–12), that recognize different epitopes. Lanes are rabbit skeletal muscle (1, 4, 7, and 10), chicken skeletal muscle (2, 5, 8, and 11), and chicken cardiac muscle (3, 6, 9, and 12).

whether the skeletal and cardiac muscle isoforms could be distinguished on the basis of the epitopes that they contain. In addition, these antibodies were assessed for the ability to recognize the receptor isoform expressed in mammalian skeletal muscle. As shown in Table I and Figure 2, every grouping possible was obtained, in that some antibodies reacted with the α isoform, some with the α and β isoforms, the α and cardiac isoforms, the β isoform, the β and cardiac isoforms, and the α, β, and cardiac isoforms. On the basis of these findings, the three isoforms appear to share a significant number of epitopes, but there is no recognition pattern that indicates a closer relationship between any two of the proteins. The lower molecular weight polypeptides visible in this gel are not precipitated in a specific manner by the Mabs since they are present in negative control precipitations in the absence of antibody (data not shown).

Tryptic Digestion of the Chicken Striated Muscle Ryanodine Receptor Isoforms Yields Unique Peptide Maps for Each Protein. In the next series of studies, we compared the peptide maps produced by tryptic digestion of the chicken skeletal and cardiac muscle ryanodine receptors. Proteolysis was carried out in two ways. In the first, each isoform was selectively immunoprecipitated and the precipitated complex digested. This approach was possible due to the large size of the receptor subunits which effectively isolated these polypeptides from other contaminating proteins. Second, following partial purification of the receptors, the unwanted receptor isoform was removed by immunoprecipitation, and the isoform remaining in the supernatant was digested. Unique peptide maps were obtained for each of the receptor isoforms expressed in chicken striated muscle in each set of experiments. The results obtained for the first method are shown in Figure 3.

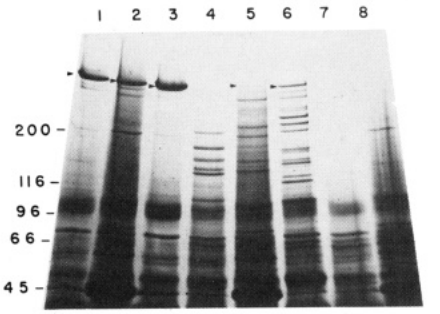

FIGURE 3: Tryptic digestion of chicken skeletal and cardiac ryanodine receptors produces isoform-specific peptide maps. CHAPS-solubilized ryanodine receptor isoforms were separated by immunoprecipitation with either 54D (anti-α) or 110E (anti-β and anti-cardiac) monoclonal antibodies. After digestion with trypsin, the receptor–antibody complex polypeptides were resolved on a 4–20% gel. Lanes 1 and 4 are chicken skeletal precipitated with 54D (anti-α), lanes 2 and 5 are chicken cardiac precipitated with 110E, lanes 3 and 6 are chicken skeletal precipitated with 110E (anti-β), and lane 7 is chicken skeletal and lane 8 is chicken cardiac with monoclonal antibody omitted. The samples in lanes 1–3 are undigested precipitates, while those in lanes 4–8 have been digested with trypsin.

Table II: Phosphorylation of Microsomal Membrane Preparations by CaM Kinase

microsomal membranes	cpm[a]	relative[b] area	counts/area
dog skeletal	1150	12591	0.091
dog cardiac	10532	17581	0.600
chicken cardiac	887	6632	0.133
chicken skeletal α	787	17313	0.045
chicken skeletal β	1992	7521	0.265
frog skeletal α	1458	14880	0.123
frog skeletal β	506	16210	0.031

[a] cpm = counts associated with ^{32}P incorporation into the polypeptide bands excised from the gels after autoradiography, and assessed by liquid scintillation counting. [b] Relative area = the area under the peaks of the densitometric scans of the SDS gels in arbitrary units and is a measure of the quantity of protein present.

In addition, digestions with different concentrations of trypsin for different lengths of time [cf. Airey et al. (1990)] also yielded different maps for each isoform. The receptor isoforms exhibit different susceptibilities to trypsin. The α isoform is the most susceptible, the cardiac receptor intermediate, and the β-receptor isoform the least sensitive to trypsin. This order of sensitivity was found in a number experiments, regardless of the time of digestion or the amount of receptor protein used.

Ryanodine Receptor Isoforms in Chicken Skeletal and Cardiac Muscle and in Frog Skeletal Muscle Are Differentially Phosphorylated by CAM Kinase. In mammalian muscle, the cardiac ryanodine receptor is phosphorylated to a greater extent than the skeletal muscle receptor protein by CaM kinase (Takasago et al., 1991; Witcher et al., 1991). Therefore, we investigated whether the chicken cardiac and skeletal isoforms could be distinguished on this basis.

SR membrane vesicles prepared from avian and dog cardiac and skeletal muscles and frog skeletal muscle were utilized in these studies. In Table II, the amount of protein in the receptor bands in the SDS gel (Figure 4a), quantitated densitometrically, is compared to the level of phosphorylation of each protein (Figure 4B,C), quantitated by scintillation counting. In Figure 4, the ryanodine receptor polypeptides are indicated by arrows. A polypeptide that migrates with a slightly greater mobility than the ryanodine receptor subunit polypeptides was phosphorylated to a significant extent. The latter polypeptide is labeled with an asterisk in Figure 4 so that it will not be confused with the ryanodine receptor subunits. We

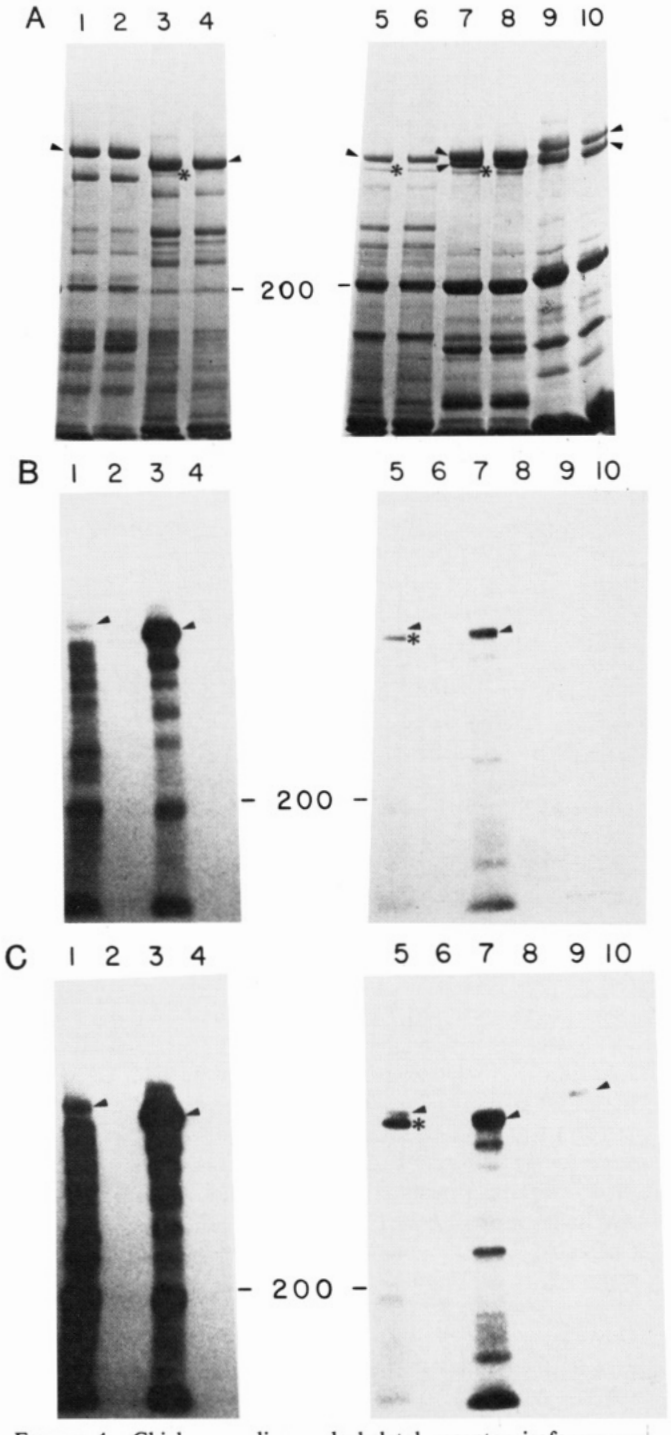

FIGURE 4: Chicken cardiac and skeletal receptor isoforms are phosphorylated to different extents by CaM kinase. Microsomal membranes incubated with or without CaM kinase for 2 min at 30 °C were run on a 4–10% gel (panel A) and then subjected to autoradiography (panels B and C). The latter two panels are two different exposures of the same autoradiograph. The complete reaction mixture was loaded on 4–10% gradient gels; microsomal protein amounts were chicken skeletal, 23 μg; chicken cardiac, 75 μg; frog skeletal, 42 μg; dog skeletal, 32 μg; and dog cardiac, 47 μg. Lanes 1, 3, 5, 7, and 9 are in the presence of kinase; lanes 2, 4, 6, 8, and 10 are in the absence of kinase. Lanes 1 and 2 are dog skeletal muscle; 3 and 4 dog cardiac; 5 and 6 chicken cardiac; 7 and 8 chicken skeletal; 9 and 10 frog skeletal. Arrowheads indicate ryanodine receptor isoforms.

have not determined the identity of this polypeptide. The following order of relative phosphorylation of the ryanodine receptor isoforms in chicken by CaM kinase was observed in five experiments: chicken skeletal β > chicken cardiac > chicken skeletal α. The observation that the chicken skeletal

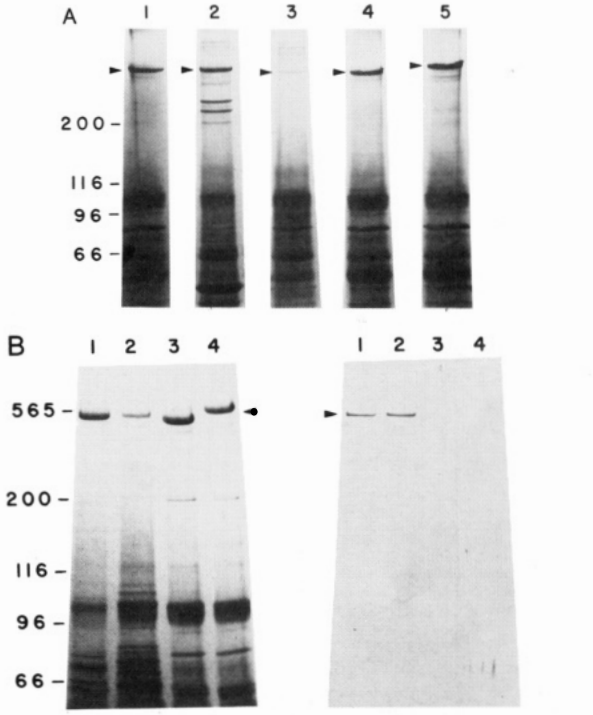

FIGURE 5: Immunoprecipitation and Western analysis show that a polyclonal serum against the mammalian cardiac ryanodine receptor CaM kinase phosphorylation site reacts with the chicken cardiac muscle but not skeletal muscle ryanodine receptor isoforms. Panel A shows precipitation of CHAPS-solubilized microsomal protein with the polyclonal antiserum (Witcher et al., 1991) and selected monoclonal antibodies. Lanes 1–3 are solubilized protein precipitated with the peptide-specific polyclonal serum: lane 1, rabbit cardiac; lane 2, chicken cardiac; lane 3, chicken skeletal. Lanes 4 and 5 are chicken skeletal muscle precipitated with receptor-specific monoclonals: lane 4, 110E (anti-β); lane 5, 54D (anti-α). Panel B shows 4–20% SDS–PAGE of immunoprecipitated CHAPS-solubilized muscle microsomal proteins, and panel C is a Western blot of an identical gel. In both panel B and panel C, lanes are as follows: 1, rabbit cardiac (300 μg of protein); 2, chicken cardiac (600 μg of protein); 3 and 4, chicken skeletal (150 μg of protein). Lanes 1–3 were immunoprecipitated with 110E (anti-cardiac and β) and lane 4 with 54D (anti-α).

β-receptor isoform was phosphorylated more extensively than the avian cardiac isoform was unexpected, since the dog skeletal receptor has been found to be phosphorylated to an insignificant extent by CaM kinase in comparison to the dog cardiac receptor (Witcher et al., 1991; Takasago et al., 1991). We also investigated receptor phosphorylation in frog skeletal muscle to examine whether the ryanodine receptor proteins were phosphorylated by this kinase in another nonmammalian vertebrate skeletal muscle. In contrast to the chicken skeletal muscle receptors, the frog α-receptor isoform was phosphorylated to a greater extent than the β isoform.

Sites Phosphorylated by CaM Kinase in Chicken Skeletal β and Cardiac Muscle Ryanodine Receptor Isoforms Are Not Equivalent. Witcher and co-workers (Witcher et al., 1991) prepared a polyclonal serum against a synthetic peptide containing an amino acid sequence identified as a CaM kinase phosphorylation site in the mammalian cardiac receptor. This serum recognizes the mammalian cardiac, but not the mammalian skeletal ryanodine receptor isoform (Witcher et al., 1991). Since there is significant phosphorylation of the chicken skeletal β isoform by CaM kinase, we tested whether this antiserum recognizes any of the chicken isoforms. As shown in Figure 5A, both the rabbit and chicken cardiac receptors are precipitated by this antiserum. In lane 3, precipitation of chicken skeletal muscle with the polyclonal

Table III: Relative Binding of Azido[^{125}I]calmodulin to Chicken Ryanodine Receptor

microsomal membranes	cpm[a]	relative area[b] (gray units)	counts/area $\times 10^3$
		+Ca	
chicken skeletal α	1385	184671	7.50
chicken skeletal β	465	119929	3.88
chicken cardiac	200	124703	1.60
		-Ca	
chicken skeletal α	345	171582	2.01
chicken skeletal β	150	199531	1.49
chicken cardiac	95	123095	0.77

[a] cpm = counts associated with ^{125}I incorporation into polypeptide bands excised from the gels measured in a γ counter. [b] Relative area is that estimated using densitometric imaging of the polypeptide band in the SDS gel and reflects the quantity of protein present. Units are arbitrary gray units.

antibody, a faint band that has a similar mobility to the β isoform can be seen. This suggests that the epitope containing the phosphorylation site is present in the cardiac isoform and is either significantly altered or absent in the skeletal muscle isoforms. In Western analyses, this antiserum reacts with the avian cardiac muscle receptor isoform, but does not recognize either the avian α- or the avian β-skeletal muscle receptors (Figure 5B). The higher level of phosphorylation of the chicken skeletal β isoform by CaM kinase, yet the lack of, or low level of, recognition of this isoform by the serum directed against a phosphorylation site sequence present in mammalian (and presumably chicken) cardiac muscle receptors, suggests that the amino acid sequences of the sites phosphorylated by CaM kinase in the chicken cardiac and skeletal β isoforms differ.

Chicken Skeletal and Cardiac Ryanodine Receptor Isoforms Bind Calmodulin to a Different Extent. Chicken skeletal and cardiac muscle microsomes were incubated with azido[^{125}I]calmodulin, a derivative of calmodulin that can be cross-linked using UV light. In these experiments, calmodulin binding was quantitated by γ counting of the ryanodine receptor polypeptide bands excised from the SDS gels, while the relative levels of each isoform were obtained by quantitative densitometry. The data in Table III relate these values and indicate that both of the skeletal isoforms bind azidocalmodulin to a greater extent than the cardiac isoform, and that the α isoform has a greater binding than the β isoform. Four separate trials conducted at two temperatures, 0 and 30 °C, showed the same order of relative binding. In agreement with previous results (Seiler et al., 1984), we find that in the absence of calcium that there is a low level of calmodulin binding to the ryanodine receptor isoforms. The order of relative binding for the photoaffinity-labeled calmodulin between the isoforms is not the same order as that observed for phosphorylation. Again, these data suggest that there is not a simple relationship between the isoforms and that no two isoforms share the same biochemical characteristics.

DISCUSSION

Although we have shown previously that avian skeletal muscle contains two different ryanodine receptor isoforms (Airey et al., 1990), we had not determined the relationship between the avian cardiac and skeletal receptor isoforms. A very recent report by Murayama and Ogawa (1992) suggests in bullfrog that the two skeletal muscles appear to differ in the calcium dependency of binding. This is further evidence that the skeletal muscle isoforms are different. The data presented in this report, obtained from several different assessments of the biochemical properties of the receptor isoforms, are consistent with the existence of three unique ryanodine receptor proteins in avian striated muscles.

The difference in mobility on SDS–PAGE of the three isoforms from avian striated muscle was initial evidence that the cardiac isoform was a different protein from those expressed in skeletal muscle. The avian cardiac isoform shares a number of epitopes with both of the chicken skeletal receptor isoforms and a smaller number with both rabbit skeletal and cardiac, and frog and fish skeletal muscle receptor isoforms. The presence of these epitopes in CHAPS-solubilized receptor proteins, which are thought to be in a native conformation (Lai et al., 1989), suggests that regions of the surface of the protein are conserved both within and between species. These conserved domains may represent regions that are important for ryanodine receptor function.

The tryptic maps obtained for the three chicken ryanodine receptor isoforms are significantly different. Previously we have shown that the α and β isoforms of the avian skeletal ryanodine receptor have different proteolytic maps (Airey et al., 1990). We extended this analysis to show that the cardiac isoform has a different tryptic map from either of the skeletal isoforms. Few, if any, common high molecular weight peptides were generated by digestion. Additionally, differences in sensitivity of the isoforms to trypsin indicate that the proteins are dissimilar. The lability of the α isoform to proteases has been noted previously (Airey et al., 1990), and the chicken skeletal β isoform was much more resistant to trypsin than either of the other two avian isoforms. As the proteolysis experiments utilized CHAPS-solubilized protein, which retains a native tetrameric conformation (Lai et al., 1989), these results suggest that the surface of the β isoform has significant differences from either the skeletal α or the cardiac isoform due to amino acid sequence and/or protein folding.

The data in Table II and Figure 4 (panels B and C) show that all three chicken isoforms are phosphorylated by CaM kinase. The more extensive phosphorylation of the β-skeletal isoform than the chicken cardiac isoform differs from the situation described in mammals where the cardiac isoform is phosphorylated to a much higher level than the skeletal isoform (Witcher et al., 1991; Takasago et al., 1991). It has been suggested that the mammalian cardiac receptor may be regulated by phosphorylation by CaM kinase (Witcher et al., 1991). The significantly lower level of phosphorylation of the avian cardiac ryanodine receptor suggests that this isoform may not be regulated in a manner similar to the mammalian cardiac receptor.

In contrast to chicken skeletal muscle, the frog α-skeletal isoform is a better substrate for CaM kinase than the lower molecular mass frog skeletal β isoform. These data suggest that the frog skeletal α isoform could be the counterpart of the chicken β isoform. Differences between the receptor isoforms in frog and chicken skeletal muscles are reflected by the different patterns of recognition for frog and chick skeletal receptors by several monoclonal antibodies (Olivares et al., 1991).

It has been demonstrated that the ryanodine receptor isoforms in canine skeletal and cardiac muscle are labeled to similar extents by the photoaffinity probe azido[^{125}I]-calmodulin (Seiler et al., 1984). This is in contrast to the situation in avian striated muscle where the three isoforms are photolabeled to different levels. Calmodulin has been suggested to play several roles in the regulation of the ryanodine receptor, including phosphorylation (Witcher et al., 1991),

degradation of the receptors (Brandt et al., 1992), and inhibition of calcium release (Meissner, 1986; Smith et al., 1989). In agreement with the latter findings, in preliminary experiments we have shown that calmodulin inhibits calcium release in chicken ryanodine receptors incorporated into planar lipid bilayers (unpublished observations).

The dissimilar binding of azidocalmodulin to the three isoforms in chicken as well as the differences in phosphorylation is consistent with the avian ryanodine receptor isoforms being unique proteins and suggests that the isoforms have the potential to be regulated in a different manner. Studies of the phosphorylation levels and calmodulin effects attained in situ will be necessary to assess the significance of these events to the actual regulation of receptor function. We have demonstrated previously (Airey et al., 1990) that both skeletal proteins are expressed in the same fibers in mature avian skeletal muscle and both are localized to triad junctions. In addition, the α and β isoforms are expressed initially at markedly different times during the development of embryonic chick skeletal muscle (Sutko et al., 1991). These observations suggest that both receptor isotypes contribute in different ways to muscle cell function, ones which may require differential regulation. We are currently comparing the ligand binding and ion channel characteristics of the three receptor proteins found in nonmammalian vertebrate muscles to establish the functional properties of these proteins.

As noted under Results, there is no recognition pattern for our monoclonal antibodies that indicates a closer relationship between any two of the patterns. In addition, the unique peptide maps, the disparate resistance to trypsin, and the differences in the relative levels of phosphorylation and in the relative levels of binding of azidocalmodulin suggest that there is not an apparent close relationship between any two of the isoforms. Two genes encoding ryanodine receptors have been identified in mammalian tissues (Otsu et al., 1990). The existence of three receptor isoforms in nonmammalian vertebrate muscles raises questions as to the genomic relationships between these proteins. The experimental approach we have utilized to demonstrate that these proteins differ biochemically does not permit speculation as to whether one of the skeletal muscle receptor isoforms is produced by posttranscriptional modification of a product of either the skeletal or the cardiac muscle gene, or if it is a product of a third, as yet unidentified, ryanodine receptor gene. The genetic relationships of ryanodine receptor gene expression in avian skeletal and cardiac muscles are also being investigated.

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