J. Biochem. Mol. Biol. Vol. 28, No. 2 pp. 129~137 March 31, 1995

Reconstitution of Sarcoplasmic Reticulum-Ca^{2+} Release Channels into Phospholipid Vesicles: Investigation of Conditions for Functional Reconstitution

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(Received October 4, 1994)

Abstract: The ryanodine-receptor Ca^{2+} release channel protein in the sarcoplasmic reticulum membrane of rabbit skeletal muscle plays an important role in muscle exitation-contraction (E-C) coupling. Various types of detergents were tested, including Chaps, cholate, octylglucoside, Zwittergents, Mega-9, Lubrol PX, and Triton X-100 for solubilization of this protein. Among these, Chaps and Triton X-100 were found to optionally solubilize the channel complex. Optimum conditions for this solubilization were pH 7.4 with a salt concentration of 1 M. The addition of phospholipid in the solubilization step helped in stabilizing the protein. The purification of the receptor was performed using sucrose density gradient centrifugation. Various methods [dilution, freeze-thaw, adsorption (Biobeads), and dialysis] were investigated to incorporate the Chaps-solubilized and purified Ca^{2+} release channel protein into liposomes made from different types of phospholipids. Of these, a combined method consisting of a dialysis, freeze-thaw and sonication steps yielded the best results. Reconstituted vesicles produced by this method with 95% phosphatidylcholine (from soybean extract) had good function.

Key words: Ca^{2+} release channel, reconstitution, ryanodine receptor, sarcoplasmic reticulum.

The Ca^{2+} release channel protein of the sarcoplasmic reticulum (SR) membrane is an integral protein which is also known as the ryanodine receptor (RyR), due to its ability to bind ryanodine. Ryanodine is a kind of plant neutral alkaloid (Jenden and Fairhurst, 1969) with high affinity and specificity (Lai and Meissner, 1989). The SR Ca^{2+} release channel plays an important role in skeletal muscle excitation-contraction (E-C) coupling by releasing, in response to a muscle action potential, Ca^{2+} necessary to cause muscle contraction.

The channel complex consists of four subunits, each of which contains 5,037 amino acids (Numa *et al.*, 1989). To understand the structure and function of membrane bound proteins, particularly the proteins related to transport, one of the best approaches is the reconstitution procedure. This procedure refers to the reincorporation of detergent-solubilized and purified membrane proteins into natural and artificial membranes (Razin, 1972; Racker, 1979). Although there are many conditions which affect the reconstitution of a functional protein, and many methods in membrane reconstitution, these can be optimized through broad approaches. This means that the reconstitution approach is, in general, totally empirical.

In this study conditions for the functional reconstitution of Ca^{2+} release channel proteins purified from SR membranes in rabbit skeletal muscle are described. First, the parameters which result in optimum solubilization and purification of the channel, such as pH, temperature, salt-concentration, and the kind and concentration of detergent were examined. Second, the kinds of phospholipids and reconstitution techniques for the best functional reconstitution were investigated.

Materials and Methods

Materials

Diisopropyl fluorophosphate (DIFP), dithiothreitol (DTT), PIPES, leupeptin, iodoacetamide, aprotinin, phenylmethylsulfonylfluoride (PMSF), and pepstatin were purchased from Sigma Chemical Co. $^{45}Ca^{2+}$ and [^{3}H]ryanodine were obtained from ICN Pharmaceuticals and Du Pont-New England Nuclear, respectively. Ryanodine and detergents were obtained from Calbiochem, and 95% and 45% phosphatidylcholine (PC) (from soy bean), pure PC (from bovine heart), PE (from bovine heart), and PS (from bovine brain) were supplied by Avanti Polar

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Lipids Inc. (Alabaster, USA). All other chemicals used were of analytical reagent grade.

Preparation of sarcoplasmic reticulum (SR)

A heavy SR vesicle fraction enriched in [^{3}H]ryanodine binding and Ca^{2+} release activities was isolated from rabbit skeletal muscle in the presence of protease inhibitors (0.2 mM PMSF, 100 mM aprotinin, 1 μM pepstatin, 1 mM benzamidine, and 1 mM iodoacetamide) as described by Meissner (1984).

Solubilization of the Ca^{2+} release channel protein

Heavy SR vesicles were solubilized in a 20 mM NaPipes buffer solution containing 1 M NaCl, 100 μM EGTA, 200 μM Ca^{2+}, 5 mM AMP, 1 mM DTT, 1 mM DIFP, 1 μM leupeptin, 5 mg/ml of phospholipid and various types and concentrations of detergent. Phospholipids used in this study were 95% PC, 95% PC/45 % PC(5/1), 45% PC, pure PC, and pure PC/PE/PS (10/2/1). The same phospholipids were used in the purification and reconstitution steps. Detergents used were cholate, $C_{12}E_9$, octyl glucoside, Lubrol-PX, Triton X-100, and Zwittergent 3-12. Unsolubilized material was removed by centrifugation in a Beckman Ti 75 rotor at 30,000 rpm for 30 min. The supernatant was analyzed for [^{3}H]ryanodine binding activity and protein concentration.

Purification of the Ca^{2+} release channel protein

The Ca^{2+} release channel protein was purified, with modifications, as described by Lai *et al.* (1988). Ca^{2+} release channel protein solubilized in the presence and absence of [^{3}H]ryanodine was placed at the top of sucrose gradients. The gradients consisted of a solution of 5% sucrose (7 ml), and a linear 10 to 20% sucrose solution of two layers in the same volume. Both solutions contained 1 M NaCl, 100 μM EGTA, 200 μM Ca^{2+}, 5 mM AMP, 0.5 mM DTT, 0.5 mM DIFP, 0.5 μM leupeptin, 5 mg/ml of phospholipid [95% PC, 95% PC/45% PC (5/1), 45% PC, pure PC, pure PC/PE/PS (10/2/1)], and 20 mM NaPipes (pH 7.4). The 5% sucrose solution contained 1% detergent, whereas the linear gradient contained 0.5% detergent.

Gradients were centrifuged in a Beckman SW41 rotor at 38,000 rpm for 7.5 h, or a SW28 rotor at 26,000 rpm for 16 h. Fractions were collected from the bottom and assayed for radioactivity, protein concentration, and purity of the Ca^{2+} release channel protein by SDS-polyacrylamide gel electrophoresis.

Reconstitution of the Ca^{2+} release channel protein

To incorporate Ca^{2+} release channel protein contained in sucrose gradient fractions into liposomes, several methods were tried with them in itself and with the concentrated form of them (by Centri-Prep 30), and with and without extra addition of phospholipids. In the dialysis method, dialysis was performed for 1 day and 2 days at 4℃ in a 100-fold excess of buffer solution composed of 0.5 M NaCl, 0.1 mM EGTA, 1.1 mM Ca^{2+}, 0.5 mM DTT, 0.5 mM DIFP, 10 mM NaHepes (pH 7.4) with and without 0.3 M sucrose, and of 0.5 mM DTT, 0.5 mM DIFP, and 200 mM NaHepes (pH 7.4) with five changes. The dialysate contained proteoliposomes.

In the dilution method, samples were diluted quickly and slowly at 4℃ and 22℃ with 20 to 50-fold dilutions in a buffer solution composed of 0.5 M NaCl, 0.1 mM EGTA, 1.1 mM Ca^{2+}, 0.5 mM DTT, 0.5 mM DIFP, and 10 mM NaHepes (pH 7.4), and in a d-H_2O, then centrifuged in a Beckman Ti 75 rotor at 30,000 rpm for 30 min. The pellet containing proteoliposomes was resuspended.

In the adsorption method, Biobeads SM2, SM4, and SM16 adsorbent (200 mg/ml) was added directly to the samples in a small column and incubated for 3 h at 4℃ and 22℃ using a rocker, then centrifuged at 3,000 rpm for 10 min, exchanged with new beads, and incubated for another 1 h. Thereafter, the eluent containing proteoliposomes was separated from the mixture by centrifugation (3,000 rpm×10 min).

Other methods attempted for functional reconstitution were: [1. adsorption (by Biobeads)/freeze-thaw, 2. adsorption/freeze-thaw/sonication, 3. adsorption/freeze-thaw/dialysis, 4. adsorption/freeze-thaw/adsorption, 5. freeze-thaw/adsorption, 6. adsorption/freeze-thaw/dilution/sonication, 7. dilution/sonication, 8. adsorption/dilution, 9. adsorption/dilution/freeze-thaw, 10. dialysis/freeze-thaw, and 11. dialysis/freeze-thaw/sonication]. In these combination methods, freeze-thaw was done twice by quick freezing in a dry ice/acetone bath followed by a slow thaw at room temperature. Sonication was done three times for 20 sec at maximum power with a 30 second interval in a bath type sonicator (Laboratory Supplies Co.). Other conditions of adsorption, dilution, and dialysis were basically the same as above. In each method, if necessary, vesicles were concentrated by centrifugation in a Beckman Ti 75 rotor at 60,000 rpm for 2 h.

In the dialysis/freeze-thaw/sonication combination method (Lee *et al.* 1994), the sucrose gradient fractions containing the Ca^{2+} release channel protein were dialyzed for two days at 4℃ in a buffer solution composed of 0.5 M NaCl, 100 μM EGTA, 200 μM Ca^{2+}, 5 mM AMP, 0.5 mM DTT, 0.5 mM DIFP, and 10 mM NaPipes (pH 7.4) to remove the detergent and to form

the proteoliposomes. The dialysis buffer solution was changed after 2 h and, thereafter, every 8 h. After dialysis the dialysate was diluted with same volume of 100 μM EGTA, 200 μM Ca^{2+}, 0.5 mM DTT, 0.5 mM DIFP, and 10 mM NaPipes (pH 7.4), then centrifuged (Beckman Ti 75 rotor, 50,000 rpm, 2 h) to concentrate the vesicles. The pellets were resuspended in 10 mM NaPipes (pH 7.4) containing 0.25 M NaCl, 100 μM EGTA, 200 μM Ca^{2+}, and 0.5 mM DTT.

The resuspended proteoliposomes were frozen quickly in a dry-ice/acetone bath and thawed slowly at room temperature, then sonicated for 1 min at 0 to 2℃ using a bath-type sonicator. Control vesicles without the Ca^{2+} release channel protein were formed by the same procedures. The concentration and the function of the Ca^{2+} release channel protein were assessed by [^{3}H]ryanodine binding and Ca^{2+} efflux activities. Vesicles were separated according to their buoyant density by centrifugation on 5 to 20% sucrose linear gradients (Beckman SW41 rotor, 38,000 rpm, 7.5 h). Sucrose gradient fractions were analyzed for protein and phosphorus contents and Ca^{2+} efflux activity.

Ca^{2+} efflux assay

Ca^{2+} efflux from proteoliposomes was measured by the Millipore filtration method (Meissner G., 1984). Reconstituted vesicles were incubated for 2 h at room temperature in a medium of 0.25 M NaCl, 100 μM EGTA, 428 μM $^{45}Ca^{2+}$, and 10 mM NaPipes (pH 7.4). The incubation was started by the addition of $^{45}Ca^{2+}$. For observing the effects of ryanodine (100 nM and 200 μM) on Ca^{2+} release from vesicles, ryanodine was added after reconstituted vesicles were incubated for 30 min with $^{45}Ca^{2+}$. Vesicles were further incubated for 1.5 h. After 2 h of incubation with $^{45}Ca^{2+}$, $^{45}Ca^{2+}$ efflux from vesicles was started by diluting vesicles 100-fold in either an isotonic quench medium [0.25 M NaCl, 10 mM NaPipes (pH 7.0), 10 mM Mg^{2+}, 2 mM EGTA, 50 μM ruthenium red] or an isotonic release medium [0.25 M NaCl, 10 mM NaPipes (pH 7.0), 1 mM EGTA, 1.05 mM Ca^{2+}]. $^{45}Ca^{2+}$ released and not trapped by the vesicles was removed by placing the vesicle suspensions on a Millipore (0.22 μm, type GS) filter and washing with the quench medium. Maximum amounts of $^{45}Ca^{2+}$ trapped by the vesicles were estimated by incubating vesicles for 2 h with a low concentration (0.1 μg/ml) of A23187, a Ca^{2+} ionophore.

Ryanodine binding assay

High-affinity [^{3}H]ryanodine binding to reconstituted RyRs was measured as described by Meissner *et al.* (1989). Vesicles were incubated in media containing 5 mM AMP and ryanodine ([^{3}H]ryanodine+ryanodine, 100 nM and 200 μM) for 8 h at room temperature. One aliquot of sample was used to determine total [^{3}H] ryanodine. The remaining sample was diluted 20 times with ice-cold water and filtered through a Whatman GF/B filter previously soaked in a 5% polyethyleneimine solution. The filter was rinsed 3 times with ice-cold water (5 ml, each) and counted for [^{3}H]ryanodine bound to RyR (Ca^{2+} channel) in the vesicles by liquid scintillation counting.

SDS polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed using the Laemmli (1970) buffer system. Gels consisted of a 3% stacking gel and a 5 to 12% linear separating gel. Protein bands were identified with Coomassie blue or silver staining.

Other biochemical assays

Protein determination was done by the method of Kaplan and Pederson (1985) using a 0.45 μm Millipore Filter (type HA) and amido black. Phosphorus was determined by the method of Chen *et al.* (1956).

Results

Effect of detergent concentration on solubilization of RyR

Heavy SR vesicles at a concentration of 1.0 mg protein/ml were solubilized with various types of detergent [cholate, $C_{12}E_9$, octyl glucoside, Lubrol-PX, Triton X-100, Chaps, and Zwittergent 3-12 (Lai *et al.*, 1989)] in a 20 mM NaPipes buffer solution (pH 7.4) containing 1 M NaCl, 100 μM EGTA, 200 μM Ca^{2+}, 5 mM AMP, and 1 mM DIFP, and the detergents at concentrations ranging from 0.75% to 2%. Samples were centrifuged and solubilization of the ryanodine receptor was assessed by determining ryanodine binding in the supernatant fraction using 10 nM [^{3}H]ryanodine.

Two different methods were tried. First, SR vesicles were incubated with [^{3}H]ryanodine for 2 h at room temperature in the absence of the detergent. Then, the detergent was added and incubation continued for one more hour. Second, the detergent was added to the SR vesicles, followed by 1 h incubation at room temperature and one additional hour incubation after [^{3}H] ryanodine was added. The values of bound radioactivities in the supernatant between the two methods were similar. Hence, the former method was used for solubilization experiments (Fig. 1).

Chaps was the best solubilizer at a concentration of 1.5%. Triton X-100 was also effective at 1.0%, but, all other detergents did not efficiently solubilize RyR in the vesicles.

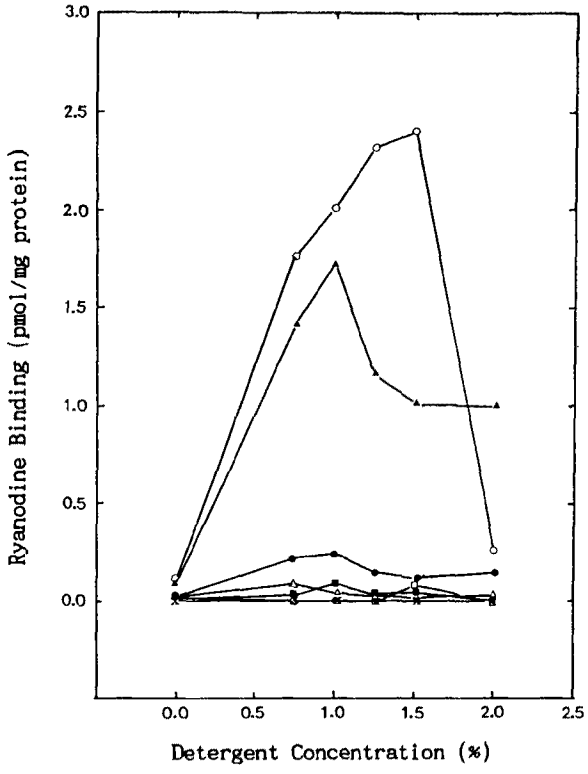

Fig. 1. Effect of various kinds of detergent on solubilization. Each sample was incubated with 10 nM [^{3}H]ryanodine for 2 h and incubated with each detergent for 1 h more at room temperature and then centrifuged. The radioactivity of [^{3}H]ryanodine in supernatant was measured by filtration assay. ○, Chaps; ▲, Triton X-100; ●, MEGA-9($C_{12}E_9$); △, cholate; ■, lubrol PX; □, octylglucoside; X, Zwittergent 3-12.

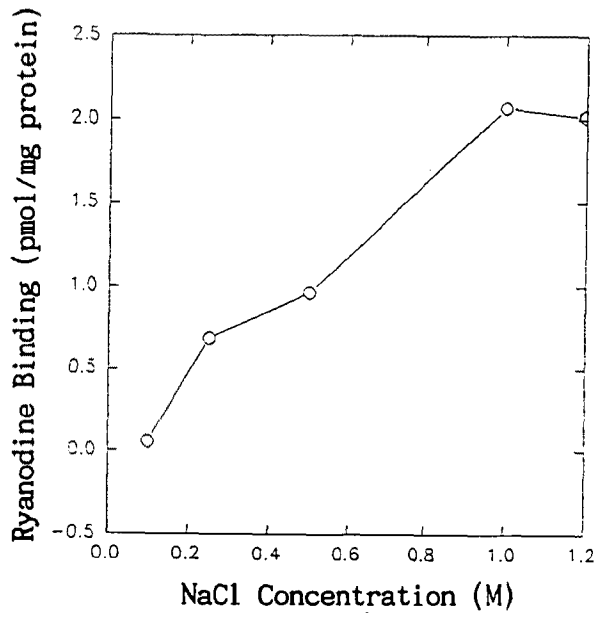

Fig. 2. Effect of NaCl concentration on solubilization. All conditions were the same as in Fig. 1. except for the concentration of NaCl.

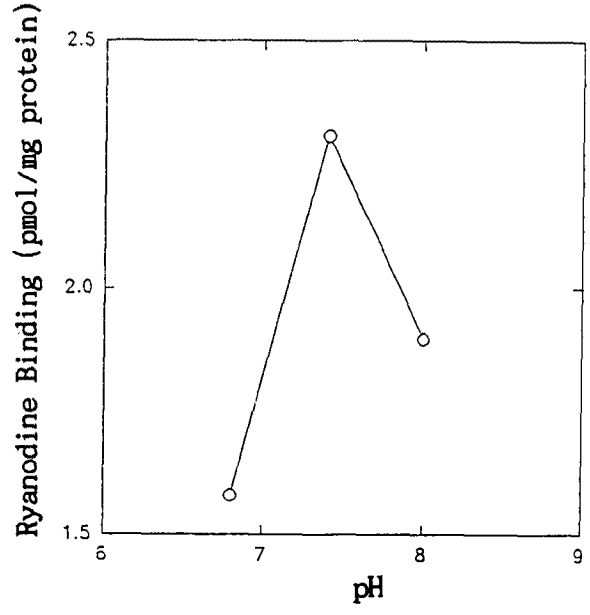

Fig. 3. Effect of pH change on solubilization. All conditions were the same as in Fig. 1. except for the pH change.

Effects of salt concentration, pH, and phospholipids on solubilization of RyR

The effect of the salt concentration on solubilization of the Ca^{2+} release channel protein was assessed using [NaCl] ranging from 0.1 to 1.2 M. Vesicles were solubilized in the presence of 1.5% Chaps. As shown in Fig. 2, 1 M NaCl was the most effective concentration. With 1.5% Chaps and 1 M NaCl the effect pH on solubilization was examined at pH 6.8, pH 7.4, and pH 8.0. Fig. 3 shows that [^{3}H]ryanodine binding activity is optionally recovered in the supernatant at pH 7.4.

Although detergents are most often used for solubilization of membrane proteins, one drawback of their use is that they often cause deterioration of the function of proteins as long as detergents remain bound to the protein. Hence, it is important to maintain the stability of the protein until detergents are removed after solubilization.

To determine whether phospholipids are an important factor (like pH, temperature, the kind and concentration of detergent) in protein stability, 5 mg/ml of phospholipid was added to the protein solution together with detergent. As shown in Table 1, the [^{3}H]ryanodine binding activity of the sample with phospholipid was 3 times higher than without phospholipids, suggesting that phospholipids stabilize the detergent-solubilized RyR. This increase in stability was observed regardless of the type of phospholipid used.

Table 1. Effect of phospholipid addition on solubilization. All conditions were the same as Fig. 1. except for with and without addition of 95% phosphatidylcholine (from soybean).

	1.5% Chaps	
	(−) PC	(+) PC
Ryanodine binding (pmol/mg protein)	0.622± 0.031	1.775± 0.053

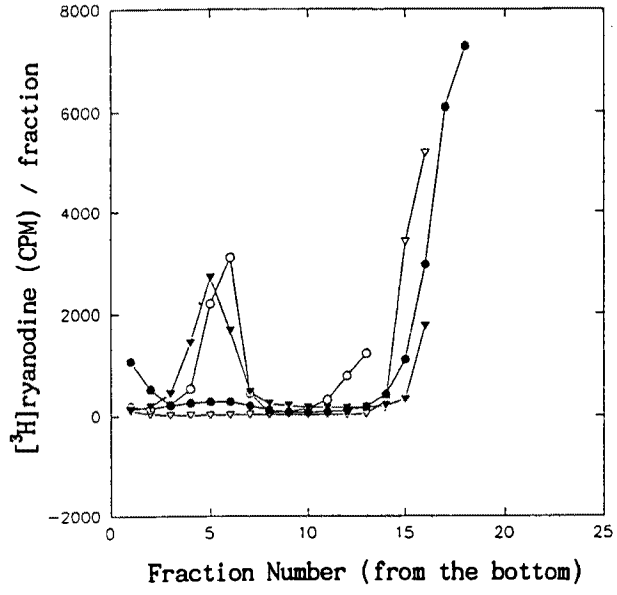

Fig. 4. Distribution of radioactivity of [^{3}H]ryanodine after sucrose gradient centrifugation of ryanodine receptor in the presence of various detergents. In these experiments, detergent used in solubilization was the same as one contained in the sucrose gradient solution. ○, Chaps; ●, MEGA-9; ▼, Triton X-100; ▽, octylglucoside.

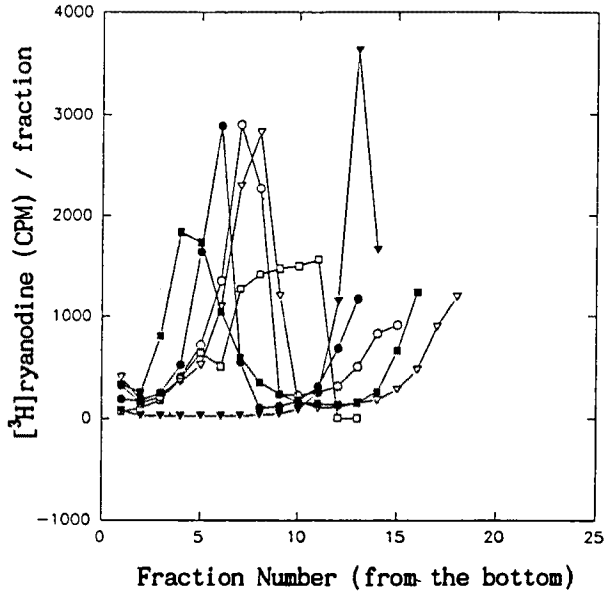

Fig. 5. Distribution of radioactivity of [^{3}H]ryanodine in sucrose gradient fractions. SR vesicles were solubilized with 1.5% Chaps and then centrifuged into 5~20% sucrose gradients containing 0.5% of the following detergents. ■, Triton X-100; ○, MEGA-9; ●, cholate; ▽, octylglucoside; □, Zwittergent 3-16; ▼, Zwittergent 3-12.

Effect of the kind of detergent on purification of RyR

To investigate whether the detergents which solubilize RyR can also be used for protein purification, heavy SR (final conc. 1 mg/ml) containing 1 M NaCl, 100 μM EGTA, 200 μM Ca^{2+}, 5 mM AMP, 0.1 M DTT, 1 mM DIFP, 20 mM NaPipes (pH 7.4), and 1.6 nM [^{3}H]-ryanodine was incubated for 2 h at room temperature. It was then solubilized for 1 h after addition of various detergents (1.5% Chaps, 1% $C_{12}E_9$, 1% octyl glucoside, and 1% Triton X-100) with 5 mg/ml of phospholipid and 0.1 mg/ml of BHT (butyrated hydroxytoluene), then centrifuged (Beckman Ti 75 rotor, 30,000 rpm, 30 min).

After centrifugation a portion of solubilized sample was saved for a ryanodine binding study, and the remaining sample was loaded on top of a 5 to 20% sucrose linear gradient buffer solution containing various 0.5% detergents (each detergent corresponded to the solubilization step) and 5 mg/ml of phospholipid, then centrifuged (Beckman SW28 rotor, 26,000 rpm, 16 h).

After centrifugation the [^{3}H]radioactivity of the gradient fractions was determined by liquid scintillation counting. In Fig. 4 the detergent used in the solubilization step was the same as in the purification step. In this case, Chaps (Lai *et al.*, 1988) and Triton X-100 produced a [^{3}H]ryanodine peak in the lower half of the gradient, suggesting the presence of [^{3}H]ryanodine labelled RyR. In the case of $C_{12}E_9$ and octyl glucoside purification of RyR using these detergents was impractical because there were no peak fractions. Radioactivity in the upper fractions of the gradient was caused by nonspecific binding and free [^{3}H]ryanodine.

The possibility that the detergent used in the solubilization step could be exchanged with a detergent in the purification step was investigated. Chaps (1.5%) was used for solubilization. The Chaps-solubilized sample was loaded on top of a 5 to 20% sucrose linear gradient buffer solution containing 5 mg/ml of phospholipid and 0.5% detergent (Triton X-100, Zwittergent 3-12, Zwittergent 3-16, $C_{12}E_9$, cholate, or octyl glucoside), then centrifuged. After centrifugation the radioactivity distribution in each fraction was determined (Fig. 5). All gradients, except those with Zwittergent 3-12 and 3-16, exhibited a radioactivity peak fraction. The radioactivity in the upper fractions of the gradient was due to nonspecific binding and free [^{3}H]ryanodine. This result suggested that the detergent used in the

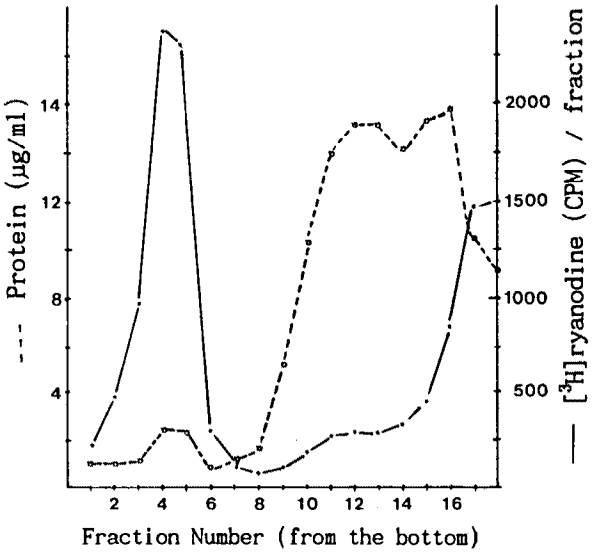

Fig. 6. Relationship between protein content and [^{3}H]ryanodine binding of gradient fractions after sucrose gradient centrifugation.

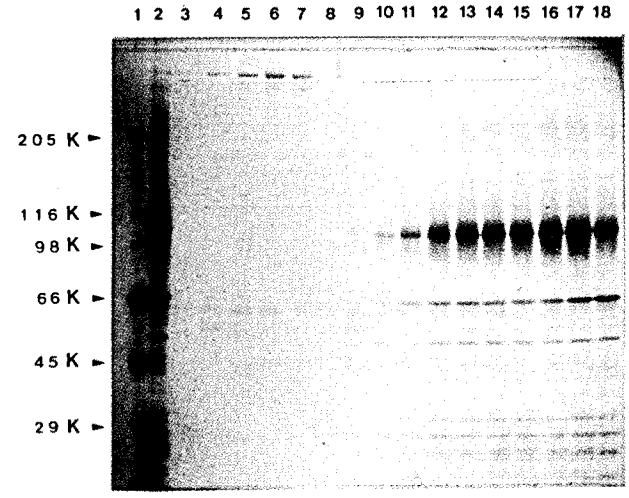

Fig. 7. SDS-PAGE analysis of gradient fractions after sucrose gradient centrifugation of Chaps-solubilized SR vesicles. Electrophoresis was done on a 5~12% linear gradient gel and the gel was siver-stained. Lane 1 : molecular standard; myosin (205 KDa), β-galactosidase (116 KDa), phosphorylase (97.4 KDa), Bovine serum albumin (66 KDa), ovalbumin (45 KDa), carbonic anhydrase (29 KDa); Lane 2 : heavy SR vesicles; Lane 3~18 : gradient fractions collected from the bottom after centrifugation.

solubilization step could be exchanged with the detergent in the purification step. This is sometimes important in regard to the reconstitution step, which depends on the ease of detergent removal.

To identify whether the gradient peak fractions contained the RyR/Ca^{2+} channel complex protein, the radioactivity distribution, the protein content, and the protein composition were determined by SDS-PAGE. Fig. 6 shows that comigration of [^{3}H]-ryanodine and the protein in the lower position (Fr. 2~Fr. 6) of the gradient fractions, and it also shows that the comigrated protein is coincident with the Ca^{2+} release channel protein (30S, ryanodine receptor) which appears with a high purity as a high molecular weight band on the gel (Fig. 7). The radioactivity of [^{3}H]ryanodine in the upper position of the gradient was due to nonspecific binding and free [^{3}H]ryanodine. These patterns of [^{3}H]ryanodine binding and bands on the gel using Triton X-100 were similar to those (Fig. 6 and 7) using Chaps.

Reconstitution of RyR

The gradient peak fractions containing 5 mg/ml of phospholipid and RyR solubilized and purified using Chaps and Triton X-100 were used in the next reconstitution step. Reconstituted vesicles were constructed by several methods of detergent removal [dilution, adsorption(Biobeads SM2, SM4, and SM16), and dialysis]. The protein content in vesicles and the $^{45}Ca^{2+}$ flux from vesicles were measured to assess functional reconstitution.

These methods did not make functionally good vesicles. Problems involved protein loss, lack of reproducibility, and $^{45}Ca^{2+}$ flux without pharmacological effect. Vesicles from these dilution methods lacked $^{45}Ca^{2+}$ release, probably due to denaturation of the Ca^{2+} release channel protein because there was almost no [^{3}H]ryanodine binding. Vesicles from the adsorption method did not show reproducibility and suffered protein loss. Biobeads probably absorbed protein, as well as detergent.

Vesicles from the dialysis method lacked reproducibility in $^{45}Ca^{2+}$ flux measurements and in the pharmacological effects of ryanodine where a high concentration (μM) of ryanodine inhibits the Ca^{2+} channel, and a low concentration (nM) activates the channel (Meissner, 1986). The following combination methods were tested: 1. adsorption (by Biobeads)/freeze-thaw, 2. adsorption/freeze-thaw/sonication, 3. adsorption/freeze-thaw/dialysis, 4. adsorption/freeze-thaw/adsorption, 5. freezethaw/adsorption, 6. adsorption/freeze-thaw/dilution/sonication, 7. dilution/sonication, 8. adsorption/dilution, 9. adsorption/dilution/freeze-thaw, 10. dialysis/freeze-thaw, and 11. dialysis/freeze-thaw/sonication. Reconstituted vesicles made from any of these methods, except dialysis/freeze-thaw/sonication (Lee *et al.*, 1994) were not functionally good vesicles, lacking reproducibility in $^{45}Ca^{2+}$ release measurements and the pharmacological effects of ryanodine on the Ca^{2+} release channel. Vesicles made from 95% PC by the dialysis/freeze-thaw/brief sonication method showed good Ca^{2+} release and good ryanodine-sensitivity, but vesicles from pure PC did not (Table 2).

Table 2. $^{45}Ca^{2+}$ efflux from 95% PC-vesicles and pure PC-vesicles in the presence of and the absence of ryanodine after reconstitution with (+) and without (−) ryanodine receptor (RyR) by dialysis/freeze-thaw/sonication method. Total indicates maximal trapped $^{45}Ca^{2+}$. Data are represented as nmol Ca^{2+} released/0.1 mg protein. Standard deviations were below± 0.3 with RyR-containing vesicles and below± 0.1 with control vesicles. (nmol/0.1 mg protein)

		Rinse			Release			Total
		[ryanodine]			[ryanodine]			
		0	100 nM	200 μM	0	100 nM	200 μM	
95% PC-vesicles	After reconstitution							
	+RyR	16.8	13.8	17.7	7.1	7.1	15.4	194.7
	control (−RyR)	5.0	5.1	6.3	3.0	4.6	5.1	163.9
	After 2nd Gr. centrifugation Fr.4 (Gr.peak)							
	+RyR	7.3		8.4	2.6		7.0	120.7
	control (−RyR)	1.0		1.1	1.0		1.1	98.4
Pure PC-vesicles	After reconstitution							
	+RyR	54.8	51.4	51.4	49.1	47.0	48.8	175.5
	control (−RyR)	13.4	12.7	13.1	11.3	10.4	11.3	146.7
	After 2nd Gr. centrifugation Fr.5 (Gr.peak)							
	+RyR	36.1		36.9	34.2		34.5	130.8
	control (−RyR)	6.5		6.5	5.6		5.8	144.4

Effect of different phospholipids on functional reconstitution

The chaps-solubilized and purified RyR/Ca^{2+} channel protein (mixture of gradient peak fractions No. 3, No. 4, and No. 5 in Fig. 7) was incorporated into liposomes made separately from 95% PC and pure PC by the dialysis/freeze-thaw/brief sonication method. A certain portion of these reconstituted vesicles was used for comparison of the degree of $^{45}Ca^{2+}$ flux (Table 2). The remaining sample was fractionated by size using 5 to 20% sucrose density linear gradient centrifugation. The protein and phospholipid contents in each fraction, and the $^{45}Ca^{2+}$ release amounts of protein rich gradient fractions containing proteoliposomes were determined and compared (Fig. 8 and Table 2). As shown in Fig. 8, the patterns for distribution of phospholipid content and protein content in each gradient fraction containing 95% PC vesicles corresponded with each other, but not with the pattern for pure PC vesicles. The disagreement observed for pure PC vesicles indicates that RyR/Ca^{2+} channel proteins were not inserted well into the vesicles. Also, control vesicles made without RyR were good for Ca^{2+} flux measurements because they gave similar patterns for phospholipid distribution as the RyR-containing vesicles.

$^{45}Ca^{2+}$ efflux data illustrated in Table 2 shows that 95% PC vesicles released more than half of their initial $^{45}Ca^{2+}$ contents through channel(s) within 25 seconds under normal conditions (minus ryanodine), whereas pure PC vesicles released only a small fraction (approximately 10%) of their $^{45}Ca^{2+}$ content. The result obtained with pure PC vesicles shows that either fewer Ca^{2+} channels were incorporated into vesicles or, that not all incorporated channels funtioned properly.

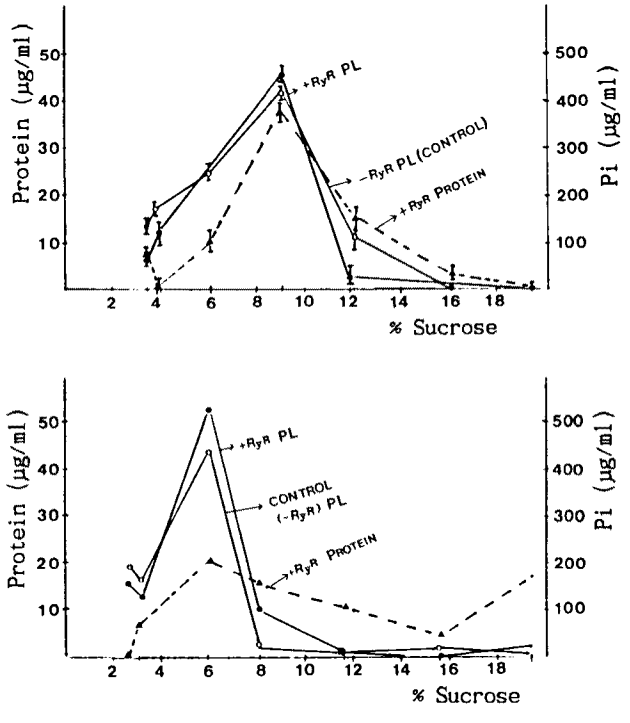

Fig. 8. Distribution of protein and phospholipid concentration in sucrose gradient fractions containing 95% PC- and pure PC-vesicles after 2nd gradient centrifugation of reconstituted vesicles. % sucrose was determined with the use of a refractometer. A: 95% PC vesicles, B: Pure PC vesicles.

The Ca^{2+} channel protein in SR vesicles is activated

Table 3. Ca^{2+} release from reconstituted vesicles exchanged with cholate and deoxycholate in the purification step after Chaps-solubilization of ryanodine receptor. Total indicates maximal trapped $^{45}Ca^{2+}$. Data are represented as nmol Ca^{2+} released/0.1 mg protein. Standard deviations were below± 0.3. (nmol/0.1 mg protein)

	Rinse			Release			Total
	[ryanodine]			[ryanodine]			
	0	100 nM	200 μM	0	100 nM	200 μM	
Reconstituted vesicles from cholate	3.6	4.1	4.6	3.0	3.6	5.1	83.1
Reconstituted vesicles from deoxycholate	13.4	13.5	14.5	11.7	11.2	12.1	98.6

by low concentrations (nM) of ryanodine and inhibited by high concentrations (μM) of ryanodine (Meissner, 1986). The data shown in Table 2 reflects these effects, at two different concentrations of ryanodine (100 nM and 200 μM), on Ca^{2+} channels in 95% PC vesicles. However, the effects are not seen in the case of pure PC vesicles. Table 2 also shows a similar inhibitory effect of a high ryanodine concentration (200 μM) on the Ca^{2+} channel function of vesicles in a protein peak gradient fraction (fraction 4 from the bottom, Fig. 8) after 95% PC vesicles were fractionated in a 5 to 20% sucrose linear gradient.

In the case of pure PC vesicles (fraction 5 from the bottom, Fig. 8), no significant Ca^{2+} channel activity was observed. In the case of control vesicles without RyR/Ca^{2+} channels, there seemed to be some small Ca^{2+} movement from 95% PC and pure PC vesicles under normal conditions (minus ryanodine). However, there was no effect due to the two different ryanodine concentrations, and, after sucrose gradient centrifugation, there was no Ca^{2+} release from 95% PC control vesicles (phospholipid peak gradient fraction). There was also almost no release from pure PC control vesicles, and there was no ryanodine effect in either sample.

It appears that functional reconstitution can only be achieved with vesicles where the RyR/Ca^{2+} release channel complex protein is incorporated into 95% PC vesicles made by the dialysis, freeze-thaw, and brief sonication method.

Effects of cholate and deoxycholate on functional reconstitution

Cholate and deoxycholate are often used for membrane solubilization because of their ease of removal by dialysis. Therefore, with reference to the results illustrated in Fig. 5, reconstituted vesicles were made by the same method as described above except that the Chaps-solubilized Ca^{2+} channel protein was sedimented through a sucrose gradient containing either cholate (0.5%) or deoxycholate (0.3%).

Following reconstitution by the dialysis, freeze-thaw, and brief sonication method, $^{45}Ca^{2+}$ release from these vesicles was measured (Table 3). Results showed that $^{45}Ca^{2+}$ release through the Ca^{2+} channel protein from both vesicles was low under normal conditions (minus ryanodine), and that the effect of the ryanodine concentration on the Ca^{2+} channel protein in both vesicles was not as clear as that in vesicles reconstituted from Chaps containing gradient fractions. Only the Chaps-solubilized sample resulted in functionally good vesicles with reproducibility of results (Table 2). In addition to these detergents, Triton X-100 was good for solubilization and purification, but was not dializable by this method. Hence, reconstituted vesicles from Triton X-100 did not give good results.

Discussion

Membrane protein purification and reconstitution represent an important step in the determination of the structure and function of membrane proteins, in particular, proteins that transport ions and other molecules, such as ion channels and transporters. In this study three steps were considered : SR membrane solubilization, RyR purification, and RyR reconstitution into phospholipid vesicles. In these steps there are many factors which could affect the purification and reconstitution of a functional RyR. Hence, it is important to determine the best conditions for functional reconstitution.

In the solubilization step it was necessary to choose a detergent which solubilized the Ca^{2+} channel protein without irreversible loss of function. In general, the selection of a detergent is largely empirical. Therefore, to choose a good solubilizer for the RyR/Ca^{2+} release channel complex protein in SR of skeletal muscle, some characteristic detergents (structural analogy, high CMC, ionic and nonionic) were examined (Fig. 1). Chaps and Triton X-100 were good solubilizers, but Chaps was selected because Triton X-100 did not result in good Ca^{2+} release from vesicles after reconstitution using the dialysis, freeze-thaw, and brief sonication techniques. A likely reason for this is the difficulty of Triton X-100 removal by dialysis owing to its low CMC, though it has an advantage as a nonionic detergent

causing little loss of [^{3}H]ryanodine binding. Although the utility of Triton X-100 in other reconstitution techniques has been demonstrated by its removal using Biobeads (SM2, SM4 and SM16, produced by Bio-Rad) (Holloway, 1973), Triton X-100 was unacceptable because Ca^{2+} release from vesicles made by the adsorption method did not exhibit reproducible results. Furthermore, adsorption using Biobeads, especially SM2, caused a distinct loss of protein when new Biobeads were repeatedly treated to remove Triton X-100 (data not shown).

The results shown in Figs. 2 and 3 show that a pH value of 7.4 and 1 M NaCl yielded optimum results. This agrees with the results of membrane protein investigations. The result that phospholipid can serve as a stabilizer for detergent-solubilized Ca^{2+} release channel protein agrees with Inui *et al.* (1987).

In the purification step detergent-exchange was attempted because Chaps-solubilized RyR transferred into buffers containing Triton X-100, Lubrol PX, MEGA-9, cholate, digitonin, or octylglucoside can maintain a high affinity of ryanodine binding (Lai *et al.*, 1989). The detergent used for solubilization of RyR could be replaced by another detergent, using the sucrose gradient centrifugation procedure. This observation greatly facilitates purification and reconstitution of the channel by allowing the use of conditions which are optimum for both steps. However, the utility of Triton X-100, cholate, and deoxycholate in the detergent-exchange method is excluded due to the difficulty of detergent removal by, at least, the dialysis, freeze-thaw, and brief sonication method (Table 3).

In the reconstitution step there are several techniques, such as adsorption, dilution, dialysis, freeze-thaw, sonication, and their combined use to make proteoliposomes. There are many factors to be decided in each method. Among them, the type of phospholipid is also important because phospholipids around membrane proteins in intact membranes generally affect their function. Hence, reconstitution was attempted with different phospholipids, such as 45% PC, 95% PC/45% PC (5/1), pure PC, and pure PC/PE/PS (10/2/1) as a lipid source for proteoliposomes. However, none of these phospholipid compositions resulted in a better quality of reconstituted vesicles for the RyR/Ca^{2+} release channel compared with 95% PC made using the dialysis, freeze-thaw, and brief sonication method of Lee *et al.* (1994).

From these results, and the fact that functionally active vesicles could be only obtained from continuous empirical try and error, further improvements in the reconstitution of a functional RyR/Ca^{2+} release channel protein might be made with other phospholipids, other detergents, or other reconstitution techniques.

Acknowledgement

This article is offered to commemorate the retirement of professor Chung-No Joo from the Department of Biochemistry, College of Science, Yonsei University.

We appreciate the helpful discussion and critical review of this manuscript by Dr. Gerhard Meissner of Department of Biochemistry and Biophysics in University of North Carolina at Chapel Hill. This paper was supported (in part) by the NON-DIRECTED RESEARCH FUND, Korea Research Foundation, 1991.

References

Chen, P. S., Toribara, T. T. and Warner, H. (1956) *Anal. Chem.* **28**, 1756.

Holloway, P. W. (1973) *Anal. Biochem.* **53**, 304.

Inui, M., Saito, A. and Fleischer S. (1987) *J. Biol. Chem.* **262**, 1740.

Jenden, D. J. and Fairhurst, A. S. (1969) *Pharmacol. Rev.* **21**, 1.

Kaplan, R. S. and Pederson, P. L. (1985) *Anal. Biochem.* **150**, 97.

Laemmli, U. K. (1970) *Nature* **227**, 680.

Lai, F. A., Erickson, H. P., Rousseau. E., Lia, Q. Y. and Meissner G. (1988) *Nature* **331**, 315.

Lai, F. A. and Meissner, G. (1989) *J. Bioenerg. Biomembr.* **21**, 227.

Lai, F. A., Misra, M., Xu, L., Smith, H. A. and Meissner, G. (1989) *J. Biol. Chem.* **264**, 16776.

Lee, H. B., Xu, L. and Meissner, G. (1994) *J. Biol. Chem.* **269**, 13305.

Meissner, G. (1984) *J. Biol. Chem.* **259**, 2365.

Meissner, G. (1986) *J. Biol. Chem.* **261**, 6300.

Meissner, G., Rousseau, E. and Lai, F. A. (1989) *J. Biol. Chem.* **264**, 1715.

Numa, S. K., Takeshima, H. and Nishimura, S. (1989) *Nature* **339**, 439.

Racker, E. (1979) *Method in Enzymol.* **55**, 699.

Razin, S. (1972) *Biochim. Biophys. Acta* **265**, 241.