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[^{3}H]Ryanodine Binding Sites of SR Vesicles of the Chicken Pectoral Muscle

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To investigate the properties of ryanodine binding sites of the bird skeletal SR vesicles, SDS PAGE, purification of RyR, and [^{3}H]ryanodine binding study were carried out in the SR vesicles prepared from the chicken pectoral muscle. The chicken SR vesicles have two high molecular weight (HMW) protein bands as in eel SR vesicles on SDS PAGE. The HMW bands on SDS PAGE were found in the [^{3}H] ryanodine peak fraction (Fr_{3-5}) obtained from the purification step of the ryanodine receptor protein. Bmax and K_D of the chicken [^{3}H]ryanodine binding sites were 12.52 pmol/mg protein and 14.53 nM, respectively. Specific [^{3}H]ryanodine binding was almost maximal at 50~100 μM Ca^{2+}, but was not increased by 5 mM AMP and not inhibited by high Ca^{2+}. Binding was significantly inhibited by 20~100 μM ruthenium red and 1 mM tetracaine, but slightly inhibited by Mg^{2+}. From the above results, it is suggested that chicken SR vesicles have the ryanodine binding sites to which the binding of ryanodine is almost maximal at 50~10 μM Ca^{2+}, is significantly inhibited by ruthenium red and tetracaine, slightly inhibited by Mg^{2+}, but not affected by AMP and not inhibited by high Ca^{2+}.

Key Words: Ca-release channel, Ryanodine receptor, Sarcoplasmic reticulum, Chicken

Abbreviation: AMP, Adenosine-5'-monophosphate; DIFP, Diisopropyl fluorophosphate: EGTA, Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; PMSF, Phenylmethysulfonyl fluoride; Chaps, 3-[(3-cholamidopropyl) dimethyl-ammonio]-1- propane sulfonate; RyR, Ryanodine receptor; EDTA, Ethylenediamine tetraacetic acid; PAGE, Polyacrylamide gel; SDS, Sodium dodecyl sulfate

INTRODUCTION

For muscular contraction sufficient amount of calcium should be influxed from extracellular fluid (ECF) or released from intracellular repository, sarcoplasmic reticulum (SR). Calcium influx from ECF occurred through sarcolemmal Ca-channel, dihydropyridine receptors. And calcium release from SR is done through IP_3 receptor (Donaldson et al, 1988; Rojas & Hidalgo, 1990) or Ca-releasing channel, so called ryanodine receptor (RyR)(Fleischer & Inui, 1989; Lai & Meissner, 1989). It has been known that RyRs consist of four ~550 kDa polypeptides in the form of homotetramer (Fleisher et al, 1989; Lai et al, *1989*). But according to recent electrophoretic studies, RyRs could be observed as single protein band with different mobility (Inui et al, 1987; Lai et al, 1988) or two protein bands with homooligmer (Airey et al, 1990; Olivares et al, 1991; Murayama & Ogaea,

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1992; Airey et al, 1993; Seok et al, 1995) according to organ (brain, skeletal muscle, cardiac muscle etc.) or species.

RyRs in the mammalian cardiac and skeletal muscle SR show single protein band on the SDS PAGE and have similar characteristics but the former release calcium more rapidly than the latter, and the latter show slower mobility than the former (Meissner et al, 1986; Meissner & Henderson, 1987). $MgCl_2$ and calcium have different actions on the cardiac and skeletal muscle in [^{3}H]ryanodine binding to RyRs (Pessah et al, 1985; Seifert & Cassida, 1986; Michalak et al, 1988; Zimany & Pessah, 1991). On the other hand, SR RyRs of the fishes (Olivares et al, 1991; Seok et al, 1995), birds (Airey et al, 1990; Airey et al, 1993) and amphibians (Olivares et al, 1991; Murayama & Ogaea, 1992) are having two high molecular weight (HMW) band, while those of mammalian skeletal and cardiac muscle are having single HMW band. The properties of RyRs of fishes and amphibians have been reported (Murayama & Ogaea, 1992; Seok et al, 1995), however, in birds there are few reports on the properties of RyRs except immunologic studies (Airey et al, 1990; Airey et al, 1993).

Therefore to investigate the properties of ryanodine binding sites in bird skeletal muscle, we isolated chicken pectoral SR vesicles and carried out SDS PAGE, purification of RyRs and [^{3}H]ryanodine binding study.

METHODS

Isolation of SR vesicles

After sacrificing chicken, about 40 g of chicken pectoral muscle was excised, minced, and homogenized with a Polytron (Kinematica) for 90 sec (3 × 30 sec) at the middle setting in 7.5 volumes of the medium containing 0.1 M NaCl, 20 mM K/Pipes, pH 6.8, 0.1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 10 mM aprotinin, 1 μM leupeptin, 1 μM pepstatin, 1 mM benzamidine and 1 mM iodoacetamide. Further isolation of SR vesicles was followed as described in Seok et al. (1995) and Nam & Seok (1996). Final collect of SR vesicles were resuspended in 0.3 M sucrose, 5 mM K/Pipes, pH 7.0 and stored at −70°C

SDS-polyacrylamide gel electrophoresis and isolation of RyR

SR vesicles were analysed in SDS PAGE which was performed in the Laemmli buffer system (Laemmli, 1970) using 3~12% linear polyacrylamide running gels. About 50~100 μg of SR from skeletal muscle of rabbit, chicken and eel, and canine cardiac muscle were treated by sample buffer and then electrophoresed for 16 hr. After electrophoresis the gel was fixed in the solution of 12% trichloroacetic acid and were stained by colloidal Commassie Blue staining methode (Neuhoff et al, 1988).

On the other hand, to confirm which band binds to [^{3}H]ryanodine, they were purified using rate density gradient centrifugation as described by Lai et al. (1988). Pectoral muscle SR vesicles (2.5 mg/ml) were solubilized in the solution of 1.5% Chaps (1 M NaCl, 20 mM Na/Pipes, pH 7.4, 100 μM $CaCl_2$, 5 mM AMP, 5 mg/ml phosphatidylcholine, 100 μM dithiothreitol, and 1 μM leupeptin and 1.5% Chaps) for 1 hr at room temperature and for 1 hr at 4°C, loaded on 7~20% linear sucrose gradient and centrifuged for 16 hr at 121,500 × g (Sorvall; AH 629 rotor). 3 nM of [^{3}H]ryanodine was added to solubilized solution and used as an indicator. After centrifugation they were separated into 16 fractions, 2 ml of each, from the bottom. To find the peak [^{3}H]ryanodine radioactivity among them, 50 μl of each fraction was removed and checked by β-counter (LS6500, Beckman, USA) to evaluate radioactivity.

[^{3}H]Ryanodine binding

[^{3}H]Ryanodine binding was followed essentially as described by Seok et al (1995). After SR vesicles (30~50 μg) were incubated for 2 hr at 37°C in high concentration of KCl solution (1 M KCl, 20 mM K/Pipes, pH 7.4, 100 μM $CaCl_2$, 5 mM AMP, 0.2 mM PMSF, 2 μM leupeptin, 2 mM dithiothreitol and 2~128 nM [^{3}H]ryanodine) diluted with 40 volumes of cold water, filtered through Whatman filter (GF/B; pore size 1 μm) soaked with 2 % polyethylenimine, and washed with cold water (5 ml × 3). The washed filter papers were put into plastic vial containing scintillation cocktail, and their radioactivity were determined by β-counter (Beckman). Nonspecific binding was estimated using a 1,000-fold excess of unlabeled ryanodine. The specific binding (B) in each

concentration was the value measured by subtracting nonspecific binding from the total binding and the value of K_D and Bmax of ryanodine binding were determined by applying to the equation, $B = K_D \times B/F + Bmax$ (F = [^{3}H]ryanodine free concentration). Another binding experiments were carried out in the same way as described above after adding 16 nM [^{3}H]ryanodine, to evaluate the effect of Ca^{2+} (1 μM~1 mM), AMP (5 mM), tetracaine (1 mM), ruthenium red (10~100 μM) and $MgCl_2$ (0.1~5 mM) on [^{3}H] ryanodine binding.

Materials

[^{3}H]Ryanodine was purchased from Dupont-New England Nuclear, AMP and Protease inhibitors from Sigma. All other chemicals were of analytical grade. The significances of the results were calculated using Students' *t*-test.

RESULTS

Identification of RyR

SDS gel analysis of Chaps-solubilized, purified fraction of SR vesicles of chicken pectoral muscle was shown in Fig. 1. It was found that chicken pectoral SR vesicles had two separated HMW bands (lane 3) like eel SR vesicles (lane 5), differently from mammalian skeletal muscle (lane 2) and cardiac muscle (lane 4) having single HMW band, but interval between each band was narrower than eel's. When radioactivity of each of 16 fractions obtained after purification procedure with [^{3}H]ryanodine was measured, peak was appeared in fraction numbers from 3 to 5 (Fig. 2), and HMW protein band (not shown) was found in the fraction 3 to 5 after electrophoresis of each of 16 fractions.

[^{3}H]Ryanodine binding to SR vesicles

Ryanodine binding site and K_D value: [^{3}H] Ryanodine binding of chicken SR vesicles was increased in proportion to [^{3}H]ryanodine concentration, which is nearly saturated at 32 nM. Data were analyzed by Scatchard plot (Fig. 3). The maximum value of specific high affinity [^{3}H]ryanodine binding of chicken SR vesicles was 12.52 pmol per mg protein and the value of K_D was 14.53 nM (Table 1).

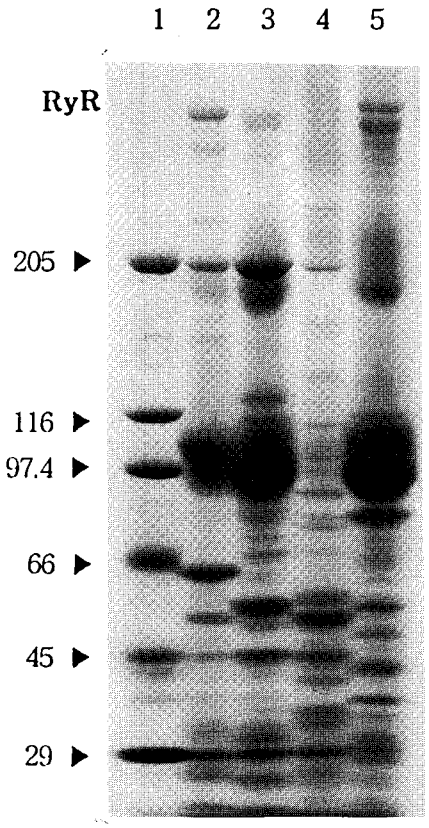

Fig. 1. SDS-gel of SR vesicles of rabbit, chicken skeletal, canine cardiac, and eel tail muscle. SDS-polyacrylamide gel (3~12%) electrophoresis of molecular weight standard (lane 1), rabbit (lane 2), chicken (lane 3), canine (lane 4), and eel (lane 5). Molecular weight values (x 10^{-3}) of standard proteins are indicated on the left. RyR = ryanodine receptor

Effect of calcium and AMP on the binding to chicken's SR vesicles: When free calcium concentration was elevated from 1 μM to 1 mM under the 16 nM [^{3}H]ryanodine, its binding to SR vesicles was increased from 0.193 pmol/mg protein to 3.945 pmol/mg protein. In case of elevation of free calcium more than 10 μM, the specific binding was increased significantly compare to that in 1 μM Ca^{2+} ($P<0.001$). [^{3}H]Ryanodine binding was nearly saturated when Ca^{2+} concentration was more than 50 μM. Above this concentration, there was no inhibitory effect on [^{3}H]ryanodine binding (Table 2).

When 5 mM AMP was added in the reaction mixture containing 100 μM Ca^{2+}, the amount of

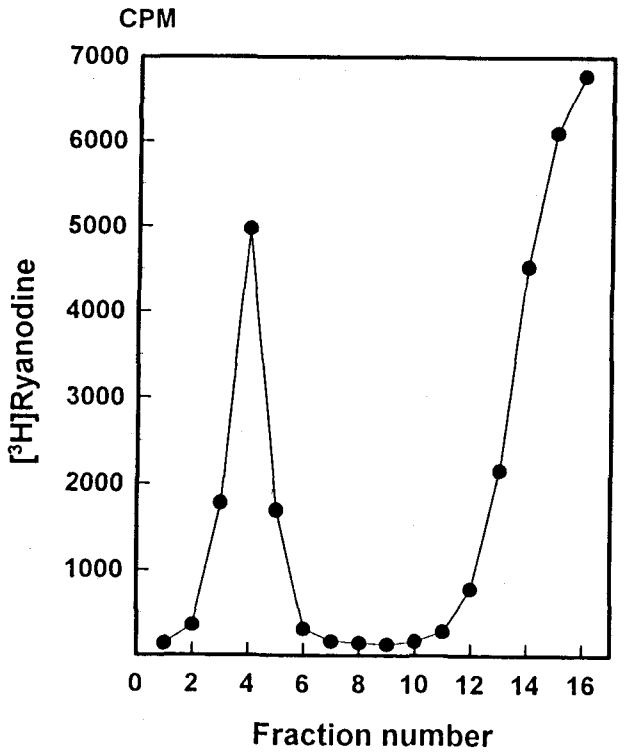

Fig. 2. Sedimentation profile of Chaps-solubilized, purified chicken ryanodine receptor. Chicken SR vesicles (2.5 mg of protein/ml) were solubilized with Chaps (1.5%) in a medium containing 1.0 M NaCl, 20 mM Na/Pipes, pH 7.4, 100 μM $CaCl_2$, 5 mM AMP, 5 mg/ml phosphatidylcholine, 100 μM dithiothreitol, 1 μM leupeptin, 200 μM PMSF, and 3 nM [^{3}H]ryanodine. The solubilized proteins were loaded onto a linear 7~20% sucrose gradient in the above medium containing 1% Chaps and centrifuged at 4°C in a Sorvall AH629 rotor for 16 hr at 26,000 rpm,. 16 fractions of 2 ml were collected and analyzed for [^{3}H]ryanodine radioactivity.

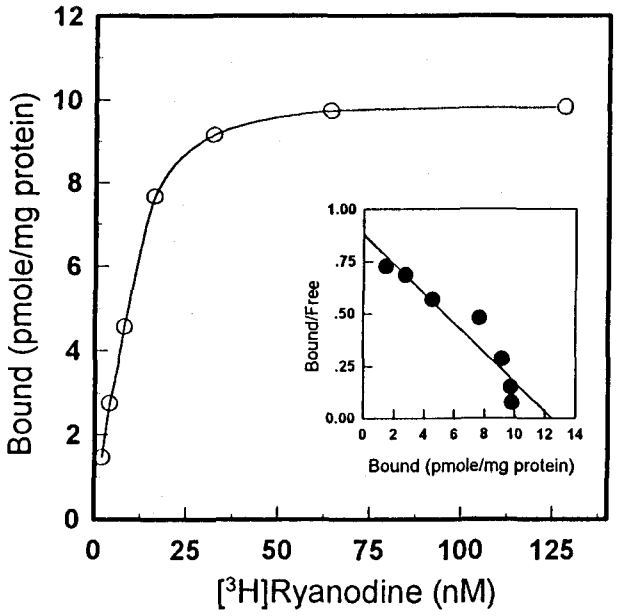

Fig. 3. Specific binding of [^{3}H]ryanodine to chicken's pectoral muscle SR vesicles. [^{3}H]ryanodine binding was carried out in the medium containing 1 M KCl, 20 mM K/Pipes, pH 7.4, 100 μM $CaCl_2$, 5 mM AMP, 200 μM PMSF, 100 μM dithiothreitol, 1 μM leupeptin and 2 - 128 nM [^{3}H]ryanodine. Nonspecific binding was assessed using a 1,000-fold excess of unlabeled ryanodine. *Inset ; Scatchard plot* of the saturation data

Table 1. Ryanodine binding sites and K_D values of SR vesicles of the chicken skeletal muscle

Bmax (pmol/mg protein)	K_D (nM)
12.52±0.96	14.53±1.44

Data (mean±SE) were obtained from Scatchard analysis of 6 binding experiments. [^{3}H]ryanodine binding to the SR vesicles of the chicken skeletal muscle was done for 2 h at 37°C in the solution containing 1 M KCl, 20 mM K/Pipes, pH 7.4, 100 μM $CaCl_2$, 5 mM AMP, 0.2 mM PMSF, 100 μM dithiothreitol, 1 μM leupeptin, and 2~128 nM [^{3}H]ryanodine. Nonspecific binding was assessed using a 1,000-fold excess of unlabeled ryanodine.

[^{3}H]ryanodine binding was slightly increased to 3.888 pmole/mg protein compared to that (3.793 pmol/mg protein) in the presence of calcium only (Table 3), but there was no statistical significance.

Effects of ruthenium red, $MgCl_2$ and tetracaine on the [^{3}H]ryanodine binding to chicken's SR vesicles: To evaluate the effect of ruthenium red on specific binding (3.888 pmole/mg protein) in the reaction mixture containing 100 μM Ca^{2+}, when ruthenium red was increased from 10 μM to 100 μM in the binding solution, [^{3}H]ryanodine binding was changed from 3.770 to 1.680 pmol/mg protein. Its effect was statistically significant ($P<0.001$), when ruthenium red is more than 20 μM (Table 4).

When $MgCl_2$ was increased from 0.1 mM to 2 mM, there was no effect on [^{3}H]ryanodine binding in

Table 2. Effect of calcium on the [^{3}H]ryanodine binding to chicken SR vesicles

Specific binding of [^{3}H]ryanodine in the presence of Ca^{2+}					
1 μM	10 μM	50 μM	100 μM	300 μM	1 mM
0.193±0.023	2.340±0.019*	3.785±0.058*	3.888±0.071*	3.935±0.079*	3.945±0.090* pmol/mg protein

[^{3}H]Ryanodine binding was carried out for 2 hr at 37 °C in the medium containing 1 M KCl, 20 mM K/Pipes, pH 7.0, 200 μM PMSF, 100 μM dithiothreitol, 1 μM leupeptin, 5 mM AMP and 16 nM [^{3}H]ryanodine in the various concentrations of $CaCl_2$. Data (mean±SE) were obtained from 6 binding experiments. Nonspecific binding was assessed using a 1000-fold excess of unlabeled ryanodine. * : Significantly different from the corresponding value of 1 μM Ca^{2+} ($P<0.001$).

Table 4. Effect of ruthenium red on the [^{3}H]ryanodine binding to chicken SR vesicles

Specific binding of [^{3}H]ryanodine in the presence of Ca^{2+}+ruthenium red				
100 μM	10 μM	20 μM	50 μM	100 μM
3.888±0.071	3.770±0.090	3.278±0.082*	2.310±0.012*	1.680±0.027* pmol/mg protein

[^{3}H]Ryanodine binding was carried out for 2 hr at 37 °C in the medium containing 1 M KCl, 20 mM K/Pipes, pH 7.0, 200 μM PMSF, 100 μM dithiothreitol, 1 μM leupeptin, 5 mM AMP and 16 nM [^{3}H]ryanodine in the presence of 100 μM $CaCl_2$. Nonspecific binding was assessed using a 1000-fold excess of unlabeled ryanodine. Data were obtained from 6 experiments. * : Significantly different from the corresponding value of 100 μM Ca^{2+} ($P<0.001$).

Table 3. Effect of AMP on the [^{3}H]ryanodine binding to chicken SR vesicles

In the absence of AMP	In the presence of AMP
3.793±0.085	3.888±0.071 pmol/mg protein

Data (mean±SE) were obtained from 6 binding experiments. [^{3}H]Ryanodine binding to the SR vesicles of the chicken skeletal muscle was done for 2 h at 37°C in the solution containing 1 M KCl, 20 mM K/Pipes, pH 7.4, 100 μM $CaCl_2$, 0.2 mM PMSF, 100 μM dithiothreitol, 1 μM leupeptin, and 16 nM [^{3}H]ryanodine. Nonspecific binding was assessed using a 1,000-fold excess of unlabeled ryanodine.

the reaction mixture containing 100 μM Ca^{2+}, but 5 mM $MgCl_2$ significantly inhibited [^{3}H]ryanodine binding ($P<0.05$) (Table 5).

When 1 mM tetracaine was added in the reaction mixture containing 100 μM Ca^{2+}, [^{3}H]ryanodine binding was inhibited significantly ($P<0.05$)(Table 6).

DISCUSSION

Since RyRs have been known as Ca-releasing channels of SR which increase the concentration of intracellular calcium, several studies (Ikemoto et al, 1985; Meissner et al, 1986; Rousseau et al, 1986; Meissner & Henderson, 1987) of general functional characteristics of Ca-channels in skeletal and cardiac

Table 5. Effect of $MgCl_2$ on the [^{3}H]ryanodine binding to chicken SR vesicles

Specific binding of [^{3}H]ryanodine in the presence of Ca^{2+} + $MgCl_2$					
100 μM	0.1 mM	0.5 mM	1 mM	2 mM	5 mM
3.888±0.071	3.990±0.058	3.893±0.083	3.878±0.092	3.820±0.070	3.517±0.017* pmole/mg protein

[^{3}H]Ryanodine binding was carried out for 2 hr at 37 °C in the medium containing 1 M KCl, 20 mM K/Pipes, pH 7.0, 200 μM PMSF, 100 μM dithiothreitol, 1 μM leupeptin, 5 mM AMP and 16 nM [^{3}H]ryanodine in the presence of 100 μM $CaCl_2$ and/or various $MgCl_2$. Nonspecific binding was assessed using a 1000-fold excess of unlabeled ryanodine. Data were obtained from 6 experiments. * : Significantly different from the corresponding value of 100 μM Ca^{2+} ($P<0.005$).

Table 6. Effect of tetracaine on the [^{3}H]ryanodine binding to chicken SR vesicles

In the presence of 100 μM Ca^{2+}	In the presence of 1 mM tetracaine
3.888±0.071	3.260±0.114* pmol/mg protein

Data (mean±SE) were obtained from 6 binding ex periments. [^{3}H]Ryanodine binding to the SR vesicles of the chicken skeletal muscle was done for 2 h at 37°C in the solution containing 1 M KCl, 20 mM K/Pipes, pH 7.4, 100 μM $CaCl_2$, 0.2 mM PMSF, 100 μM dithiothreitol, 1 μM leupeptin, and 16 nM [^{3}H]ryanodine. Nonspecific binding was assessed using a 1,000-fold excess of unlabeled ryanodine. Data were obtained from 6 experiments. *: Significantly different from the corresponding value of 100 μM Ca^{2+} ($P<0.05$).

muscle show that Ca-channels are activated by calcium and adenine nucleotides but inhibited by $MgCl_2$, ruthenium red or tetracaine. Addition to these functional studies, what made SR Ca-channel research profoundly was only after the ryanodine had been used as experimental tools for refinement of Ca-releasing channel. Ryanodine has been popularly known since it was used for Ca-channel studies by many researchers (Pessah et al, 1985; Sutko et al, 1985; Campbell et al, 1987; Lai et al, 1988). Ryanodine is neutral plant alkaloids which is isolated from *Ryania Speciosa* in South America, and has prominent pharmacologic action on muscular system (Jenden & Fairhust, 1969). Ryanodine has binary action (Meissner, 1986; Alderson & Feher, 1987; Lattanzio et al, 1987), in low concentration it opens Ca-channels whereas in high concentration (more than 10 μM) it closes Ca-channels.

Ryanodine binding to Ca-channel is influenced by Ca-releasing substances, whose actions depend on SR of various tissue type (Pessah et al, 1985; Seifert & Cassida, 1986; Michalak et al, 1988; Zimanyi & Pessah, 1991). For example, in contrast with RyR of cardiac muscle, ryanodine binding to skeletal receptors was optimal at 10~100 μM Ca^{2+}, was inhibited at Ca^{2+} concentrations >1 mM (Pessah et al, 1985; Michalak et al, 1988; Zimanyi & Pessah, 1991), and was effectively inhibited by millimolar Mg^{2+} (Pessah et al, 1985; Michalak et al, 1988). But in cardiac receptors, ryanodine binding was not inhibited at high Ca^{2+} concentration (Pessah et al, 1985; Seifert & Cassida, 1986; Michalak et al, 1988; Zimanyi & Pessah, 1991) as well as at millimolar Mg^{2+} (Pessah et al, 1985; Seifert & Cassida, 1986; Michalak et al, 1988). The reason why calcium and Mg^{2+} have different affinity by tissue type was not understood. But according to these findings and that birds have two separated bands of ryanodine binding protein (Airey et al, 1990; Olivares et al, 1991; Airey et al, 1993) in contrast with mammalia, we could think that ryanodine binding to birds receptors is different from that of mammalia.

The present study indicated that chicken's pectoral muscular SR vesicles have two HMW bands. Bmax and K_D of chicken ryanodine binding sites were 8

12.52 pmol/mg protein and 14.53 nM (Table 1) and were in the range of those of mammalian skeletal and cardiac muscular SR vesicles (4～25 pmol/mg protein and 4～200 nM)(Fleischer et al, 1985; Pessah et al, 1985; Lattanzio et al, 1987; Lai et al, 1988). When free calcium concentration was elevated from 1 μM to 1 mM in the reaction mixture containing 16 nM [^{3}H]ryanodine, the optimal calcium concentration of [^{3}H]ryanodine binding was at 50 μM to 1 mM (Table 2). Compare this result with that of mammalian skeletal (optimal range; 10～100 μM)(Pessah et al, 1985; Michalak et al, 1988; Zimanyi & Pessah, 1991) and cardiac muscle (optimal range; 10 μM～1 mM) (Pessah et al, 1985; Seifert & Cassida, 1986; Michalak et al, 1988; Zimanyi & Pessah, 1991), RyR of chicken SR was similar to that of cardiac muscle rather than skeletal muscle. In contrast with the reports of the effect of adenosine nucleotides on mammalian skeletal and cardiac SR vesicles by Michalak et al (1988) and Pessah et al (1985), AMP (5 mM) did not increase [^{3}H]ryanodine binding to chicken SR (Table 3). Ruthenium red (20～100 μM) effectively blocked the [^{3}H]ryanodine binding which was seen in mammalian skeletal (El-Hayek et al, 1993) and cardiac (Holmberg & William, 1990) SR vesicles. $MgCl_2$ do not inhibit ryanodine binding at 0.1 mM to 2 mM, but inhibited it at 5 mM (Table 5), which was also seen in mammalian cardiac SR vesicles (Pessah et al, 1985; Seifert & Cassida, 1986; Michalak et al, 1988; Zimanyi & Pessah, 1991).

Although the eel SR vesicles have two HMW bands (Seok et al, 1995), [^{3}H]ryanodine binding was not effectively inhibited by tetracaine and ruthenium red, but binding in chicken's pectoral SR vesicles was effectively inhibited by them (Table 4, Table 6). According to these findings, though we could not confirm in present study, we think that although some RyRs have two HMW bands, [^{3}H]ryanodine binding properties could be different from each other. By these data (interval between two bands after electrophoresis is wider in eel, *etc*), we could only guess that there are different aminoacid sequences in protein bands, therefore many other immunologic and molecular biologic studies must be performed to define these structures.

In conclusion, from the above results, it is suggested that chicken SR vesicles have the ryanodine binding sites to which ryanodine binding is almost maximal at 50～100 μM Ca^{2+}, is significantly inhibited by ruthenium red and tetracaine, and slightly inhibited by Mg^{2+}, but not inhibited by high Ca^{2+}.

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