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Early over-expression of low-affinity [^{3}H]ryanodine receptor sites in heavy sarcoplasmic reticulum fraction from dystrophic chicken pectoralis major

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Heavy sarcoplasmic reticulum (SR) membranes enriched in [^{3}H]ryanodine receptor have been isolated from pectoralis major (PM) of normal line 412 and dystrophic line 413 chickens paired at various stages during post-hatch development. Normal PM 2 days ex ovo yields 17% lower protein recovery in the heavy SR subfractions compared to preparations from paired dystrophic PM (0.80 vs. 0.96 mg/g PM, respectively). By 2 weeks ex ovo, protein recovery in normal SR subfractions decreases over 3-fold to 0.24 mg/g PM, whereas yields from dystrophic PM increase to 1.67 mg/g PM. Dystrophic preparations consistently give 7–9-fold higher recoveries of protein in heavy SR subfractions compared to normal PM when examined at 2, 4, and 5.5 weeks ex ovo. [^{3}H]Ryanodine binding to normal SR from PM 2 days ex ovo exhibits nonlinear Scatchard plots which resolve into high-($K_{d,app}$ = 18 nM; B_{max} = 1.7 pmol/mg protein) and low-($K_{d,app}$ = 532 nM; B_{max} = 2.6 pmol/mg protein) affinity binding sites, whereas dystrophic preparations exhibit only high-affinity [^{3}H]ryanodine binding sites ($K_{d,app}$ = 31 nM; B_{max} = 2.1 pmol/mg protein). Both normal and dystrophic PM have similar capacities to bind [^{3}H]ryanodine (2.6 vs. 2.0 pmol/g PM, respectively) at 2 days ex ovo. However, at 2, 4, and 5.5 weeks ex ovo the density of high-affinity [^{3}H]ryanodine binding sites in normal SR drops dramatically to 3.5, 1.2, and 0.4 pmol/mg protein, respectively, and corresponds with disappearance of the low-affinity binding sites. In marked contrast, heavy SR membranes from dystrophic PM 2, 4, and 5.5 weeks ex ovo, maintain their high-affinity binding sites for [^{3}H]ryanodine and exhibit high densities of low-affinity binding sites ($K_{d,app}$ = 725–4500 nM; B_{max} = 15.4–25.1 pmol/mg protein). Early developmental over-expression of [^{3}H]ryanodine binding capacity in dystrophic PM ranges from 34-fold to 388-fold that of normal PM at 2 weeks and 5.5 weeks, respectively, and correspond to the intensity with which high molecular weight doublets of M_r 340 000 and 320 000 stain on SDS-PAGE. Low-affinity [^{3}H]ryanodine binding sites of dystrophic SR exhibit 4–6-fold higher sensitivity to activation by Ca^{2+} and altered sensitivity to activation by caffeine and adenine nucleotides. These results demonstrate that over-expression of junctional SR and [^{3}H]ryanodine receptors having altered radioligand binding properties is a very early event in the post-hatch development of dystrophic PM. Since the [^{3}H]ryanodine receptor is a specific marker for the SR Ca^{2+} release channel at the muscle triad and a key component of excitation-contraction coupling, abnormal expression of [^{3}H]ryanodine receptors may be of fundamental importance to the etiology of muscular dystrophy in the chicken.

Introduction

[^{3}H]Ryanodine binds selectively to sites within or near Ca^{2+} and adenine nucleotide activated Ca^{2+}-permeable pores localized at the triad junction of skeletal and cardiac muscle [1–11]. Recent recognition that high molecular weight protein oligomers not only make physical connections between the transverse tubule and the terminal cisternae of sarcoplasmic reticulum (SR)

Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IC_{50}, concentration giving 50% inhibition; k_{obs}; pseudo-first-order association rate constant; K_d, apparent equilibrium constant; K_{dCa}; apparent affinity for receptor activation by calcium; PM, pectoralis major; SR, sarcoplasmic reticulum.

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(i.e., span the triad junction) but also appear to comprise the [^{3}H]ryanodine receptor has permitted significant advances in our understanding of the structure and function of a key component critical to excitation-contraction coupling in muscle [12]. [^{3}H]Ryanodine binding sites are unmasked in the presence of effectors which stimulate Ca^{2+} release from SR, including Ca^{2+}, adenine nucleotides, caffeine, and anthraquinones [13,14]. In contrast, ligands which inhibit Ca^{2+} release from SR such as Mg^{2+} and ruthenium red, also inhibit Ca^{2+}-activation of [^{3}H]ryanodine binding sites [13]. Thus [^{3}H]ryanodine binding studies have proved extremely useful not only in purification of the Ca^{2+} release channel, but have also served to probe receptor function.

Chicken dystrophy is an inherited (autosomal recessive) myogenic disease which primarily afflicts fast-twitch fibers and has proved a valuable animal model for human dystrophies [15]. The disease produces a progressive decrease in tetanic strength and prolongs relaxation times [16]. The etiology of inherited dystrophy in the chicken has remained obscure. The altered mechanical properties of dystrophic muscle may reflect a lesion in excitation-contraction coupling [16,17]. For example, muscle microsomes from dystrophic chicken are reported to have altered Ca^{2+} transport properties; especially evident is a lower capacity of the SR to sequester Ca^{2+} [18–21]. Marked proliferation of T-tubular and junctional SR membranes are prominent features of dystrophic chicken muscle fibers and are accompanied by a high yield of 'low-density' membrane vesicles in the microsomal fraction [22–24].

However, noted difficulties have confounded interpretation of observed differences in Ca^{2+} transport properties of normal and dystrophic SR preparations including shifts in fiber type composition and isolation of membrane subfractions of defined origin especially from dystrophic muscle [17,25–26]. Martonosi has suggested that abnormal Ca^{2+} transport observed with dystrophic SR may arise from a combination of factors that are not primary features of the disease [26].

The present study examines the functional development of [^{3}H]ryanodine receptors, a highly specific marker of junctional SR, from normal and dystrophic chicken pectoralis major (PM). A dramatic over-expression of low-affinity [^{3}H]ryanodine binding sites appears early during post-hatch development of dystrophic PM and contrasts with significant down regulation of the receptor early in the development of normal PM. We further report that [^{3}H]ryanodine receptors from dystrophic PM exhibit altered radioligand binding properties in response to modulatory ligands including Ca^{2+}, caffeine, and adenine nucleotides when compared to receptors from normal muscle.

Experimental Procedures

Materials. [9,21-^{3}H]Ryanodine (98–99% radiochemical purity, 54.7 Ci/mmol) was obtained from New England Nuclear, Wilmington, DE. Caffeine, and cAMP were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest purity available.

Avian pectoralis major. All animals used in this study were obtained from the Department of Avian Sciences, University of California, Davis, CA. PM was dissected from outbred (New Hampshire Red) homozygous normal line 412 and the respective homozygous dystrophic line 413 paired from the same hatch at ages ranging from 2 days to 5.5 weeks ex ovo. In separate experiments, PM was dissected from highly inbred congenetic (White Leghorn) normal line 003 and dystrophic line 433 paired at ages of 2 and 10 weeks ex ovo. Muscle tissue pooled according to age and genetic line was rapidly frozen immediately following dissection and stored at −80°C for a time not exceeding 8 weeks.

Isolation of junctional sarcoplasmic reticulum membranes. SR vesicles from chicken PM were isolated by a minor modification of the method of Saito and coworkers [27] as modified by Inui et. al. [5]. Equal amounts of normal and dystrophic PM were prepared using the same buffer stocks and centrifuged simultaneously in the same rotors to minimize differences due to technique. Briefly, thawed muscle was ground and homogenized in at least five volumes ice-cold 5 mM imidazole (pH 7.4) and 0.3 M sucrose (Buffer A) for 1 min at maximum speed. Homogenates were centrifuged in a Sorvall GSA rotor at $8000 \times g_{max}$ for 10 min. The pellets were rehomogenized in five volumes Buffer A containing 0.5 mM Mg-ATP for 1.5 min and centrifuged at $6000 \times g_{max}$ for 20 min. The supernatant (crude microsomal fraction) was filtered through cheesecloth and the crude SR pellet obtained by centrifugation at $60\,000 \times g_{max}$ for 60 min. The resulting supernatant (light microsomes) was not further purified while the crude SR fraction was resuspended in 10 ml Buffer A per 50 g original tissue and layered (10 ml/gradient) on top of a four-step discontinuous sucrose gradient (7 ml of each 27%, 32%, 38%, and 48% sucrose (w/v)). Sucrose gradients were centrifuged in a Beckman SW 28 rotor for 16 h. Five membrane fractions were collected (I: 10%/27% interface; II: 27%/32% interface; III: 38%; IV: 38%/48% interface; and V: the pellet at the bottom of the tube). All gradient fractions were diluted in Buffer A, pelleted, and resuspended in 20 mM Hepes or Tris maleate (pH 7.1), 115 mM KCl, and 15 mM NaCl (Buffer B) at 4–6 mg protein/ml. Protein was determined in triplicate by the method of Lowry et. al. [28] with bovine serum albumin as a standard. In initial

experiments samples from each centrifugation step were saved for protein determination and receptor assay.

[^{3}H]Ryanodine binding studies. All equilibrium binding studies were incubated at 37°C for 180 min in Buffer B containing 100 μg muscle protein per ml (1 ml, final volume). Binding reactions were quenched by rapid filtration through Whatman GF/B glass fiber filters using a cell harvester (Brandel, Gaithersburg, MD). The filters were immediately washed with ice cold Buffer B (2 × 2.5 ml), dried under vacuum, and radioactivity counted by liquid scintillation in Bio-Safe cocktail (RPI Corp., Mount Prospect, IL).

Binding isotherms were determined in Buffer B containing 50 μM $CaCl_2$ and 10 nM [^{3}H]ryanodine, by titrating varying concentrations of unlabelled ryanodine ranging from 10 nM to 10000 nM. [^{3}H]Ryanodine concentrations below 10 nM were obtained by direct additions of a 100 × stock of diluted radioligand (specific activity = 54.7 Ci/mmol). Nonspecific binding was determined in the presence of 100 μM unlabelled ryanodine and averaged 5% and 1–2% of specific binding at 10 nM and 1–5 nM, respectively.

Activation and inactivation of the [^{3}H]ryanodine binding site was measured in Buffer B containing 100 μM $CaCl_2$. Free Ca^{2+} was adjusted by titrating EGTA based on an apparent stability constant for Ca: EGTA of $4.39 \cdot 10^6$ M^{-1} and calculated using the SPECS computer program [29]. Ca^{2+} concentrations > 100 μM were obtained by direct addition of aliquots of a 100 × stock of $CaCl_2$. To assess the sensitivity of high- and low-affinity [^{3}H]ryanodine binding sites to Ca^{2+} (or other modulators described below), experiments were performed at 10 nM and 250 nM (10 nM radioligand and 240 nM unlabelled ryanodine; specific activity = 2.2 Ci/mmol) [^{3}H]ryanodine, respectively. At 250 nM [^{3}H]ryanodine less than 2% of the high-affinity sites were detected as specifically bound radioligand in normal PM preparations (i.e., these sites were saturated by unlabelled ryanodine) and hence allowed direct assessment of the low-affinity sites.

The influence of Mg^{2+}, cAMP, and caffeine, at optimal (50 μM) and suboptimal (0.8 μM) Ca^{2+} was determined at 10nM and 250 or 500 nM [^{3}H]ryanodine to assess the response of high and low affinity binding sites, respectively. Binding was assessed under competitive conditions by adding SR membranes last to the assay medium to initiate the reaction.

Measurement of association kinetics. Association of [^{3}H]ryanodine with high (measured at 10 nM [^{3}H]ryanodine) and low (measured at 250 nM [^{3}H]ryanodine) affinity receptor sites was assessed at free Ca^{2+} ranging from 1 to 100 μM. Binding was initiated by addition of SR at times ranging from 320 min to 5 min prior to rapid filtration as described above.

Analysis of binding data. Equilibrium binding isotherms and association kinetics were analyzed by non-linear regression analysis using the Enzfitter program (Elsevier-Biosoft, Cambridge, U.K.). Each analysis consisted of triplicate determinations at each concentration of ligand or time point. Single and multiple apparent dissociation constants ($K_{d,app}$ and B_{max} values and their respective standard errors were calculated directly from the non-linear regression. The influence of Ca^{2+} on activation and inactivation of [^{3}H]ryanodine binding was analyzed by the method of Hill [30] by plotting the log of fractional receptor occupancy (log $(B/B_{ns} - B)$); where B_{ns} the occupancy at low (10 nM) or high (250 or 500 nM) concentrations of [^{3}H]ryanodine under optimal Ca^{2+}) versus log [Ca^{2+}]. Values from 10% to 90% B_{ns} were used in the analyses of activation or inactivation and resulted in linear Hill plots whose intercepts with the abscissa measure the apparent affinity of the activator site for Ca^{2+} (K_{dCa}) or the concentration of Ca^{2+} which causes 50% inhibition (IC_{50}), respectively.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SR membranes were solubilized under reducing conditions in sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.125% Bromophenol blue. Proteins were electrophoresed at constant voltage in the discontinuous buffer system of Laemmli [31] on 3–17% linear gradient gels having a 3% stacking gel. Protein standards ranged from unreduced α-macroglobulin (M_r = 340000) to carbonic anhydrase (M_r = 29000). Gels were stained with Coomassie blue.

Results and Discussion

Over-expression of heavy SR in dystrophic PM

Sucrose gradient purification of crude SR microsomes from normal line 412 and dystrophic line 413 PM each result in two membrane bands in the 38% sucrose layer (fractions III and IV) which comprise approx. 98% of the total measurable [^{3}H]ryanodine receptor. Sucrose gradient fractions I, II, and V, as well as the light microsomal fraction and MgATP pellet contain negligible [^{3}H]ryanodine binding sites (< 0.1 pmol/mg protein). Separation of fraction III from IV in initial experiments resulted in nearly identical [^{3}H]ryanodine binding densities and affinities (not shown) and these fractions were combined in subsequent experiments. Enrichment of fractions III and IV in [^{3}H]ryanodine receptor (a specific marker for terminal cisternae of junctional SR) and the appearance of high molecular weight junctional proteins on SDS-PAGE (Fig. 1) provides supporting evidence that these heavy SR fractions are enriched in markers of the terminal cisternae relative to the other fractions isolated.

Table I shows the protein distribution among various membrane subfractions from PM preparations 4 weeks ex ovo. Compared to dystrophic line 413, normal line

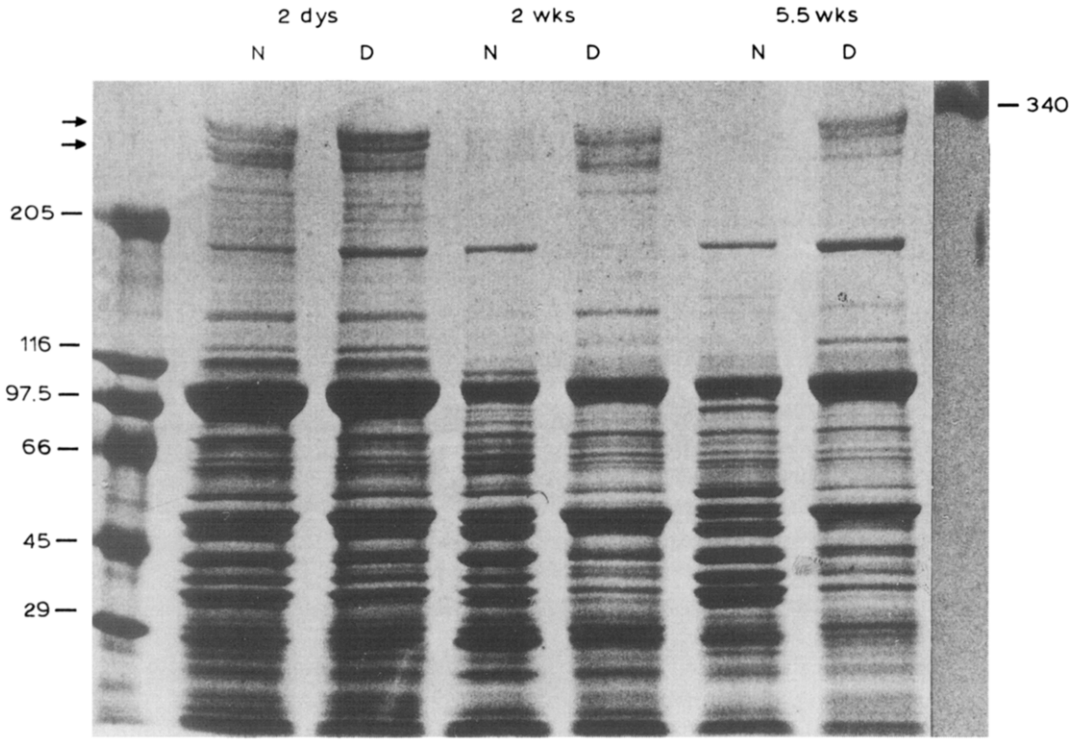

Fig. 1. SDS-PAGE of heavy SR membrane preparations from normal line 412 (N) and dystrophic line 413 (D) pectoralis major (PM) paired at 2 days, 2 weeks, and 5.5 weeks, ex ovo. Junctional membranes were denatured under reducing conditions as described in Experimental Procedures and 20 μg protein applied to each lane. The high molecular weight doublet of the putative [^{3}H]ryanodine receptor are indicated by arrows. Molecular weight standards are shown in outside lanes (unreduced α-macroglobulin is shown in the right lane) and their molecular weights are given in k.

412 yields 32% and 25% less protein in the crude microsomal and the light microsomal fractions, respectively. More significant is the nearly 4-fold higher protein recovery in the crude SR fraction from line 413 when compared to identical preparations with normal line 412. Although heavy SR fractions account for approx. 65% of the proteins recovered from the sucrose gradients of both normal and dystrophic preparations at this age, dystrophic PM gives 8.6-fold greater yield in the heavy SR fraction (1.21 vs. 0.14 mg/g PM, respectively).

Recovery of protein in fractions III and IV from normal line 412 PM is highly age dependent. Two day normal PM yields 0.80 mg of heavy SR protein per g tissue (Table II). By 2 weeks ex ovo the yield decreases over 3-fold (to 0.24 mg/g) and remains depressed at 4 weeks (0.14 mg/g) and 5.5 weeks ex ovo (0.28 mg/g).

TABLE I

Protein distribution among various membrane subfractions obtained from 4 week ex ovo line 412 normal and line 413 dystrophic pectoralis major (PM)

PM membranes were prepared as described in Experimental Procedures and protein concentrations determined in each fraction [25]. Crude microsomes were recovered from the MgATP supernatant. Light microsomes were recovered from the 60000 × *g* supernatant. Sucrose gradient factors recovered from the crude SR pellet obtained at 60000 × *g*. Results are averages of triplicate determinations whose standard deviations varied less than 10%.

Line	Protein recovery (mg protein/g PM)					
	crude microsomes	light microsomes	Sucrose gradient fractions			
			I	II	III and IV	V
412 Normal	501	4.20	0.03	0.01	0.14	0.30
413 Dystrophic	732	5.61	0.11	0.35	1.21	0.18

TABLE II

Age-dependent recovery of heavy SR (mg protein/g PM) from pooled sucrose gradient fractions III and IV obtained from normal and dystrophic PM and their total [^{3}H]ryanodine binding capacity (pmol/g PM)

The total [^{3}H]ryanodine binding capacity is calculated from nonlinear regression analysis of binding isotherms and the number of classes of receptor sites detected is given in parentheses. Results represent the average of two experiments each in triplicate.

Age ex ovo	Line 412 normal		Line 413 dystrophic	
	Protein (mg/g)	Capacity (pmol/g)	Protein (mg/g)	Capacity (pmol/g)
2 day	0.80	2.6(≥ 2)	0.96	2.0(1)
2 week	0.24	0.84(1)	1.67	28.2(≥ 2)
4 week	0.14	0.17(1)	1.21	33.0(≥ 2)
5.5 week	0.28	0.11(1)	1.89	42.7(≥ 2)

This is in marked contrast to recovery of protein in heavy SR fractions from dystrophic line 413 which significantly increases with age from 0.96 mg/g PM at 2 days ex ovo to 1.89 mg/g PM at 5.5 weeks (Table II). Initial experiments with highly inbred normal line 003 and dystrophic line 433 at 10 weeks post-hatch exhibit even more dramatic differences in protein recovery from heavy SR fractions yielding 0.06 (±0.02) and 0.58 (±0.05) mg/g PM, respectively. Regardless of the phenotypic expression of chicken dystrophy, hypertrophied PM in line 413 or atrophied PM in line 433, subfractionation of diseased PM ≥ 2 weeks ex ovo consistently exhibits 7–10-fold greater yield of heavy SR protein recovered from sucrose gradient fractions III and IV.

The yields of heavy SR from normal line 412 chicken PM > 2 days ex ovo are consistent with those previously obtained with preparations from rabbit fast-twitch skeletal muscle [5,27]. For example, rabbit skeletal muscle preparations yield an average 0.3 mg of junctional SR per g of tissue [27] while normal PM yields an average of 0.24, 0.14 to 0.28 mg/g PM at 2, 4, and 5.5 weeks ex ovo, respectively (Table II). Higher recoveries of protein in the heavy SR membrane fractions obtained from newly hatched (2 day ex ovo) normal chicks may reflect proliferation of the junctional SR during embryonic development. Apparently this embryonic character is rapidly lost (within two weeks) in normal PM but is maintained in dystrophic PM where junctional SR (measured by [^{3}H]ryanodine binding capacity, shown below) proliferates throughout the first 5.5 weeks examined in this study. Kosk-Kosicka and coworkers using PM from genetic lines 412/413 identified an abundance of 'low density' vesicles in the microsomal fraction of immature normal and dystrophic PM [24]. Based on ultrastructural studies, it was suggested that these vesicles were of junctional T-tubule origin [24]. Interestingly, the greatest yield of 'low density' vesicles were found in preparations from immature (1–2 week ex ovo) normal and dystrophic PM. Whereas recovery of low density vesicles where drastically reduced by 4 weeks ex ovo in normal PM, dystrophic PM maintained a high yield of 'low density' vesicles in their microsomal fraction. The present results further suggest that proliferation of junctional SR is one of the earliest lesions expressed in chicken dystrophy and appears to reflect a juvenile condition.

Over-expression of [^{3}H]ryanodine receptors in dystrophic PM

Concomitant with early loss of protein recovered in heavy SR fractions from normal PM is the disappearance of low-affinity [^{3}H]ryanodine binding sites and a significant decrease in the density of high-affinity binding sites for [^{3}H]ryanodine (Tables II and III). Normal SR fractions at day 2 have a total capacity of [^{3}H]ryanodine-binding sites of 2.6 pmol/g PM (Table II) distributed among high- and low-affinity sites ($K_{d,app}$ = 18 nM, B_{max} = 1.7 pmol/mg protein and $K_{d,app}$ = 532 nM, B_{max} = 2.6 pmol/mg protein) (Table III, Fig. 2). [^{3}H]Ryanodine binding capacity at 2 weeks decreases to 0.84 pmol/g PM (Table II) and is limited to a single class of high-affinity binding sites ($K_{d,app}$ = 17 nM; B_{max} = 3.5 pmol/mg protein). Down regulation of [^{3}H]ryanodine receptor capacity continues with normal development of line 412 PM reaching 0.17 and 0.11 pmol/g PM at 4 and 5.5 weeks ex ovo, respectively (Table II). In marked contrast, results from line 413 demonstrate that dystrophic junctional SR preparations from 2 day chicks initially lack low-affinity [^{3}H]ryanodine binding sites but have nearly equal density of high-affinity sites ($K_{d,app}$ = 31 nM; B_{max} = 2.1 pmol/mg protein; capacity = 2.0 pmol/g PM). However, by 2 weeks ex ovo dystrophic PM exhibits significant densities of high- and low-affinity [^{3}H]ryanodine-binding sites ($K_{d,app}$ = 17 and 725 nM; B_{max} = 1.5 and 15.4 pmol/mg protein, respectively) resulting in a marked 34-fold higher capacity for binding [^{3}H]ryanodine compared to normal line 412 (28.2 vs. 0.84 pmol/g PM, respectively). Elevated capacity to bind [^{3}H]ryanodine by dystrophic SR is augmented during post-hatch development by 194- and 388-fold compared to normal junctional SR at 4 and 5.5 weeks, respectively. Initial studies with 10 week atrophic PM from line 433 also reveal approx. 200-fold higher capacity to bind [^{3}H]ryanodine compared to preparations from the congenetic normal line 003 (data not shown).

Recent purification of the [^{3}H]ryanodine receptor by numerous laboratories has provided evidence for its association with a high molecular weight subunit which ranges from M_r 300000 to 450000 depending on the method of preparation [3–11]. Down regulation of [^{3}H]ryanodine receptors during normal post-hatch de-

TABLE III

Apparent affinities ($K_{d,app}$) and receptor densities (B_{max}) of [^{3}H]ryanodine binding sites found in heavy SR preparations at various developmental stages

n.d. refers to preparations where low-affinity classes of binding sites were not detected. Values are the results of triplicate determinations ± the standard error of the estimate. Each experiment was duplicated at least once (in triplicate) and gave similar results.

Age ex ovo	Line 412 normal		Line 413 dystrophic	
	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)
2 day	18±2	1.7±0.2	31±2	2.1± < 0.1
	532±133	2.6±0.1	n.d.	n.d.
2 week	17±1	3.5± < 0.1	17±8	1.5±0.4
	n.d.	n.d.	725±110	15.4±0.8
4 week	17±5	1.2±0.2	22±0.1	2.2± < 0.1
	n.d.	n.d.	2680±210	25.1±0.1
5.5 week	20±4	0.4± < 0.1	16± <1	2.8± < 0.1
	n.d.	n.d.	4498±801	16.5± < 0.1

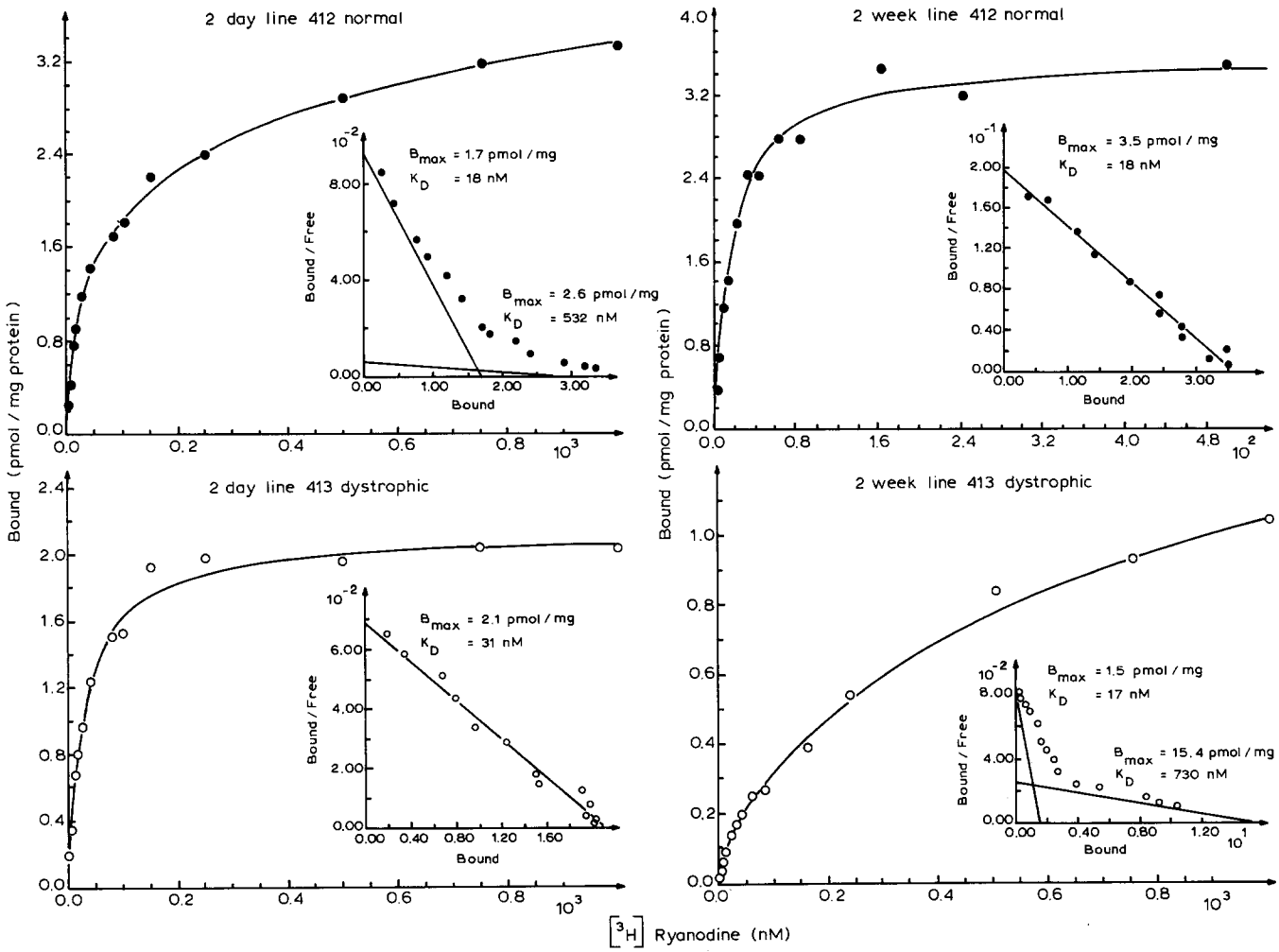

Fig. 2. [^{3}H]Ryanodine binding isotherms for heavy SR receptor from normal line 412 and dystrophic line 413 PM at 2 days and 2 weeks ex ovo. Binding assays were performed as described in Experimental Procedures in the presence of 50 μM $CaCl_2$. Each point represents the mean of triplicate determinations which varied <10%. Best-fit curves were plotted by non-linear regression analysis.

velopment of chicken PM from line 412 coincides with a decrease in the intensity with which the high molecular weight doublet of M_r 340000 and 320000 stains on SDS-PAGE. Preparations from dystrophic PM which maintain a high expression of [^{3}H]ryanodine receptors also exhibit prominent high molecular weight doublets at the ages examined in this study (Fig. 1). Additional differences are evident in the intensity of bands of lower molecular weight. Preparations from normal PM consistently display darker bands below approx. 50000 relative to dystrophic SR. These bands may represent glycolytic proteins of transverse tubule origin since initial experiments (Pessah, I.N., unpublished data) have revealed a nearly 3-fold higher capacity to bind [^{3}H]nitrendipine, a specific marker for voltage-gated slow inward Ca^{2+} channels of the transverse tubule in normal versus dystrophic SR preparations at 2 weeks ex ovo. This may reflect a higher yield of intact triads in normal preparations. However, one cannot discount the possibility that the high molecular weight junctional foot protein from normal SR is more sensitive to proteolytic cleavage by endogenous protease activity [9].

The present study demonstrates the existence of multiple classes of [^{3}H]ryanodine receptor sites in skeletal muscle SR. Vertebrate skeletal muscle preparations examined thus far have demonstrated essentially linear Scatchard plots with a single class of high-affinity [^{3}H]ryanodine binding sites having $K_{d,app}$ values ranging from 5 to 100 nM, depending on assay conditions [1–13]. However, two recent reports suggest the presence of low affinity ($K_{app} \approx 3$–10 μM) binding sites for [^{3}H]ryanodine in skeletal muscle SR membranes when assayed in very high salt (1 M KCl or NaCl) [32–33]. A significant observation in the present study is that under identical conditions including physiologically relevant concentrations of monovalent cations, expression of multiple classes of [^{3}H]ryanodine binding sites appears to predominate in immature and dystrophic PM and coincides with the relative intensity of the high molecular weight doublet (i.e., the putative [^{3}H]ryano-

dine receptor). Normal mature vertebrate cardiac muscle contains prominent high- and low- ($K_{d,app} > 300$ nM affinity receptor sites [1,4,34–36]. Interestingly, fetal cardiac muscle, like normal juvenile PM, expresses a significantly higher density of ryanodine receptors when compared to mature cardiac muscle [37]. The distribution, location, and functional significance of low-affinity [^{3}H]ryanodine receptor sites in SR remains to be determined. However, it has become clear that major differences exist in the gating properties of Ca release channels and in the modulation of [^{3}H]ryanodine binding sites by relevant ligands (e.g., sensitivity to metal ions) in skeletal and cardiac muscle.

Ca^{2+} activation/inactivation of normal and dystrophic [^{3}H]ryanodine receptor sites

[^{3}H]Ryanodine binding to receptor from rabbit skeletal muscle is both activated and inactivated by 1 μM and > 100 μM Ca^{2+}, respectively [1,3,8,13]. This property of [^{3}H]ryanodine receptors is preserved with receptors from both normal and dystrophic PM (Fig. 3). Ca^{2+} activation of radioligand binding at 10 nM [^{3}H]ryanodine give similar values of K_{dCa} for normal and dystrophic receptors ($K_{dCa}(\pm S.E.) = 7.5$ (± 2) vs. 7.3 (± 6) μM, respectively). Interestingly, high-affinity [^{3}H]ryanodine binding sites (measured at 10 nM radioligand) from dystrophic preparations have significantly higher sensitivity to Ca^{2+} inactivation when compared those from normal line 412 ($IC_{50}(\pm S.E.) = 386$ (± 25) vs. 644 (± 104) μM, respectively). Similar experiments at 250 nM [^{3}H]ryanodine where performed to determine the sensitivity of low-affinity binding sites detected in dystrophic (but not normal) PM to Ca^{2+} activation/inactivation. Fig. 3 demonstrates that at both 2 and 4 weeks ex ovo the low-affinity [^{3}H]ryanodine binding sites exhibit significantly greater sensitivity to Ca^{2+} activation ($K_{dCa}(\pm S.E.) = 2.9$ (± 0.5) and 1.7 (± 0.6) μM, respectively) than the high-affinity binding sites in normal PM at these ages ($K_{dCa}(\pm S.E.) = 11.3$ (± 2.5) and 9.5 (± 1.5) μM, respectively).

Ca^{2+} concentration influences the pseudo-first-order rate constant (k_{obs}) for radioligand binding to high-affinity [^{3}H]ryanodine binding sites (measured at 7 nM radioligand) from both normal and dystrophic heavy SR (Fig. 4A). Ca^{2+} at 10 μM gives average k_{obs} values of 0.057 and 0.041 min^{-1} while 100 μM Ca^{2+} gives 0.019 and 0.018 min^{-1} for normal and dystrophic preparations (S.E. < 10%), respectively. Low (1 μM) Ca^{2+} results in very slow association kinetics ($k_{obs} = 0.009$ and 0.008 min^{-1} for normal and dystrophic preparations (S.E. < 10%), respectively). Low-affinity binding sites (measured at 250 nM [^{3}H]ryanodine) in dystrophic junctional SR exhibit k_{obs} values which are less influenced by the level of Ca^{2+} (Fig. 4B). Ca^{2+} at 1, 10, and 50 μM give k_{obs} values of 0.020, 0.025, and 0.015 min^{-1} respectively. These results demonstrate that differences in sensitivity to Ca^{2+} activation observed between high-affinity normal and low-affinity dystrophic [^{3}H]ryanodine binding sites measured in equilibrium binding studies are at least in part the result of differences in association kinetics. High-affinity ryanodine binding has been demonstrated to occur with an open (Ca^{2+} conducting) state of the Ca^{2+} release channel of

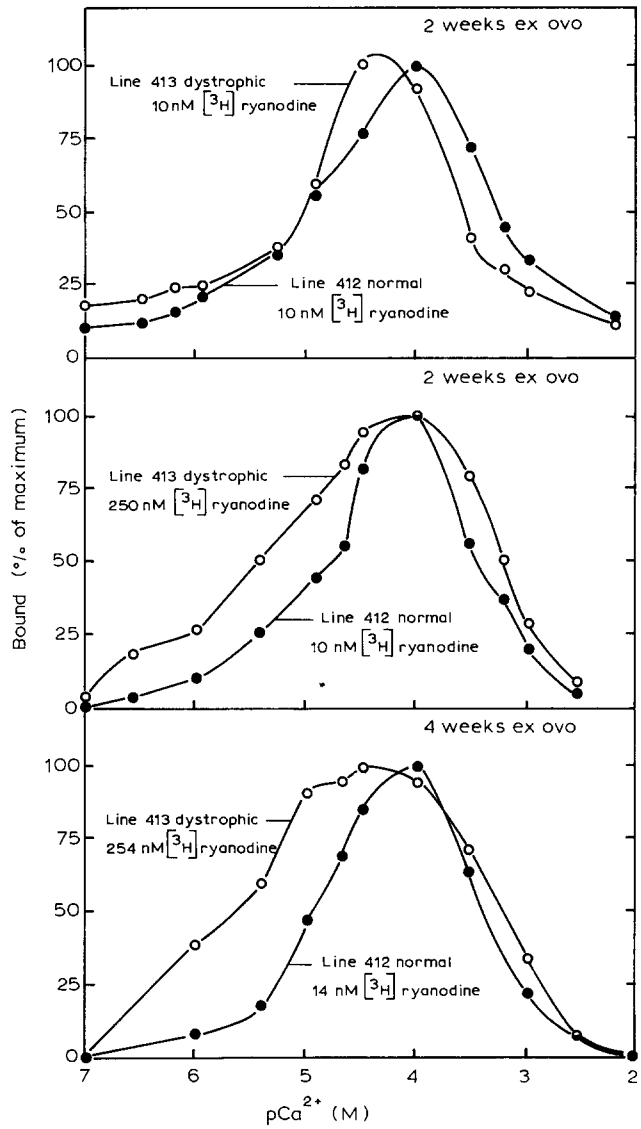

Fig. 3. Ca^{2+} activation and inactivation of [^{3}H]ryanodine binding to paired heavy SR preparations from normal line 412 (●) and dystrophic line 413 (○). The Ca^{2+} sensitivity of high-affinity [^{3}H]ryanodine binding sites was measured at approx. 10 nM radioligand while the response of low-affinity binding sites found in dystrophic preparations was assessed at approx. 250 nM radioligand. [^{3}H]Ryanodine occupancy at 100% ranged from 0.49 to 0.62 and 0.19 to 0.24 pmol/mg protein at 10 nM radioligand at 2 and 4 weeks for 412 and 413, respectively. Maximal occupancy at 2 and 4 weeks for low affinity sites measurable only in preparations from line 413 ranged from 5.1 to 7.9 pmol/mg protein. Each point represents the mean of triplicates. Each experiment was repeated a least twice and the apparent affinity for Ca^{2+} activation (K_{dCa}) and the inhibitory constant (IC_{50}) and their respective standard errors were determined by linear regression of Hill plots and are summarized in the text.

TABLE IV

Modulation of high-affinity (measured at 10 nM radioligand) and low affinity (measured at 500 nM radioligand; 10 nM [^{3}H]ryanodine + 490 nM unlabelled ryanodine) [^{3}H]ryanodine binding sites by Ca^{2+}, cAMP, and caffeine

Values are means ± standard deviations of three experiments each in duplicate. Assay conditions under which low affinity receptor sites were not detected are denoted by n.d.

Additions	412 Normal		413 Dystrophic	
	bound (pmol/mg)	activation (× control)	bound (pmol/mg)	activation (× control)
10 nM [^{3}H]ryanodine + 1 μM Ca^{2+}, 1 mM Mg^{2+}				
Alone	0.016 ± < 0.01	1.0	0.014 ± < 0.01	1.0
+ 20 mM caffeine	0.151 ± < 0.01	9.4	0.333 ± 0.022	23.8
+ 20 mM caffeine, 2 mM cAMP	0.310 ± 0.03	19.4	0.917 ± 0.046	65.5
500 nM [^{3}H]ryanodine + 1 μM Ca^{2+}, 1 mM Mg^{2+}				
Alone	n.d.	–	0.856 ± 0.017	1.0
+ 20 mM caffeine	n.d.	–	3.46 ± 0.14	4.0
+ 20 mM caffeine, 2 mM cAMP	n.d.	–	4.84 ± 0.22	5.6

junctional SR [9–13,35]. Upon forming a ryanodine-receptor complex the alkaloid binding site rapidly occludes, bound alkaloid is recalcitrant to dissociation by excess unlabelled ryanodine, and results in a persistent subconductance state [9,11,13]. The present results strongly suggest that low-affinity receptor sites over-expressed in dystrophic PM may reflect Ca^{2+} channels having significantly lower threshold to activation by Ca^{2+}. Although abnormal channel gating and Ca^{2+} permeability properties of dystrophic junctional SR remain to be directly demonstrated by ion transport measurements, [^{3}H]ryanodine binding studies suggest that early over-expression of low-affinity [^{3}H]ryanodine receptors having altered sensitivity to activator Ca^{2+} may significantly contribute to the etiology of dystrophy in the chicken.

Modulation of Ca^{2+}-activated [^{3}H]ryanodine binding by adenine nucleotide, and caffeine

When assayed at suboptimal (1 μM) Ca^{2+} with 1 mM Mg^{2+}, high-affinity [^{3}H]ryanodine binding sites from normal and dystrophic SR exhibit very low occupancy (Table IV). However, addition of 20 mM caffeine under these conditions results in much larger increase in [^{3}H]ryanodine receptor occupancy in dystrophic (23.8-fold) compared to normal (9.4-fold) SR (Table IV). The addition of 2 mM cAMP in the presence of 20 mM caffeine results in 2.1- and 2.8-fold additional activation in normal and dystrophic [^{3}H] ryanodine occupancy, respectively (i.e., 19.4 vs. 65.5-fold stimulation over controls lacking modulators, respectively) (Table IV). Low-affinity [^{3}H]ryanodine binding sites (assayed at 500 nM radioligand) from dystrophic junctional SR exhibit appreciable binding in the presence of 1 μM Ca^{2+} and 1 mM Mg^{2+} (0.86 pmol/mg protein). Addition of 20 mM caffeine alone or in the presence of 2 mM cAMP result in only 4.0 and 5.6-fold activation of radioligand binding, respectively. The significant differences in modulation of [^{3}H]ryanodine binding sites by cAMP and/or caffeine observed with normal and dystrophic muscle support the view that [^{3}H]ryanodine receptors in dystrophic muscle reflect a population of Ca^{2+} release channels which favor an open channel state despite low (1 μM) Ca^{2+} and 1 mM Mg^{2+}. Significantly larger increases in receptor occupancy observed at 10 nM [^{3}H]ryanodine in the presence of adenine nucleotide and/or caffeine with dystrophic preparations may reflect an increase in the affinity of 'low affinity' sites for the radioligand under these assay conditions. These findings demonstrate the altered sensitivity of low-affinity [^{3}H]ryanodine binding sites to chemical modulation and suggests that the level at which they are expressed (i.e., their titer) may be of significance to muscle function. The titer of low-affinity receptor may be especially important if they are converted to the high-affinity state by physiologically relevant modulators (e.g., adenine nucleotides) (Table IV). Perhaps even more significant to excitation-contraction coupling is the possible influence of activation of low-affinity ryanodine receptors by endogenous ligands on the expression of high-affinity [^{3}H]ryanodine binding sites which reflect the open conformation of the SR Ca^{2+} release channel.

In conclusion, the present study demonstrates that over-expression of junctional SR and [^{3}H]ryanodine receptor having altered radioligand binding properties is a very early event in the post-hatch development of dystrophic PM and precedes the development of muscle dysfunction in this species [38]. Since the [^{3}H]ryanodine receptor is a specific marker for the SR Ca^{2+} release

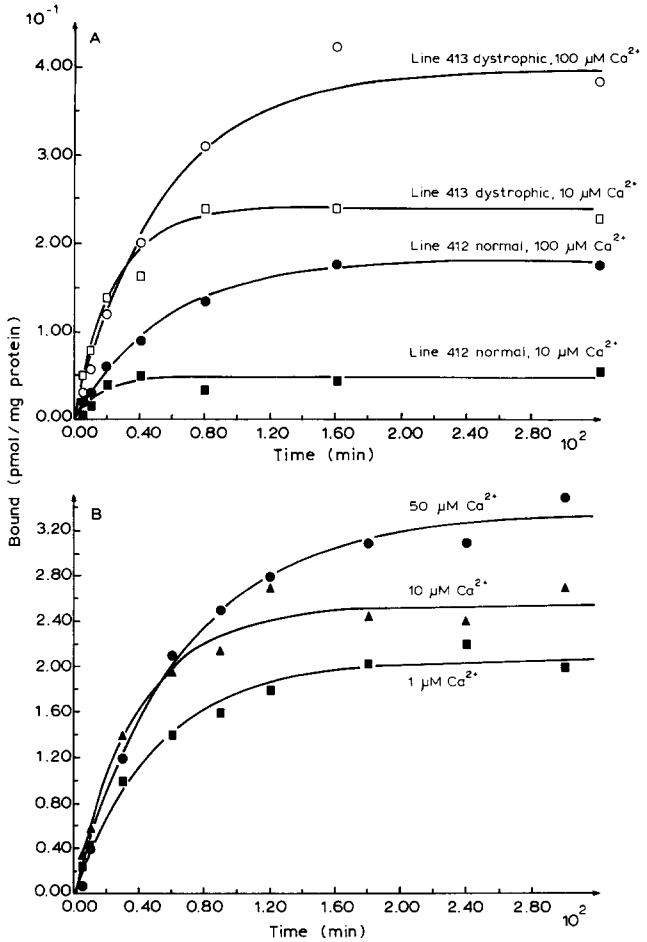

Fig. 4. Association of [^{3}H]ryanodine with heavy SR receptors prepared from normal and dystrophic PM at various free Ca^{2+}. Rates of association to high-affinity binding sites were measured at 10 nM [^{3}H]ryanodine for normal (●, ■) and dystrophic (○, □) preparations (A). Low-affinity binding sites detected only in dystrophic preparations were assessed at 250 nM [^{3}H]ryanodine (B). Each point represents the mean of triplicates which varied <10% and curves were fitted by nonlinear regression. Each experiment was repeated at least twice and the pseudo-first-order rate constants (k_{obs}) and their standard errors given in the text.

channel of the muscle triad and is a key component of excitation-contraction coupling, the abnormal expression of this receptor may be of fundamental importance to the etiology of muscular dystrophy in the chicken.

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