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Characterization of Calcium Release Channel (Ryanodine Receptor) in Sarcoplasmic Reticulum of Crustacean Skeletal Muscle

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ABSTRACT

To characterize the SR Ca-release channel protein complex of crustacean, ^{45}Ca-release, [^{3}H]ryanodine binding, and immunoblot studies were carried out in the crayfish and/or lobster skeletal sarcoplasmic reticulum.

Bmax and affinity of crayfish SR to ryanodine were lower than those of lobster SR. AMP (5mM) increased [^{3}H]ryanodine binding significantly in both vesicles ($P<0.05$). Mg^{2+} (5mM) or tetracaine (1mM) inhibited [^{3}H]ryanodine binding significantly in both vesicles ($P<0.001$), but ruthenium red (10 μM) inhibited it moderately.

In SDS polyacrylamide gel electrophoretic analysis of crayfish SR vesicles, there was a high molecular weight band that showed similar mobility with Ca-release channel protein of lobster skeletal SR, but more rapid mobility (HMWBr) than that of rabbit skeletal SR (HMWBs).

Immunoblot analysis showed that polyclonal Ab to lobster skeletal SR Ca-release channel protein was react with HMWBr of crayfish skeletal SR, but not with that of HMWBs of rabbit skeletal SR.

^{45}Ca-release from crayfish skeletal SR vesicles was increased by the increase of extravesicular calcium from 1μM to 1mM. This Ca-release phenomenon was similar, but more sensitive in the low concentration of Ca^{2+}, compared to that from lobster SR vesicles. AMP (5mM) or caffeine (10mM) did not affect to ^{45}Ca-release. ^{45}Ca-release was inhibited slightly (3~8%) by Mg^{2+} (5mM) or tetracaine (1mM), and moderately (23%) by high concentration of ruthenium red (300 μM).

From the above results, it is suggested that SR Ca-release channel protein of crustacean has different properties from that of the rabbit, and similar properties between crayfish and lobster in functional and immunological aspects, but Ca-release via crayfish channel may be more sensitive to calcium.

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

Abbreviations: AMP, adenosine-5'monophosphate : HMWB, high molecular weight band : PPO, 2, 5-diphenyloxazole : POPOP, 1,4-bis-4-methyl-5-phenyl-2-oxazoyl-benzol ; DIFP, diisopropyl fluorophosphate; EGTA, ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; Chaps, 3-[3-cholamidopropyl) dimethylammonio]-1- propanesulfonate; PVDF, polyvinylidene difluoride; RyR, ryanodine receptor

INTRODUCTION

The specific mechanisms that transduct mem

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brane potential changes to the sarcoplasmic reticulum (SR) to cause Ca-release and contraction of cardiac and skeletal muscular cells (E-C Coupling) (Ebashi 1976; Fabiato 1983; Franzini-Amstrong 1970; Schneider 1981) are unclear. In the cardiac muscle, dihydropyridine receptor (DHP receptor) has been shown to function as voltage-dependent Ca-channel which mediates Ca-influx from extracellular fluid (Cannell *et al.*, 1987; Nabauer *et al.*, 1989). The rise in intracellular calcium concentration has been suggested to induce Ca-activated Ca-release (Fabiato 1981) from SR. But in the vertebrate skeletal muscle, depolarizing currents (action potential) in the sarcolemma is conducted to T-tubule in which the L-type Ca-channels (DHP receptors) play a role for voltage sensor (Rios and Pizarro, 1988). DHP receptors directly conduct currents to SR (Schneider 1981), and then calcium is released through SR calcium release channel (ryanodine receptor = RyR) from SR.

In contrast to the vertebrate skeletal muscle, crustacean skeletal muscle fails to contract when the cell membrane is depolarized in the absence of extracellular calcium (Reuben *et al.*, 1967). Some studies suggested that the calcium ion itself which entered into the muscle cell during membrane depolarization might be sufficient for contraction (Gainer 1968; Bezanilla *et al.*, 1973; Atwater *et al.*, 1974), but others suggested that, like in the vertebrate skeletal muscle, SR Ca-release in the crustacean skeletal muscle may contribute to the increase of intracellular calcium required for muscle contraction (Ashley and Lea, 1978; Lea and Ashley, 1989; Gyorke and Palade, 1991).

The mammalian skeletal and cardiac SR Ca-release channels have been purified as 30S protein complexes composed of four Mr 565,000 polypeptides (Fleischer and Inui, 1989; Lai and Meissner, 1989; Lai *et al.*, 1989) which show differences in amino acid composition (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990; Otsu *et al.*, 1990; Nakai *et al.*, 1990). Those channels were known to be high conductive and ryanodine sensitive, and regulated by Ca^{2+}, Mg^{2+}, and ATP (Meissner and Henderson, 1987; Meissner *et al.*, 1986).

Recently, Seok *et al.* (1992a, 1992b) reported that the Ca-release channel of lobster's tail muscle SR has functional and immunological properties distinct from that of the mammalian skeletal or cardiac SR. However, properties of skeletal muscle SR Ca-release channel in other crustacean remained to be fully defined. Therefore we prepared the tail muscle SR vesicles from crayfish living in different electrolytes environment for lobster, and carried out ^{45}Ca-release, [^{3}H]ryanodine binding, and immunoblot studies.

MATERIALS AND METHODS

Isolation of SR vesicles

Isolation of SR vesicles of heavy type was followed as described by Seok *et al.* (1992b). In brief, 5~10 g of crayfish tail muscle (lobster; ~40 g) was rapidly excised, minced, and homogenized with a polytron (Kinematica) for 90 sec (3×30 sec speed at a middle setting) in 7.5 volumes of a medium-1 (0.1 M NaCl, 20 mM K/Pipes, pH 6.8, 1 mM EDTA, 0.1 mM EGTA, 0.2 mM PMSF, 100 nM aprotinin, 1 μM leupeptin, and 1 mM benzamidine). The homogenates were centrifuged at 5,000 rpm (Sorvall GSA rotor) for 30 min, and the resulting supernatant was passed through 3 layers of cheesecloth, and the suspension was centrifuged at 18,000 rpm for 30 min in Sorvall T865 rotor. Pellet was suspended in 22 ml (lobster: 132 ml) of medium-2 (0.6 M KCl, 20 mM K/Pipes, pH 7.0, 100 μM EGTA, 75 μM $CaCl_2$, 0.2 mM PMSF, and 1 μM leupeptin), and the suspension was centrifuged at 34,000 rpm for 30 min in a T865 rotor. After resuspension of pellet in 22 ml of medium-2, the suspension was loaded onto 25% and 35% discontinuous sucrose gradients in medium-2, and centrifuged for 16 h at 31,000 rpm (T865 rotor). Fraction sedimenting on 35% sucrose layer was collected, and centrifuged at 34,000 rpm for 30 min. Pellet was resuspended in solution containing 0.3 M sucrose, 5 mM K/Pipes, pH 7.0, and stored at −75℃. All procedures were carried out at 4℃.

[^{3}H]Ryanodine binding

[^{3}H]Ryanodine binding was followed essentially as described by Lai *et al.* (1989). After vesicles (30 ~50 μg) were incubated for 8 h at 20℃ in medium-3 (1 M NaCl, 20 mM Na/Pipes, pH 7.4, 0.3 mM

$CaCl_2$, 5 mM AMP, 1 mM DIFP, 5 μM leupeptin, and 1~80 nM [^{3}H]ryanodine), diluted with 40 volumes of cold water, filterd 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, in which scintillation cocktail [containing 300 ml of triton-x-100, 5 g of PPO, and 0.5 g of POPOP/(1 L of toluene)] was added. Radioactivity was determined by liquid scintillation counter (Packard, Tri CARB 300C). Nonspecific binding was estimated using a 1,000-fold excess of unlabeled ryanodine. Bmax and K_D values were obtained by Scatchard analysis of specific binding data.

Isolation of Ca-release channel complex

Lobster Ca-release channel complex was isolated by rate density gradient centrifugation as described by Lai *et al.* (1988). Rabbit SR vesicles bounded with 1.5 nM [^{3}H]ryanodine and lobster SR vesicles (1~2 mg of protein/ml) were solubilized in a medium-4 (1 M NaCl, 20 mM Na/Pipes, pH 7.4, 100 μM EGTA, 200 μM $CaCl_2$, 5 mM AMP, 5 mg/ml phosphatidylcholine, 100 μM dithiothreitol, 1 mM DIFP, 1 μM leupeptin) containing 1.5% Chaps. The solubilized proteins were loaded onto a linear 5~20% sucrose gradient in medium-4 containing 1 % Chaps, centrifuged at 24,000rpm for 14h (AH650 rotor, Sorvall), and separated into 16 fractions from the bottom. The radioactivity of each fraction from rabbit SR vesicles was counted after treatment with scintillation cocktail to find peak [^{3}H]ryanodine bound fractions with liquid scintillation counter (Packard). From the basis of these peak fractions lobster's fractions were pooled, concentrated using a Centriprep 30 concentrator (Amicon), diluted to ~5% sucrose with a buffer (0.1 M NaCl, 0.1 % Chaps, 20 mM Na/Pipes, pH 7.4), and loaded onto 10 ~ 20 % linear sucrose gradient in medium-4 containing 1 % Chaps. After centrifugation at 24000rpm for 14 h, same fractions from lobster SR vesicles as the peak fractions of [^{3}H]ryanodine radioactivity from rabbit SR vesicles, were collected, pooled, concentrated, and stored at −70℃.

Production and purification of polyclonal Ab

Antibodies to skeletal muscle SR RyR were produced as described (Seok *et al.*, 1992). Emulsion of 0.5 ml of Freund's adjuvant (complete) and 0.5 ml of saline solution containing 50 ~ 100 μg of purified lobster RyR was injected into the rabbit intradermally or subcutaneously. After two more booster injections (incomplete adjuvant + RyR) with 4-wk intervals, rabbit sera were collected at 10 to 14 d. Antisera were purified as described by Lillie and Brown (1987) using cyanogen bromide-activated sepharose column. Purified rabbit antisera were stored at −70℃ before use.

SDS -gel electrophoresis and immunoblot assay

SDS-polyacrylamide gel electrophoresis was carried out in the Laemmli buffer system using 3 ~12 % linear gradient gels (Laemmli 1970). After electrophoresis of rabbit, lobster, and crayfish skeletal muscle SR vesicles, gels were stained using colloidal Coomassie staining method as described by Neuhoff *et al.* (1988). For immunoblot analysis, the separated protein bands on the gels were electrophoretically transferred in Tris-glycine buffer (25 mM Tris, 190 mM glycine, pH 8.2~8.5) onto Immobilon PVDF membrane (Millipore Co.) for 1 h at 400 mA and 20~24 h at 1 A. Transferred membrane was blocked with 5 % non-fat dried milk proteins for 1 h or more, washed with phosphate buffer saline (PBS) (150 mM NaCl, 1.85 mM Na_2HPO_4, pH 6.7, and incubated in PBS/Tween buffer containing 1 % non-fat dried milk with primary Ab for 3 h or more. After washing with PBS/Tween buffer, membrane was incubated with peroxidase-conjugated secondary Ab for 1 h, washed with PBS/Tween buffer, and developed using 3, 3'-diaminobenzidine and H_2O_2.

^{45}Ca-release

Crayfish SR vesicles (~3 mg) were preincubated for 30 min at 4℃ in the medium-5 containing 0.1 M KCl, 20 mM K/Pipes, pH 7.0, 1 μM leupeptin, 0.1 mm EGTA, and 0.1 mM $CaCl_2$, and centrifuged for 30 min at 34,000 rpm (T865 rotor, Sorvall). The pellet was resuspened in a small volume of medium-5 and incubated with 1 mM ^{45}Ca for 2 min at 20℃ by the addition of 5 μl of 2 mM ^{45}Ca in medium-5 to 5 μl of the vesicle suspension. After incubation, ^{45}Ca-release was initiated by diluting vesicles 100-fold (1 ml) into isoosmolar efflux or rinse medium, and stopped at 25 and 60

sec by filtration on a GA/6 filter (Gelman, 0.45 μ m pore size) of filtration apparatus (Hoefer Scientific instrument). The filter paper was washed with rinse medium containing 0.1 M KCl, 20 mM K/Pipes, pH 7.0, 2 mM EGTA, and 5 mM $MgCl_2$ (1 ml × 4), and placed into plastic vial to measure radioactivity. After treatment with scintillation cocktail, radioactivity remaining in the filtered vesicles was measured using liquid scintillation counter (Packard).

Extravesicular calcium was adjusted to various concentrations by Fabiato's computer program (Fabiato 1988). Ca-release stimulants (AMP, caffeine) or inhibitors (tetracaine, ruthenium red, $MgCl_2$) were added to efflux medium containing 0.1 M KCl, 20 mM K/Pipes, pH 7.0, and various free calcium concentrations.

Materials

^{45}Ca and [^{3}H]ryanodine (54.7 Ci/mmol) were purchased from Dupont-New England Nuclear, Chaps from Boeringer Mannheim, ruthenium red from Fluka, AMP and SDS-molecular weight markers from Sigma, and peroxidase-conjugated secondary Ab from Calbiochem. All other chemicals were of analytical grade.

RESULTS

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

Scatchard analysis (Fig. 1) of [^{3}H]ryanodine binding data to lobster and crayfish SR vesicles showed the presence of high affinity binding site with a Bmax of 11.6 and 5.38 pmol/mg protein and K_D of 4.6 and 7.66 nM, respectively (Table 1).

[^{3}H]Ryanodine binding to both vesicles was increased by the increase of free calcium from 1 μM to 0.3 mM, but less slightly in 1 mM free calcium than in 0.3 mM Ca. AMP (5 mM) increased the [^{3}H]ryanodine binding significantly in both vesicles ($P<0.05$). Caffeine (10 mM) did not increase the [^{3}H]ryanodine binding, but potentiated it when combined with AMP (5 mM) (Table 2).

$MgCl_2$ (5 mM) or tetracaine (1 mM) inhibited [^{3}H]ryanodine binding significantly in both vesicles ($P<0.001$), but ruthenium red (10 μM) inhibited it moderately (15~30 %) (Table 3).

Evaluation of isolated ryanodine receptor

For the purification of crustacean skeletal SR ryanodine receptor, rabbit (bound with [^{3}H]ryan-

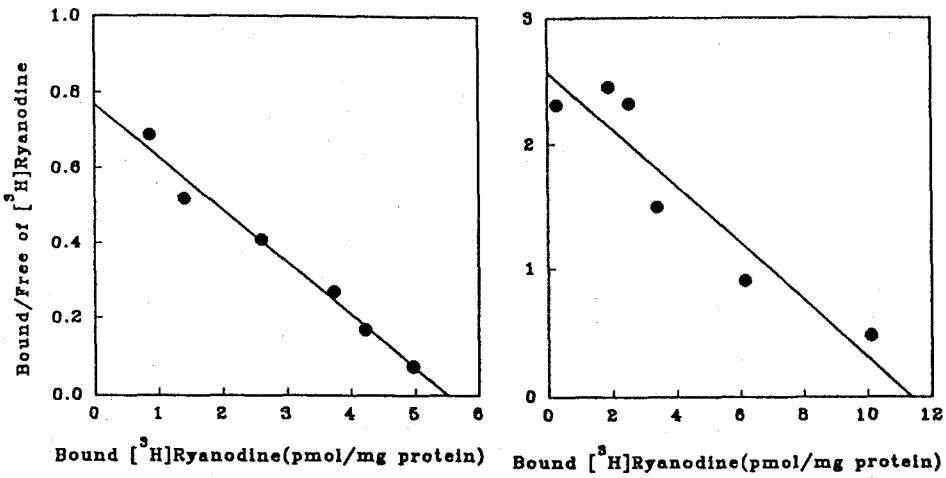

Fig. 1. Scatchard plot of [^{3}H]ryanodine binding to crayfish and lobster SR vesicles. Crayfish or lobster SR vesicles (30~50 μg) were incubated for 8 h at 20℃ in 1 M NaCl, 20 mM Na/Pipes, pH 7.4, 300 μM $CaCl_2$, 5 mM AMP, 100 μM dithiothreitol, 1 mM DIFP, 1 μM leupeptin, and 1~80 nM [^{3}H]ryanodine. Nonspecific binding was assessed using a 1,000-fold excess of unlabeled ryanodine. Specific binding data obtained from 3 duplicated experiments were analyzed by Scatchard analysis.

Table 1. Ryanodine binding sites and K_D values of crayfish and lobster SR vesicles

Bmax (pmol/mg protein)	K_D (nM)
Lobster	
11.60 ± 2.23	4.60 ± 1.21
Crayfish	
5.38 ± 1.52	7.66 ± 0.87

[^{3}H]Ryanodine binding to lobster or crayfish SR vesicles was done in the medium containing 1 M NaCl, 20 mM Na/Pipes, pH 7.4, 300 μM Ca^{2+}, 5 mM AMP, 1 mM DIFP, 100 μM dithiothreitol, 1 μM leupeptin, and 1-80 nM [^{3}H]ryanodine. Nonspecific binding was assessed using a 1,000-fold excess of unlabeled ryanodine.

Above data (mean ± SE) were obtained from Scatchard analysis of 3 binding data, respectively.

odine) and lobster SR vesicles were solubilized with 1.5 % Chaps, centrifuged after load on linear sucrose gradient, and separated into 16 fractions. The radioactivity of each fraction from rabbit SR vesicles indicated peak in $f_{3\sim5}$ (Fig. 2). SDS-gel electrophoresis of lobster fractions showed high molecular weight band (HMWB) in $f_{3\sim5}$ (Fig. 3).

From SDS-gel electrophoresis of SR vesicles (rabbit, lobster, and crayfish), it was found that crayfish SR vesicles had a similar high molecular weight band, but more rapid mobility (HMWBr) than the HMWBs of rabbit skeletal SR vesicles (Fig. 4).

Immunological cross-reaction

To evaluate the immunological cross-reaction

Table 2. Effect of some Ca-release stimulating agents on [^{3}H]ryanodine binding to lobster and crayfish SR vesicles

[^{3}H]Ryanodine binding (pmol/mg protein)					
Ca^{2+} (μM)			In the presence of 300 μM Ca^{2+}		
1	300	1000	AMP(5mM)	Caffeine(10mM)	AMP + Caffeine
Lobster					
2.87 ± 0.30	4.88 ± 0.38	3.66 ± 0.29	7.29 ±c 0.52*	4.88 ± 0.36	10.90 ± 0.85
Crayfish					
1.75 ± 0.19	2.41 ± 0.23	2.29 ± 0.21	4.75 ± 0.51*	2.35 ± 0.23	5.98 ± 0.33

[^{3}H]Ryanodine binding to lobster or crayfish SR vesicles was carried out in the binding medium containing 1 M NaCl, 20 mM Na/Pipes, pH 7.4, 1 mM DIFP, 100 μM dithiothreitol, 1 μM leupeptin, and 20 nM [^{3}H]ryanodine (8 hrs at 20℃). First, effect of extravesicular Ca^{2+} (1 μM, 300 μM, or 1 mM) on [^{3}H]ryanodine binding to SR vesicles was observed. Next, effect of AMP (5 mM) and/or caffeine (10 mM) in the presence of 300 μM Ca^{2+} was observed. Values [specific binding (mean ± SE)] were obtained from 4 experiments. *; Significantly different from [^{3}H]ryanodine binding data in the presence of 300 μM Ca^{2+} ($P<0.05$).

Table 3. Effect of some Ca-release inhibitors on [^{3}H]ryanodine binding to lobster and crayfish SR vesicles

[^{3}H]Ryanodine binding (pmol/mg protein)							
Ca^{2+}	Ca^{2+} + AMP	Mg^{2+}	Mg^{2+} + AMP	T	T + AMP	RR	RR + AMP
Lobster							
4.88 ± 0.37	7.29 ± 0.52	0.65 ± 0.05*[a]	0.84 ± 0.07*[b]	0.29 ± 0.03*[a]	0.76 ± 0.06*[b]	3.66 ± 0.38	6.19 ± 0.58
Crayfish							
2.41 ± 0.21	4.75 ± 0.01	0.45 ± 0.01*[a]	1.07 ± 0.18*[b]	0.11 ± 0.02*[a]	0.45 ± 0.05*[b]	1.97 ± 0.29	3.30 ± 0.62

[^{3}H]Ryanodine binding to lobster or crayfish SR vesicles was done in the binding medium containing 300 μM Ca^{2+} and/or 5 mM AMP, and some SR Ca-release channel inhibitors[5 mM $MgCl_2$, 1 mM tetracaine(T), or 10 μM ruthenium red=RR]. Data show specific [^{3}H]ryanodine binding (mean ± SE) obtained from 4 experiments. *: Singnificantly different from 300 μM Ca^{2+} effect(a) or 300 μM Ca^{2+}+5 mM AMP effect(b) on [^{3}H]ryanodine binding to lobster and crayfish SR vesicles, respectively ($P<0.001$).

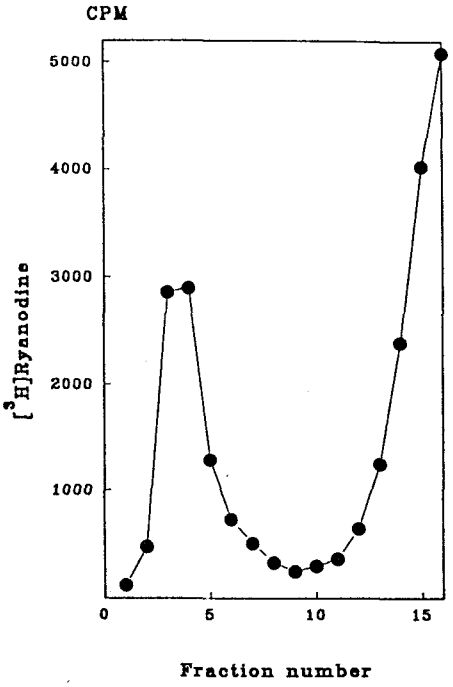

Fig. 2. Fractionation of Chaps-solubilized ryanodine receptor of rabbit vesicles. Rabbit SR vesicles (1.6 mg of protein/ml) were solubilized with Chaps (1.5%) in medium containing 1.0 M NaCl, 20 Na/Pipes, pH 7.4, 200 μM Ca^{2+}, 5 mM AMP, 5 mg/ml phosphatidylcholine, 100 μM dithiothreitol, 1 μM leupeptin, 1 mM DIFP, and 1.5 nM [^{3}H]ryanodine. The solubilized proteins were loaded onto a linear 5~20% sucrose gradient in the above medium containing 1% Chaps and centrifuged. Each fraction of about 0.30 ml was collected, and radioactivity of 16 fractions (50 μl each) were measured.

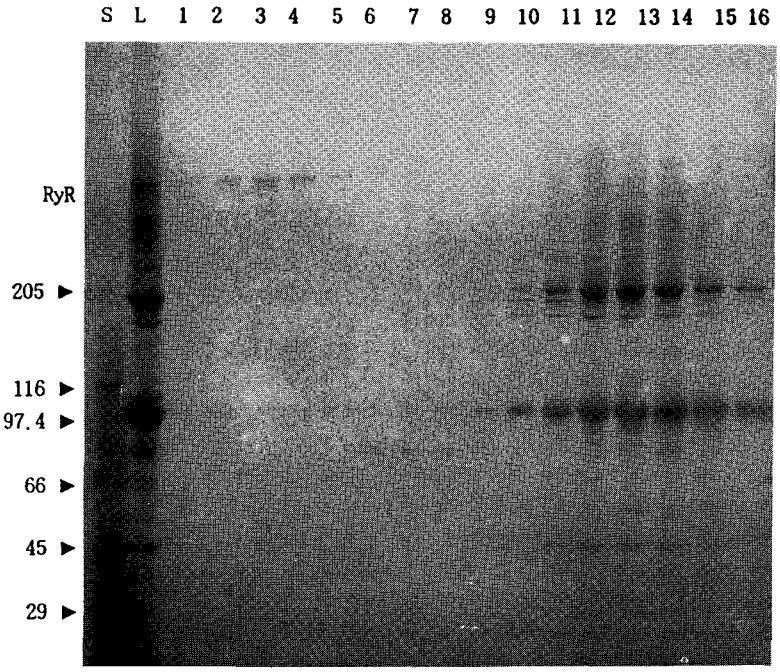

Fig. 3. SDS gel analysis of Chaps-solubilized, purified fraction of lobster SR vesicles. Chaps-solubilized fraction (1~16) from lobster SR vesicles were electrophoresed through a linear 3~12% SDS polyacrylamide gel. Molecular weight values ($\times 10^{-3}$) of standard proteins were indicated on the left. Solubilization of SR vesicles, centrifugation of solubilized protein, and fractionation were same as in Fig. 2. RyR=ryanodine receptor S = standard marker protein L=lobster SR vesicles

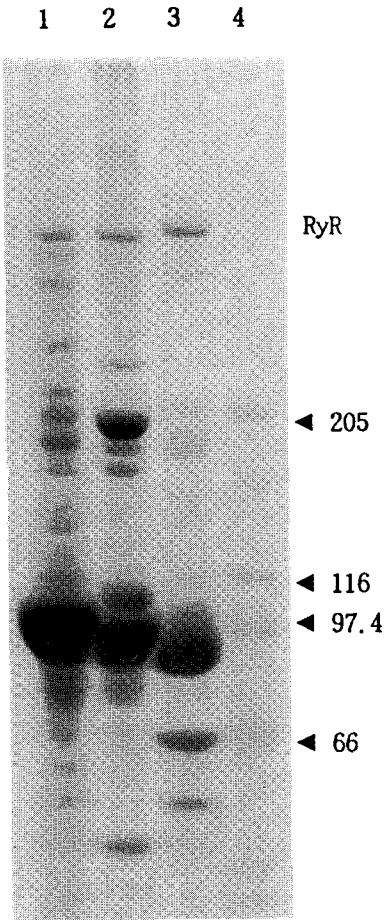

Fig. 4. SDS-gel of SR vesicles of rabbit, lobster, and crayfish skeletal muscle. SDS-polyacrylamide gel electrophoresis of crayfish (lane 1), lobster (lane 2), rabbit (lane 3) skeletal SR vesicles (~ 40 μg of protein each), and molecular weight standards (lane 4). Molecular weight values ($\times 10^{-3}$) of standard proteins are indicated on the right.

among some RyRs, polyclonal Ab to lobster SR ryanodine receptor (HMWBr) produced from rabbit was purified using CN-Br activated sepharose column, and react with protein band on PVDF membrane transferred from the electrophoresed SDS-gel of SR vesicles (rabbit, lobster, and crayfish). The polyclonal Ab react with HMWBr of crayfish SR vesicles, but not with HMWBs of rabbit SR vesicles (Fig. 5).

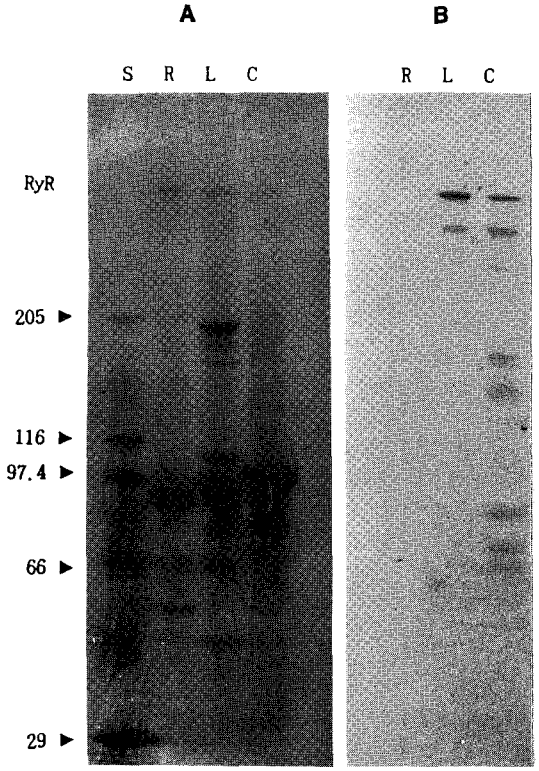

Fig. 5. Immunoblot analysis of ryanodine receptors. SR proteins (rabbit = R, lobster=L, crayfish= C) were electrophoresed through 3~12% SDS polyacrylamide gel and electrophoretically transferred onto immobilon PVDF membranes. Transfer membranes were either stained with purified lobster skeletal muscle RyR antisera (B). Molecular weight values ($\times 10^{-3}$) of standard proteins (S) are indicated on the left. RyR =ryanodine receptor.

^{45}Ca-release

Effects of Ca^{2+}, AMP, caffeine, Mg^{2+}, tetracaine or ruthenium red on the ^{45}Ca-release from crayfish skeletal SR vesicles were observed (Fig. 6~ 10).

The rate of ^{45}Ca-release (efflux rate) by rinse medium was 0.19 nmol/mg protein/sec, but it was increased by 1 μM, 10 μM, 0.1 mM, 0.3 mM, or 1 mM $CaCl_2$ to 0.52, 0.54, 0.85, 1.05 or 1.17 nmol/mg protein/sec, respectively (Fig. 6). This phenomenon was similar, but more sensitive in low concen-

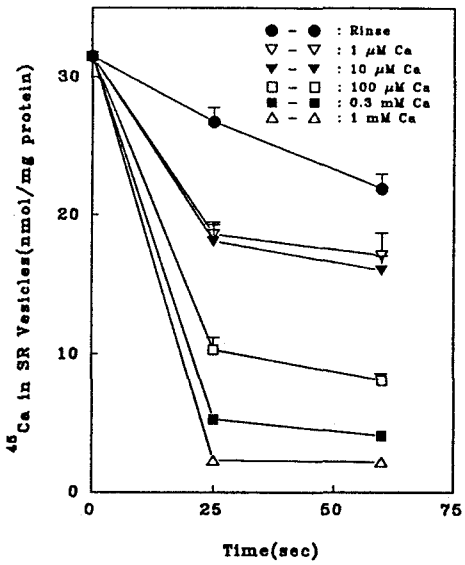

Fig. 6. Ca-induced ^{45}Ca release from crayfish SR vesicles. Crayfish vesicles were incubated for 2 min in 0.1 M KCl, 20 mM K/Pipes, pH 7.0, medium containing 1 mM ^{45}Ca and then diluted 100-fold into isoosmolar efflux medium[containing 0.1 M KCl, 20 mM K/Pipes, pH 7.0, and free Ca^{2+} (1 μM, 10 μM, 0.1 mM, 0.3 mM, or 1 mM)] and rinse solution (containing 0.1 M KCl, 20 mM K/Pipes, pH 7.0, 2 mM EGTA, and 5 mM $MgCl_2$).

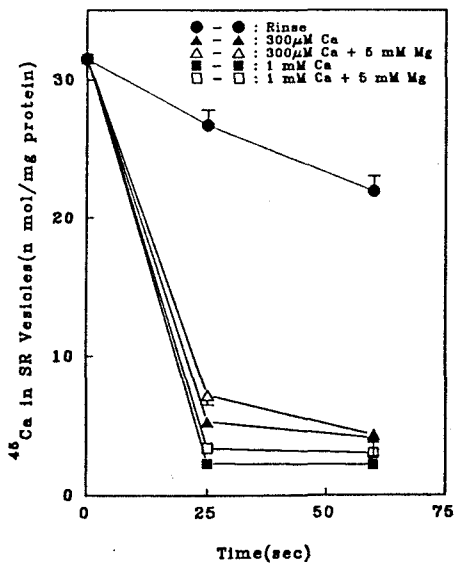

Fig. 8. Effect of Mg^{2+} on Ca-induced ^{45}Ca-release from crayfish SR vesicles. Vesicles were incubated in 0.1 M KCl, 20 mM K Pipes, pH 7.0, medium containing 1 mM ^{45}Ca. ^{45}Ca-release was done in rinse solution (containing 0.1 M KCl, 20 mM K/Pipes, pH 7.0, 2 mM EGTA, and 5 mM $MgCl_2$) and efflux medium[containing 0.1 M KCl, 20 mM K/Pipes, pH 7.0, and free Ca^{2+} (0.3 or 1 mM) with or without $MgCl_2$ (5 mM)].

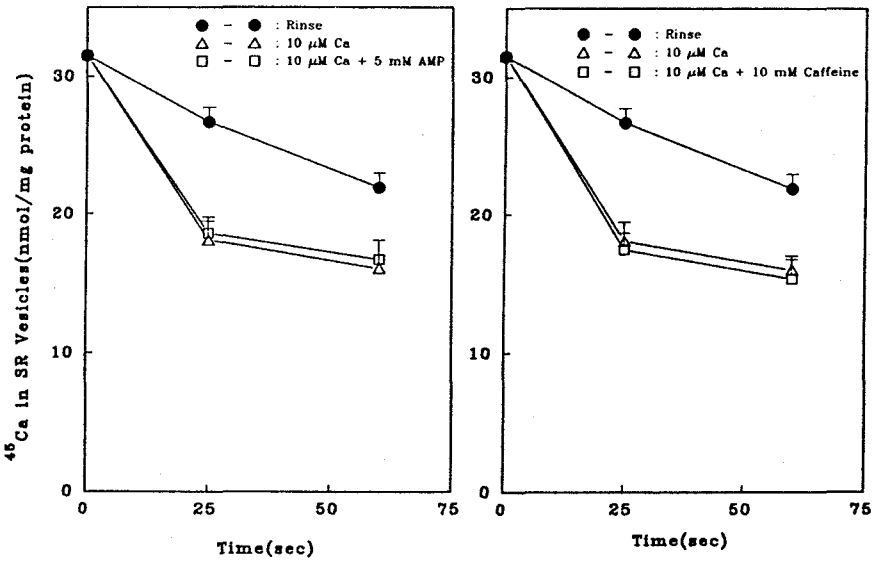

Fig. 7. Effect of AMP or caffeine on Ca-induced ^{45}Ca-release from crayfish SR vesicles. Efflux medium contained 0.1 M KCl, 20 mM K/Pipes, pH, 7.0, 10 μM $CaCl_2$, with or without AMP(5 mM) or caffeine (10 mM). Rinse contained 0.1 M KCl, 20 mM K/Pipes, pH, 7.0, 5 mM $MgCl_2$, and 2 mM EGTA.

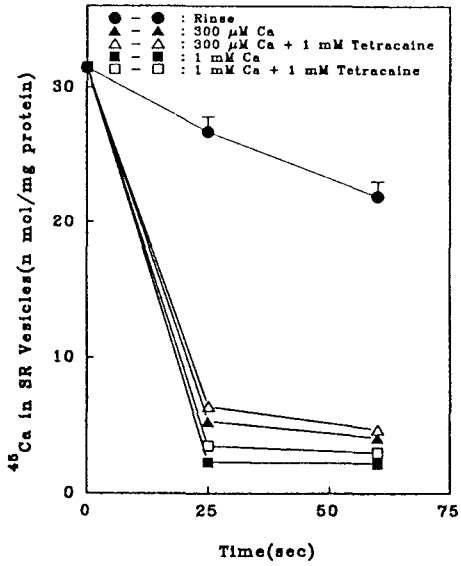

Fig. 9. Effect of tetracaine on Ca-induced ^{45}Ca-release from crayfish SR vesicles. Vesicles were incubated for 2 min in the medium containing 0.1 M KCl, 20 mM K/Pipes, pH 7.0, and 1 mM ^{45}Ca. ^{45}Ca-release were done in rinse solution (containing 0.1 M KCl, 20 mM K Pipes, pH 7.0, 2 mM EGTA and 5 mM Mg^{2+}) and efflux medium [containing 0.1 M KCl, 20 mM K/Pipes, pH 7.0, and free Ca^{2+} (0.3 or 1 mM) with or without tetracaine (1 mM)].

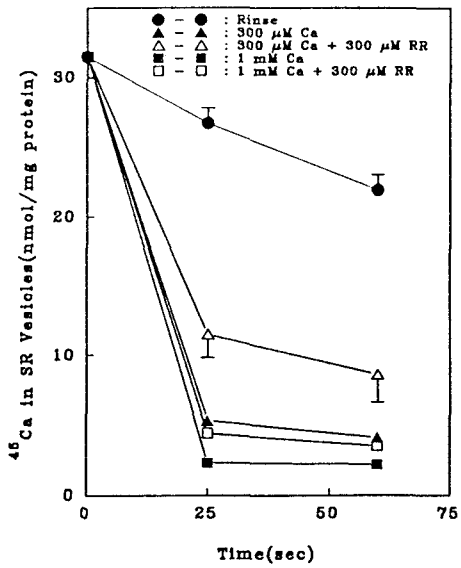

Fig. 10. Effect of ruthenium red on Ca-induced ^{45}Ca release from crayfish SR vesicles. Vesicles were incubated for 2 min in the medium containing 0.1 M KCl, 20 mM K/Pipes, pH 7.0, and 1 mM ^{45}Ca. ^{45}Ca-release was done in rinse solution (containing 0.1 M KCl, 20 mM K Pipes, pH 7.0, 2 mM EGTA, and 5 mM $MgCl_2$) and efflux medium [containing 0.1 M KCl, 20 mM K/Pipes, pH 7.0, and free Ca^{2+} (0.3 or 1 mM) with or without ruthenium red (300 μM)].

tration of $CaCl_2$, compared to that of the lobster SR vesicles (Seok *et al.*, 1992b). AMP (5 mM) or caffeine (10 mM) did not affect on the efflux rate by 10 μM $CaCl_2$ (Fig. 7). Effects of these SR Ca-release channel activator on crayfish SR vesicles were quite different from those of vertebrate SR vesicles (Meissner *et al.*, 1986).

Mg^{2+} (5 mM) inhibited slightly the efflux rate by 0.3 or 1 mM $CaCl_2$ from 1.05 or 1.17 nmol/mg protein/sec to 0.97 or 1.12 nmol/mg protein/sec, respectively (Fig. 8). Tetracaine (1 mM) also inhibited the efflux rate to similar extent as in Mg^{2+} (Fig. 9). High concentration of ruthenium red (0.3 mM) inhibited the efflux rate by 0.3 mM or 1 mM $CaCl_2$ from 1.05 or 1.17 nmol/mg protein/sec to 0.8 or 1.08 nmol/mg protein/sec, respectively (Fig. 10). These effects of inhibitors on the ^{45}Ca-release from crayfish SR vesicles were quite different from those of the vertebrate. In vertebrate skeletal SR, it has been known that ~mM Mg^{2+}, ~mM tetracaine, or ~μM ruthenium red significantly inhibit the calcium-release (Fleischer 1989; Meissner *et al.*, 1986).

DISCUSSION

Seok *et al.* (1992a, 1992b) reported that lobster skeletal muscle SR Ca-release channel has some different function from mammalian channel proteins, but it is still not clear in the various properties. So we observed ^{45}Ca-release, [^3H]ryanodine binding and immunological cross-reactivity in lobster and/or crayfish SR vesicles to find more detailed properties of crustacean calcium-release channel.

[^3H]Ryanodine binding site of crayfish SR vesi-

cles was high affinity binding site (with Bmax of 5.38 pmol/mg protein and K_D of 7.66 nM), but Bmax and K_D of crayfish SR vesicles were lower than those of lobster's (Table 1). When the fractions concomitantly obtained from purification steps of lobster RyR were analyzed electrophoretically, HMWBr was mainly appeared in $f_{3\sim5}$ in coincidence with the peak [^{3}H]ryanodine binding of rabbit SR vesicles (standard) (Fig. 3). In electrophoretical analysis high molecular weight band (HMWB) of crayfish skeletal vesicles was located in the similar position with lobster's HMWBr, but to slightly lower position than rabbit's HMWBs (Fig. 4). Therefore we could assume that crayfish SR vesicles also would have some different Ca-release channel in function from that in lobster or rabbit skeletal SR vesicles.

^{45}Ca-release of crayfish SR vesicles was increased by the increase of extravesicular calcium (Fig. 6). This pattern was similar with the Ca-release pattern in lobster SR vesicles reported by Seok *et al.*, (1992b), but ^{45}Ca-release from crayfish SR vesicles was more rapid in low calcium concentration (<1 mM) than that from lobster SR vesicles. In the vertebrate, ^{45}Ca-release through the Ca-release channel of skeletal SR vesicles was known to be peak at about 10 μM Ca^{2+} in extravesicles, and be inhibited by the increase of extravesiclular calcium (Meissner and Henderson, 1987; Meissner *et al.*, 1986). So we can assume that crustacean SR vesicles have only Ca-stimulatory site of low affinity without Ca-inhibitory site.

AMP and caffeine, which have been called to stimulating agents for SR Ca-release channel in vertebrates (Fleischer and Inui, 1989; Meissner and Henderson, 1987; Meissner *et al.*, 1986), did not activate ^{45}Ca-release in crayfish SR vesicles (Fig. 8). Mg^{2+}, tetracaine, or ruhtenium red just slightly inhibited the^{45}Ca-release in crayfish SR vesicles (Fig. 8~10), compared to the effect on the Ca-release from the vertebrate skeletal SR vesicles (Fleischer and Inui, 1989; Meissner *et al.*, 1986).

The effects of some agents (Ca^{2+}, AMP, caffeine, Mg^{2+}, or tetracaine), which have been known to stimulate or inhibit the Ca-release in vertebrate SR vesicles, on [^{3}H]ryanodine binding to crayfish or lobster SR vesicles were nearly similar (Table 2 and 3). [^{3}H]Ryanodine binding at the concentration of 0.3 mM or 1 mM Ca^{2+} was increased more than at the concentration of 1 μM Ca^{2+}, but prominent at 0.3 mM. AMP significantly increased [^{3}H] ryanodine binding to both tissues ($P<0.05$), and caffeine increased it only when combined with AMP (Table 2). Although Mg^{2+} or tetracaine slightly inhibited the ^{45}Ca-release (Fig. 8 and 9), they significantly inhibited the [^{3}H]ryanodine binding to SR vesicles of crayfish and lobster skeletal muscles ($P<0.001$) (Table 3).

In immunoblot assay polyclonal Ab to lobster's Ca-release channel protein did not react with rabbit's HMWBs, but react with crayfish's HMWBr (Fig. 5). This result suggests that crayfish HMWB is immunologically similar with that of lobster, but different from HMWBs of rabbit. However, polyclonal Ab also react faintly with some other protein bands, except the main HMWBr of lobster and crayfish. We considered this might be due to inappropriate purification of polyclonal Ab or degradation of ryanodine receptor by protease during immunoblot assay.

These results showed that crustacean SR Ca-release channel protein has functional and immunological properties distinct from the rabbit's, and crayfish and lobster SR Ca-release channel may have similar properties in functional and immunological aspects, but crayfish channel may be more sensitive to calcium.

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=국문초록=

갑각류 골격근의 Sarcoplasmic Reticulum에서 칼슘유리 Channel의 특성

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갑각류 골격근의 SR에서 칼슘유리 channel protein complex의 성격을 규명하기 위해 민물가재 및/또는 바다가재의 SR vesicles을 분리하여 ^{45}Ca 유리, [^{3}H]ryanodine 결합, 및 immunoblot 실험을 실시하여 다음과 같은 결과를 얻었다.

1. 민물가재 SR의 [^{3}H]ryanodine 결합 실험에서 민물가재 SR의 maximal binding site 및 affinity 모두 바다가재에서 보다 낮았으나, high affinity binding site이었다.
Extravesicles 칼슘농도를 증가시켰을 때 [^{3}H]ryanodine결합은 약간 증가되었으나, AMP나 AMP와 caffeine을 동시에 첨가하였을 때는 현저히 증가되었다($p<0.05$). 이런 증가 현상은 $MgCl_2$나 tetracaine으로 유의성 있게 억제되었으나($p<0.001$), ruthenium red에 의해서는 약간 억제되었다.

2. 민물가재 SR을 전기영동하였을 때 바다가재의 ryanodine receptor band (HMWBr)와 비슷하나 포유류의 것(HMWBs) 보다는 약간 빠른 mobility를 나타냈다.

3. 바다가재 HMWBr에 대한 polyclonal Ab를 이용한 민물가재, 바다가재 및 토끼 골격근의 칼슘유리 channel간의 면역학적 교차반응에서 민물가재와 바다가재의 칼슘유리 channel 간에는 교차반응이 있었으나, 포유류의 것과는 아무런 반응이 없었다.

4. 민물가재 SR에서 ^{45}Ca유리는 extravesicles의 칼슘농도 증가에 따라서 증가되었고, 낮은 외부 칼슘 농도에서 바다가재 보다 빠르게 일어났으나, AMP와 caffeine에 의해 영향을 받지 않았고, $MgCl_2$와 tetracaine으로 약간(3~8%) 그리고 고농도의 ruthenium red로 중등도(23%) 억제되었다.

이상의 실험성적으로 갑각류 칼슘유리 channel protein은 포유류의 것과는 기능적으로나 면역학적으로 매우 다른 특징을 가지고 있고, 민물가재와 바다가재 칼슘유리 channel은 서로 유사한 특징을 갖지만, 민물가재의 칼슘유리 channel이 바다가재의 것보다 외부칼슘에 예민한 기능을 갖는 것으로 사료된다.