Modulation of the Interactions of Isolated Ryanodine Receptors of Rabbit Skeletal Muscle by Na^+ and $K^{+\dagger}$

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ABSTRACT: Ryanodine receptors (RyRs) of skeletal muscle, as calcium release channels, have been found to form semicrystalline arrays in the membrane of sarcoplasmic reticulum. Recently, both experimental observations and theoretical simulations suggested cooperative coupling within interlocking RyRs. To better understand the interactions between RyRs and their modulation, the aggregation and dissociation of isolated RyRs in aqueous medium containing various Na^+ and K^+ concentrations were investigated using photon correlation spectroscopy (PCS) and atomic force microscopy (AFM). RyRs aggregated readily at low salt concentrations. However, a different behavior was observed in the presence of Na^+ or K^+. Detectable aggregates were formed in 5 μg/mL RyR sample when the concentration of Na^+ and K^+ was reduced from 1 M to below 0.28 and 0.23 M, respectively. The dissociation of RyR aggregates was also examined when raising the salt concentration. While aggregates formed in 0.15 M NaCl medium could reverse almost completely, those formed in 0.15 M KCl medium only dissolved partly. When keeping the total salt concentration at 0.15 M, the aggregation and dissociation of RyRs were seen to evidently depend on the relative concentration of Na^+ and K^+. The interaction between RyRs was strengthened with increasing Na^+/K^+ ratios in the mixed medium. Accompanying this, a decrease of [^{3}H]ryanodine binding occurred. The results obtained with PCS and AFM provide further evidence for the interaction between RyRs and suggest the importance of Na^+, K^+, and their relative composition in modulating the interaction and cooperation between RyRs in vivo.

Receptor proteins in both eukaryotic and prokaryotic cells are often found attaching to each other and forming regular lattices in the membrane (*1*, *2*). A recent theoretical analysis of a model lattice of interacting transmembrane receptors indicated that such an array could provide a novel mechanism for receptor signaling regulation in cells (*3*, *4*). The cooperative interactions between the receptors organized into an array (or cluster) dramatically increase their sensitivity to ligands as well as the dynamic response range. Such concerted modulation of membrane receptors may represent a hitherto unrecognized way of cellular regulation, which may have a vital role in certain physiological processes.

It is well-known that RyRs (calcium release channels)[1] usually form semicrystalline arrays in the membrane of the SR of various cells (*5*, *6*). In addition, by electrophysiological study, the coordinated gating was found in reconstituted RyRs isolated from both skeletal and cardiac muscle cells (*7*, *8*). These observations suggest interactions among clustering RyRs with physiological significance. Furthermore, recent investigations indicated that the interaction between RyRs might participate in some modulation processes of cytoplasmic calcium. For instance, during the occurrence of Ca^{2+} sparks, synchronized opening of a number of RyRs obviously is required (*9*−*11*). Although simultaneous activation of RyRs in a cluster may be accounted for by Ca^{2+}-induced Ca^{2+} release (*12*, *13*), other mechanisms may exist, including the cooperative coupling of clustered RyRs (*10*, *11*). More complex than the concurrent opening of RyRs, the mechanism for the termination of Ca^{2+} sparks, which needs simultaneous closing of RyRs, is an even more controversial issue. A recent simulation model indicated that

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[1] Abbreviations: RyR, Ryanodine receptor, calcium release channel; PCS, photon correlation spectroscopy; AFM, atomic force microscopy; SR, sarcoplasmic reticulum; TT, transverse tubule; HSR, heavy sarcoplasmic reticulum; e−c coupling, excitation-contraction coupling; PC, phosphatidylcholine; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; DFP, diisopropylfluorophosphate.

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the termination of Ca^{2+} release could be easily explained if the coupled interaction among clustering RyRs participates in this process (*14*).

Thus, the interactions between RyR and their modulation are fundamental issues to understand those phenomena mentioned above. However, examining the interaction between membrane proteins in vivo is technically difficult. Recently, by electron microscopy study, it was shown that isolated RyRs in aqueous medium could self-assemble into a two-dimensional (2D) array, with similar dimensions as those observed in native SR membranes (*15*). Thus, it is plausible to work with such a simple system to look at the basic features of the interaction between RyRs.

It is well-known that Na^+ and K^+ are two important monovalent cations in the organism. The modulation of monovalent cations to the e−c coupling was widely reported (*16*−*20*). However, up to now, no well-accepted mechanism has been proposed for these observed phenomena. Recently, the modulation of K^+ to the interaction among arraying RyRs was found (*15*), suggesting the possible role of monovalent cations in modulating the cooperative coupling of RyRs in vivo. This work motivated us to systematically investigate the modulation of single or mixed Na^+ and K^+ to the interaction between RyRs.

In this study, the interactions between RyRs and their modulation were studied by examining the aggregation and dissociation behaviors of isolated RyRs in aqueous medium, focusing on both dynamic process and final structure. Two techniques, PCS and AFM, were employed. PCS, also referred to as dynamic light scattering, was employed for its high sensitivity to the size of the particles in solution (*21*, *22*). AFM was exploited to image the topography of RyR aggregates. In addition, as a convenient method, the [^{3}H]-ryanodine binding assay was used to investigate the functional state of isolated RyRs in different mediums (*23*, *24*).

EXPERIMENTAL PROCEDURES

Isolation and Purification of Rabbit RyR1s. The isolation and purification of RyR1s of rabbit skeletal muscle were as described previously (*25*, *26*). In brief, the HSR vesicles were prepared by sucrose step gradient centrifugation (20/35/40%, w/w). Then, the HSR was solubilized with CHAPS, and solubilized proteins were fractionated by centrifugation on a 6−20% linear sucrose gradient. The protein composition of 1 mL fractions was monitored by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and identified by Western blot analysis. The fraction containing highly purified RyRs was used for this study. The buffer for storing the isolated RyRs contained 1 M NaCl or KCl, 20 mM Na-Pipes or K-Pipes, 100 μM EGTA, 1 mM DTT, 1 mM DFP, ∼10 mM CHAPS, ∼3 mg/mL PC, and ∼17% sucrose, and the pH was 7.1. The protein concentration was 50−100 μg/mL.

PCS. PCS experiments were performed on a Zetasizer 3000HS$_A$ (Malvern Instruments Ltd., U.K.) with a HeNe laser, operating at 633 nm. The scattering angle for size analysis was fixed at 90°. All measurements were carried out at 20 °C. Solvent and particle refractive indexes were set to 1.330 and 1.520, respectively. The solvent viscosity was set to 1.00 for analysis at 20 °C. CONTIN was chosen as the analysis method due to its suitability for describing smooth distributions. Before PCS measurement, the samples of isolated RyRs in 1 M KCl or NaCl storage buffer were diluted with a low salt solution to a final solution that contained 20 mM Pipes, 0.1 mM DFP, 1 mM CHAPS, 0.3 mg/mL PC, and 500 μM EGTA. The concentration of NaCl or KCl or their combination in the final solution varied from 0.05 to 0.40 M. The protein concentration was 5−10 μg/mL. The pH of all of the solutions was 7.1. Each sample containing a certain concentration of NaCl and/or KCl was only used once.

AFM. AFM was performed with a Nanoscope IIIa-MultiMode AFM (Digital Instruments, Santa Barbara, CA) equipped with an "E" or "J" scanner. Images were recorded in tapping mode using TESP tips (Digital Instruments) or NCH-W tips (Nanosensors, Germany). An aliquot (10 μL) of the RyR sample with different salt concentrations was applied to a piece of Parafilm and then covered by freshly cleaved mica for 3−5 min to promote protein adsorption. Afterward, the mica substrate was washed with deionized H_2O. The specimens were dried and imaged in air at a relative humidity of less than 40%.

[^{3}H]Ryanodine Binding. These experiments were carried out according to the method of Pessah et al (*23*). HSR vesicles (0.25 mg protein/mL) or isolated RyRs (2 μg/mL) were incubated at 32 °C for 5 h (for HSR) or at 22 °C for 14 h (for isolated RyRs), with various Ca^{2+} concentrations in the presence of a designed combination of Na^+ and K^+. In addition, the binding buffer contained 20 mM Pipes, 100 μM EGTA, 2 nM [^{3}H]ryanodine (Dupont), and 14 nM ryanodine (for HSR) or 3 nM (for isolated RyRs), and its pH was 7.1. The total Ca^{2+} necessary for obtaining desired free Ca^{2+} in the presence of 100 μM EGTA was calculated by a computer program, WinMaxc (*27*). The binding reaction was quenched by rapid filtration through Whatman GF/B filters mounted on a 48 well Brandel cell harvester. The filters were rinsed three times with ice-cold wash buffer, put into scintillation vials, and shaken overnight with scintillation fluid. The bound [^{3}H]ryanodine was determined with a scintillation counter (Beckman, LS 6000IC).

RESULTS

Formation and Structure of Aggregates. During the isolation and purification of RyRs, the presence of exogenous phospholipid in the medium was required for maintaining channel activity (*25*). Therefore, the RyR sample used in this study, after dilution, contained 1 mM CHAPS and 0.3 mg/mL PC. First, as a control, the size distribution of the mixture of PC and CHAPS in the absence of RyR was examined, and the representative result in 0.25 M KCl is illustrated in Figure 1A. A narrow monomodal distribution indicates that PC and CHAPS formed homogeneous particles with a diameter of about 26 nm in the aqueous medium. The monomodal distribution of the particles did not change with KCl or NaCl concentration within the range of 0.1−0.4 M (data not shown). In the presence of RyR, the size distributions were dependent on the salt concentration. At high concentrations of KCl or NaCl, such as 0.35 M KCl (Figure 1B), a monomodal distribution was also observed, although with a slight shift to a larger diameter. As revealed by cryo-EM and three-dimensional (3D) reconstruction, RyR presents an overall shape of a square prism (28 nm × 28 nm × 12 nm) (*28*). The size distribution of RyR cannot

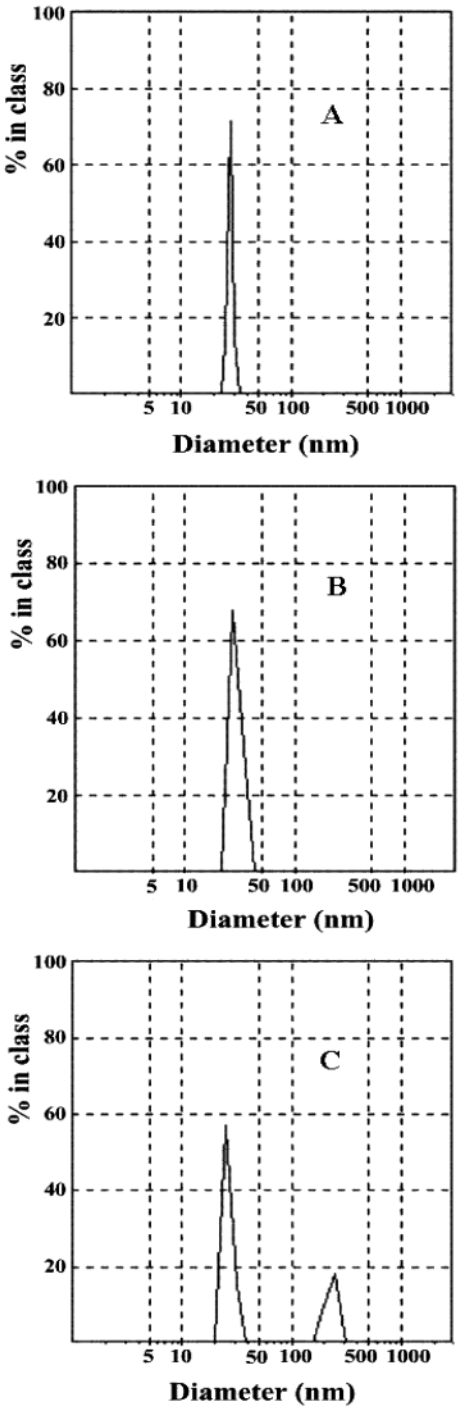

FIGURE 1: PCS determination of the size distribution of isolated RyRs in different aqueous mediums. All samples contained 0.3 mg/mL PC and 1 mM CHAPS. (A) Control: only 0.3 mg/mL PC and 1 mM CHAPS in 0.25 M KCl; (B) RyR sample: 5 μg/mL RyR in medium with 0.35 M K^+; (C) different RyR sample: 5 μg/mL RyR in medium with 0.15 M K^+. Similar results were obtained in other five experiments.

unequivocally be distinguished from that of the particles formed by PC and CHAPS, due to similar sizes. Figure 1B indicates that no detectable aggregates were formed in the medium containing a high concentration of monovalent cation. However, in the mediums with low concentrations of salt, such as 0.15 M K^+, the size distribution of the RyR sample became bimodal (Figure 1C). The appearance of the second peak with a larger diameter indicated that some aggregates were formed in solution.

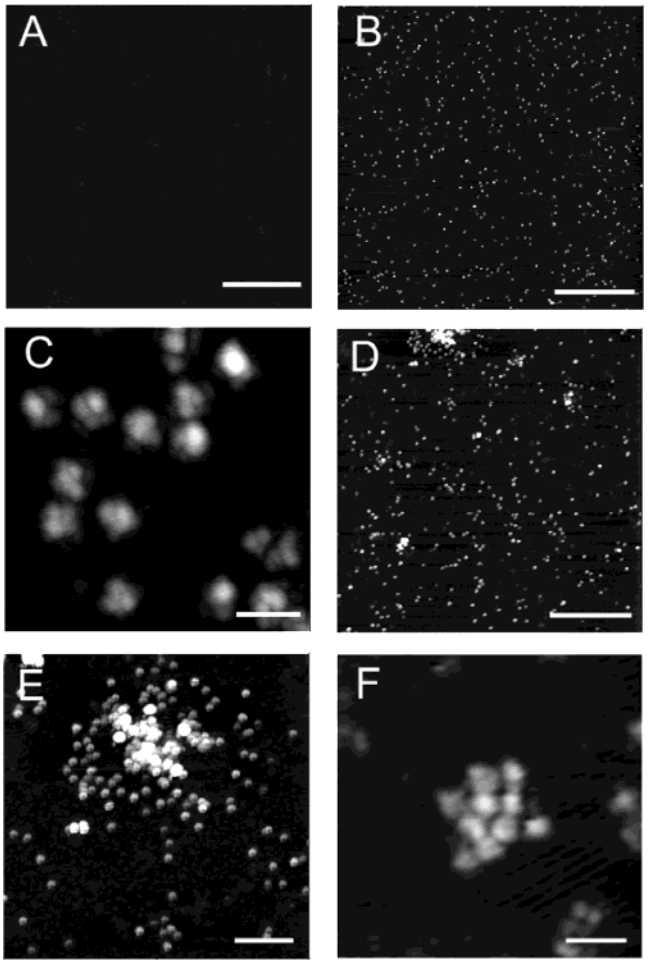

FIGURE 2: AFM images obtained in air with tapping mode. (A) Sample prepared with pure PC and CHAPS; (B,C) RyR sample prepared in 0.35 M KCl medium; (D−F) RyR sample prepared in 0.15 M KCl medium. All RyR samples contained 5 μg/mL protein. The scale bar in A, B, and D represents 1 μm; in C, it represents 60 nm; in E, it represents 300 nm; and in F, it represents 80 nm.

The structure of aggregates was revealed by AFM studies. When preparing the RyR sample for AFM observation, PC and CHAPS usually self-assembled into small patches of membrane on the surface of mica, and most of them could be removed by washing with deionized H_2O. In the control sample containing only PC and CHAPS, particles were hardly found on the surface of mica after washing (Figure 2A). However, with the RyR sample, RyR molecules still remained due to their strong adsorption to the surface of mica. The RyRs sample with 0.35 M KCl showed that the particles with even sizes were dispersed on the surface of mica (Figure 2B). With a finer scanning range, the typical tetramer structures of RyRs can be identified (Figure 2C). The appearances of individual particles were different, probably due to the different orientations of RyRs adsorbed to the mica substrate, as described previously (*26*). Similar results were obtained with a RyR sample in a high concentration of NaCl medium (data not shown). However, some aggregates and 2D patches could be found for the RyR sample in a low salt medium. The typical AFM image of the RyR sample prepared from the medium containing 0.15 M KCl is shown in Figure 2D. To avoid possible artifacts arising from preparing the AFM samples, five samples were made at each concentration of KCl and a total of 30 regions (5 μm × 5 μm) were scanned. In the samples prepared from

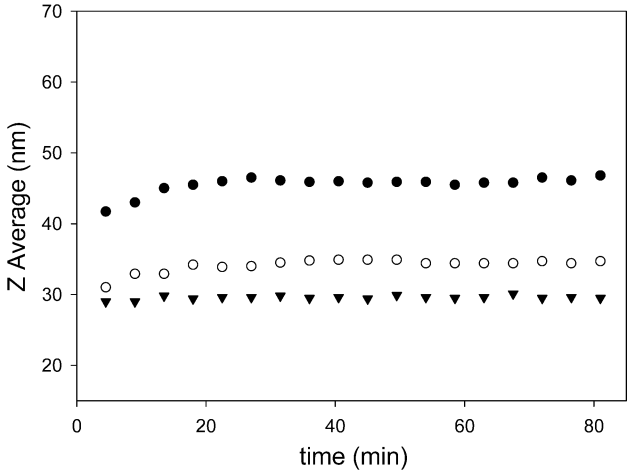

FIGURE 3: Time course of the change of average hydrodynamic diameter of the RyR sample. At 0 min, the concentration of KCl in RyR samples was decreased from 1 M to different values. The final concentration of K^+ in the buffer: 0.11 (●), 0.15 (○), and 0.35 (▼) M. All samples contained 5 μg/mL RyR. Data points are taken from one representative result of five experiments.

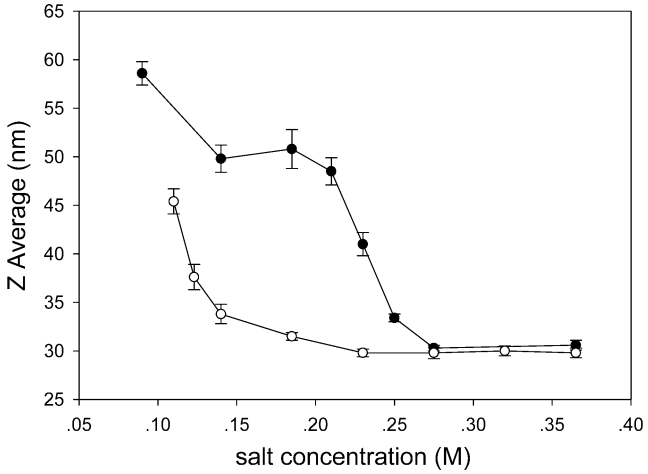

FIGURE 4: Salt concentration dependence of *Z* average in KCl(○) and NaCl mediums (●). All samples were freshly diluted from 1 M KCl or NaCl. After they were diluted, all samples were incubated at 20 °C for 30 min before PCS determination. The concentration of RyR in the final medium was 5 μg/mL. The results are presented as the mean of 3−5 independent experiments. The error bars represent the standard deviation.

the medium containing 0.35 M KCl, only one bigger round particle was seen in one of 30 regions. However, in the samples containing 0.15 M KCl, the aggregates with various appearances were found in 20 out of 30 regions. Some of the aggregates were 3D clusters, formed by linkage and stacking of RyR particles (Figure 2E). More often, 2D patches, arrayed of several square RyR particles, were observed (Figure 2F). Some smaller particles were also observed in Figure 2F. Several factors, e.g., different orientations of RyRs on the mica substrate and/or partial hydrolysis of RyRs, may be responsible for that. Similar aggregates were observed for the RyR sample containing a low NaCl concentration. The results of AFM studies provide morphological evidence for the aggregation and assembly of RyRs produced by decreasing salt concentration, consistent with PCS data.

Kinetics of Aggregation. To gain the insight into the aggregation kinetics, the time courses of average hydrodynamic diameter change of the RyR samples in the mediums containing different KCl concentrations were examined by continuous PCS determination (Figure 3). To ensure efficient solubilization of RyRs, 1 M KCl was present in the storage buffer. When the concentration of KCl was decreased to 0.35 M, the *Z* average was stable at about 30 nm, indicating no aggregation of RyRs. However, if the concentration of KCl was reduced to 0.15 M, the aggregation kinetics seems to show a biphasic time course. At the beginning, the *Z* average increased quickly, and then a slow and small increase of the *Z* average occurred in the following 20 min. Such biphasic time courses can be more clearly seen when further decreasing KCl to 0.11 M. In this case, the *Z* average first abruptly increased to about 40 nm, which was followed by a slow increase up to ∼45 nm.

It should be pointed out that because of the irregular structure of RyR aggregates, the *Z* average determined by PCS cannot represent the actual dimensions of the aggregating particles. However, it may provide us with quantitative data of the aggregations in different mediums.

Differential Effect of Na^+ and K^+ on Aggregation of RyRs. In both NaCl and KCl mediums, the aggregation of RyRs would occur at a low salt concentration. According to the aggregation kinetics (Figure 3), the aggregation of RyRs tended to be stable after 30 min. Thus, after dilution, all RyR samples were incubated at 20 °C for 30 min before PCS determination. For a detectable aggregation in the 5 μg/mL RyR sample, the salt concentration was required to decrease from 1 M to below 0.28 M for NaCl and 0.23 M for KCl, respectively (Figure 4). With a decrease to a lower salt concentration, the *Z* average gradually increased, indicating further aggregation of RyRs. According to the *Z* average data obtained at the 0.1−0.28 M salt concentrations, RyRs in NaCl medium aggregated more readily than those in KCl medium at the same salt concentration.

Because both Na^+ and K^+ are present in the cytoplasm, we examined RyRs samples containing these two monovalent cations. The total concentration of Na^+ and K^+ was kept at 150 mM. The effect of three different combinations of Na^+ and K^+ (20 mM Na^+ + 130 mM K^+, 30 mM Na^+ + 120 mM K^+, and 40 mM Na^+ + 110 mM K^+) on aggregation was examined. The mixture of 20 mM Na^+ + 130 mM K^+ is assumed to be the resting state in muscle cells. As shown in Figure 5, with the salt composition changing from 20 mM Na^+ + 130 mM K^+ to 30 mM Na^+ + 120 mM K^+ and to 40 mM Na^+ + 110 mM K^+, the *Z* average evidently increased from 36.0 ± 0.6 to 40.8 ± 1.0 nm and then to 52.1 ± 1.9 nm. The *Z* average determined in 150 mM K^+ or Na^+ mediums is also presented.

Dissociation of RyR Aggregates. To obtain more information of the interaction between RyRs, the dissociation of RyR aggregates was investigated using PCS. After the RyR sample was incubated in 0.15 M NaCl or KCl medium at 20 °C for 30 min, concentrated NaCl or KCl was added into the medium to increase the salt concentration to 0.35 M. The results illustrated in Figure 6A show that the *Z* average decreased to ∼30 nm when increasing NaCl to 0.35 M. Accompanying this, the second peak in the diameter distribution disappeared (data not shown). It indicated almost a complete reversal of the aggregation in NaCl medium. However, the aggregates formed in 0.15 M KCl medium did

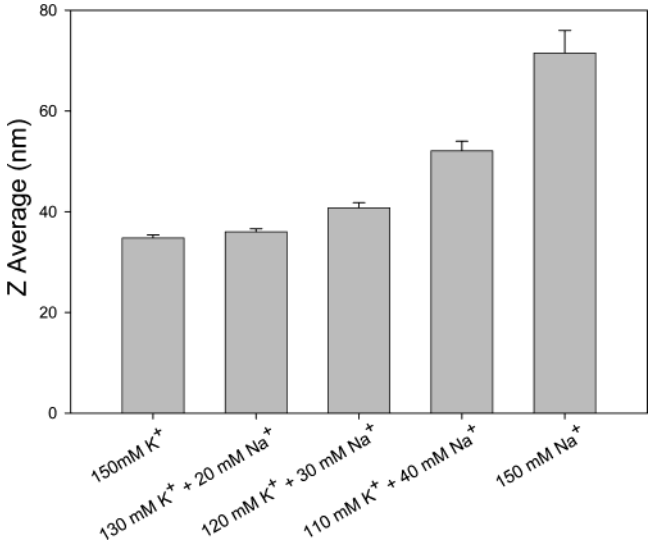

FIGURE 5: Modulation of the aggregation of RyRs by different combinations of Na^+ and K^+. The total concentration of Na^+ and K^+ was kept at 0.15 M. All samples contained 10 μg/mL RyR. The results are presented as the mean of 3−5 independent experiments. The error bars represent the standard deviation.

not dissolve in the same way. As shown in Figure 6A, there was a small drop in the *Z* average, indicating only partial reversibility in KCl medium.

To investigate if the reversibility is affected by the salt concentration used for inducing the aggregation, the salt concentration was reduced to 0.11 M (Figure 6B). It is seen that the aggregates formed in 0.11 M KCl medium almost did not dissolve, while those formed in the NaCl medium became partially reversible. To exclude that the irreversibility arises from long incubation in the medium of low salt concentration, the influence of the incubation time was examined. Reducing the incubation time to 5 min did not cause any difference to the reversibility of aggregation (data not shown).

The dissociation processes of RyRs aggregates formed in the medium containing a constant salt concentration with different combinations of Na^+ and K^+ were also examined. Although different extents of the aggregation were induced by different combinations of Na^+ and K^+ (Figure 5), the aggregates formed in the medium with more Na^+ seem to dissociate more readily when increasing the salt concentration (data not shown).

[^{3}H]Ryanodine Binding Assay. To investigate the effect of different combinations of Na^+ and K^+ on the function of purified RyRs, the [^{3}H]ryanodine binding assay was performed on both HSR vesicle (Figure 7A) and isolated RyRs (Figure 7B). Both of them showed bell-shaped [Ca^{2+}] dependence, with a peak binding at 10−100 μM [Ca^{2+}], although the curve of the [Ca^{2+}] dependence obtained from isolated RyRs was broader. It is indicated that purified RyRs kept activity when diluted to different salt solutions. The aggregation observed at the low salt concentration was not induced by denaturalization of this channel. Moreover, it is evident that [^{3}H]ryanodine binding to either HSR or isolated RyRs decreased with the increase of Na^+ in binding buffer, indicating the activity of the channel was modulated by different combinations of Na^+ and K^+ in the solution.

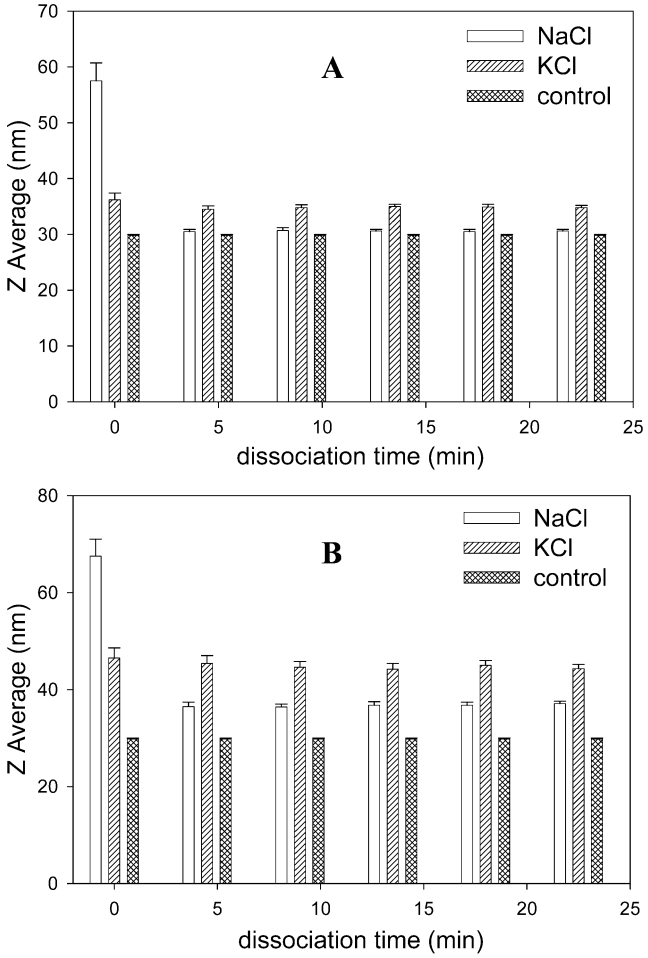

FIGURE 6: Dissociation process of RyRs aggregates formed in low NaCl medium (open bars) and in low KCl medium (bars with strips). RyRs did not form aggregates in 0.35 M KCl or NaCl mediums. The *Z* average determined in these samples was used as the control (bars with lattices). After the samples were incubated for 30 min in low salt solutions, the salt concentration was increased to 0.35 M. Five sequential measurements were performed by PCS, with each measurement of 4.5 min. (A) Dissociation of RyR aggregates formed in 0.15 M NaCl or KCl medium; (B) dissociation of RyR aggregates formed in 0.11 M NaCl or KCl medium. Samples contained 7 μg/mL RyR. The results are presented as the mean of four independent experiments. The error bars represent the standard deviation.

DISCUSSION

By electron microscopy study, it has been shown that isolated RyRs in aqueous medium could self-assemble into a 2D array, when the concentration of KCl in the medium is decreased below 0.25 M (*15*). Consistent with that, the present study with PCS clearly showed that the aggregation of RyRs was induced by similarly decreasing the concentration of KCl and NaCl. AFM studies showed that RyRs in the medium with a low salt concentration tended to form some irregular aggregates, and more often 2D small patches. In addition to that, this study interestingly found that the aggregation and dissociation of isolated RyRs were differentially affected by Na^+ and K^+.

These findings clearly indicate the effect of monovalent cations on the interaction between RyRs. This effect may be related to the modulation of e−c coupling by monovalent cations, which was first reported about 20 years ago (*16*−*18*). It was found that the contraction evoked by the action

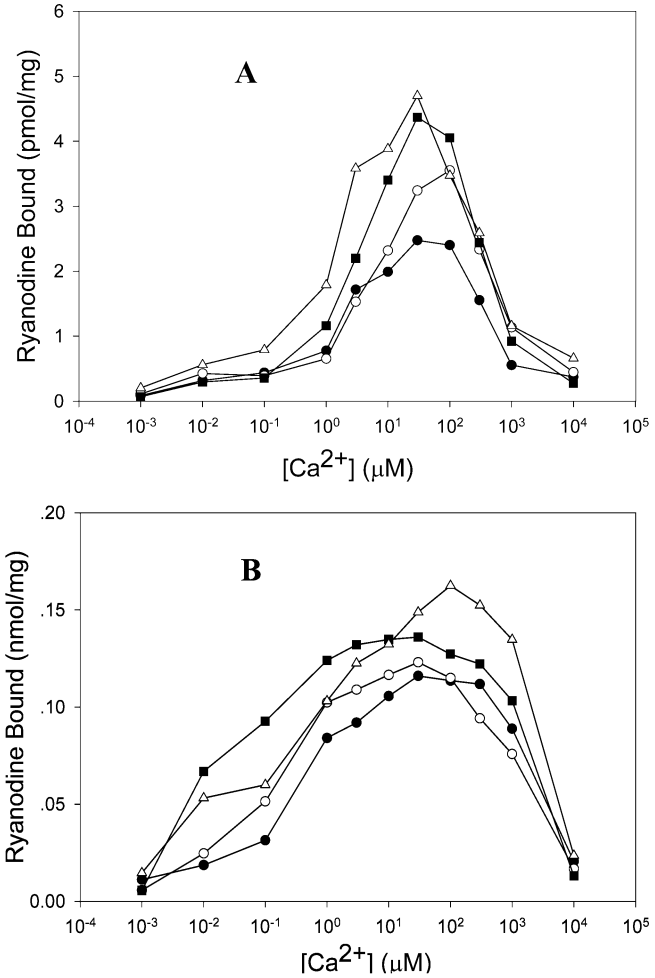

FIGURE 7: Ca^{2+} dependence of [^{3}H]ryanodine binding in the binding buffer with different combinations of Na^+ and K^+; 140 mM K^+ + 10 mM Na^+ (△), 130 mM K^+ + 20 mM Na^+ (■), 110 mM K^+ + 40 mM Na^+ (○), 90 mM K^+ + 60 mM Na^+ (●). (A) HSR vesicles; (B) isolated RyR. Each assay was performed on duplicated samples and repeated twice.

potential was increased by veratridine, an inhibitory agent of inactivation mechanism of sodium channels. In addition, they showed that the influx of Na^+ ions was partly responsible for the enhanced contraction. Then, a direct relationship between Na^+ entry and contraction in isolated frog muscle fibers was investigated (*19*). The role of Na^+ in e−c coupling was further elucidated by replacing Na^+ with Li^+ in the external solution (*20*). Because the sensitivity of the contractile apparatus to Ca^{2+} did not change with altering the Na^+ and K^+ composition (*29*), the increased contraction induced by Na^+ entry should mainly result from the increase of myoplasmic Ca^{2+}. The reversed Na^+−Ca^{2+} exchange described in cardiac muscle cells (*30*−*32*) may be partly responsible for that. However, even in cardiac muscle, the role of Na^+−Ca^{2+} exchange is still controversial (*33*, *34*). Recently, it was found that in both frog atrial cells and twitch muscle fibers Na^+ entry is able to increase the release of calcium ions from the SR in the absence of Na^+−Ca^{2+} exchange (*35*, *36*). Thus, a new mechanism was required to explain these observations.

By the [^{3}H]ryanodine binding assay, the regulation of RyRs of skeletal muscle by monovalent cations has been studied previously (*24*). According to the estimation of ryanodine binding to the HSR, the activity of RyRs in KCl medium is higher than those in NaCl at equivalent concentrations. In the present study, the assay was performed on both the HSR and the isolated RyRs in the presence of different combinations of Na^+ and K^+. It clearly showed that the ryanodine binding to either HSR or isolated RyRs was affected by different compositions of Na^+ and K^+ in binding buffer. When the total salt concentration was kept constant at 0.15 M, ryanodine binding decreased with increasing Na^+ concentration. Thus, it is unlikely that the increased release of calcium ions from the SR induced by Na^+ entry results from direct activation of RyRs.

Cooperative gating of interlocking RyRs is a newly proposed mechanism in e−c coupling. Simulation models (*14*, *37*) and various experimental observations (*7*−*11*) support that the cooperative coupling between RyRs participates in Ca^{2+} release related processes. In this work, the differential modulation of Na^+ and K^+ to the interaction between RyRs was revealed. It was also observed that the interaction between RyRs was strengthened with increasing Na^+ concentration in 0.15 M mixed Na^+, K^+ mediums. Thus, Na^+ entry may positively modulate the cooperation between RyRs and increase the release of calcium ions from the SR.

The regular array of RyRs in the SR membrane is highly conserved during evolution (C. Franzini-Armstrong, personal communication),[2] suggesting the existence of a general mechanism to modulate the cooperativity between RyRs. During e−c coupling, a change of Na^+ and K^+ levels occurs in the narrow TT−SR gap when the action potential propagates into the TT membrane (*38*, *39*). In addition, this change would not be reversed rapidly due to the limited number of Na^+/K^+ pumps on the TT membrane (*40*). RyRs are located in the SR membrane facing this gap and would sense this change just before their activation. Our work indicated the potential of Na^+ and K^+ to modulate the interaction between RyRs. Thus, we would like to propose the possibility that the change of Na^+ and K^+ concentrations in the TT−SR gap during e−c coupling is of physiological significance through modulating the interactions between RyRs. The change of Na^+ and K^+ levels in the gap accompanying the action potential may act as a signal to strengthen the interaction and coupling between RyRs, which is necessary for the subsequent vital release of calcium ions from the SR and even for concurrent closing of RyRs. After e−c coupling, the Na^+, K^+ level and the interactions among clustered RyRs return to the resting level.

In the present study, plenty of information was obtained about the interactions between RyRs and their modulation by Na^+ and K^+. The physiological relevance of these findings was discussed, especially with regard to the enhancement of contraction induced by Na^+ entry. A possible mechanism is proposed for monovalent cations to modulate the cooperative coupling among clustering RyRs during e−c coupling. However, it should be pointed out that the present work is performed with isolated RyRs in aqueous medium. Further studies are required to verify this under physiological conditions. Besides monovalent cation, the modulations by

[2] This knowledge was obtained from personal communication with C. Franzini-Armstrong. This is the written approval from Professor C. Franzini-Armstrong: "Dear Hu Xiaofang: this is to state that you have my written permission to use the personal communication material that you indicated. Sincerely, Clara Franzini-Armstrong. E-mail: armstroc@mail.med.upenn.edu.

other factors, such as FKBP, the functional state of RyR, Ca^{2+}, and Mg^{2+}, are also valuable to explore. The precise effects of these factors would be helpful for understanding the modulation mechanism in detail.

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