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Molecular nature of sulfhydryl modification by hydrogen peroxide on type 1 ryanodine receptor[1]

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Key words

Ca^{2+} release channel/ryanodine receptor; hydrogen peroxide; reactive oxygen; redox-sensitive ion channel

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Abstract

Aim: To elucidate the molecular nature of sulfhydryl modification by hydrogen peroxide on type 1 ryanodine receptor (RyR1). **Methods**: Rabbit skeletal muscle sarcoplasmic reticulum was treated with hydrogen peroxide, then RyR1 complex was isolated. The proteins in the complex were analysed by electrophoresis, Western blot and electron microscopy. **Results**: (1) Hydrogen peroxide induces inter-subunit cross-linking within the tetrameric RyR1 molecule; (2) in parallel to inter-subunit cross-linking, the RyR1 molecule changes morphology; (3) the chemical and morphological changes are reversible: upon reduction by reducing agents, the RyR1 molecule regains its original state. **Conclusion**: These findings suggest that the molecular mechanism of RyR1 channel activity in sarcoplasmic reticulum regulated by hydrogen peroxide is through inter-subunit cross-linking within the tetrameric RyR1 molecule, which in turn induces structural changes of RyR1.

Introduction

The ryanodine receptors (RyR) are a class of calcium release channels in the endoplasmic reticulum or sarcoplasmic reticulum (SR) membrane. The RyR is regulated by Ca^{2+}, Mg^{2+}, ATP, calmodulin, FK-506 binding protein, and voltage-dependent channels such as dihydropyridine receptors[1–4]. The skeletal RyR (RyR1) also contains up to 100 cysteine residues per monomer[5] and it has become abundantly clear that the redox status of some of these cysteines can regulate activity of the RyR[6–9]. Activity of the RyR can be modulated by sulfhydryl modifying agents and the nature of the modification can determine both quantitative and qualitative changes in the properties of the RyR. These agents include *N*-ethyl maleimide[10,11], diamide[10,11], glutathione[12], NOC-12[13], and nitric oxide[13–15]. Apart from glutathione and nitric oxide, however, other agents are exogenous; their relevance to the physiological and/or pathological state of muscle is not conclusive.

Hydrogen peroxide (H_2O_2) is a reactive oxygen species produced in living cells[16,17]. It has been shown that H_2O_2 promotes ryanodine binding to SR membranes and Ca^{2+} release from SR vesicles[18]. H_2O_2 activates RyR single channels reconstituted in the lipid bilayer and increases channel open probability, suggesting that the effect of H_2O_2 on SR Ca^{2+} release is directly due to an oxidation of the RyR[19,20]. Aghdasi *et al* suggested that sulfhydryl modifying agents might induce inter-subunit cross-linking between RyR monomers to form dimers of RyR subunits[10,11]. However, the hypothesis is based on data from SR membranes where hundreds of other proteins exist apart from the RyR, and the cross-linked products that are identified as dimers of RyR1 subunits are ambiguous. Moreover, the hypothesis was mainly based on experiments using diamide, an exogenous reagent, as an oxidant. Whether endogenous oxidants produced *in vivo*, such as H_2O_2, also produce the same or a similar effect remains elusive.

In this study, we treated skeletal SR membranes with

H_2O_2, isolated the RyR1 complex and investigated the molecular nature of sulfhydryl modification by H_2O_2 on RyR1. Our studies revealed that: (1) H_2O_2 induced inter-subunit cross-linking within the tetrameric RyR1 molecule; (2) in parallel to inter-subunit cross-linking, the RyR1 molecule changed morphology; and (3) the chemical and morphological changes were reversible, after reduction by a reducing agent, the RyR1 molecule regains its original state. These findings suggest that the molecular mechanism of RyR1 channel activity in SR regulated by H_2O_2 is through inter-subunit cross-linking within the RyR1 tetrameric molecule, which in turn induces structural changes of the RyR1.

Materials and methods

Materials [^{3}H]Ryanodine was obtained from Amersham Biosciences (Piscataway, NJ, USA). Anti-dihydropyridine receptor (DHPR)α1 subunit and anti-triadin monoclonal antibodies, CHAPS, Tween-20 and protease inhibitors were obtained from Sigma (St Louis, USA), and polyvinylidine difluoride (PVDF) membrane was obtained from Millipore (Billerica, MA, USA). Anti-RyR antibody, Ab2160, was raised against a consensus sequence of the C-terminus residues of RyR (peptide sequence FFPAGDCFRKQYEDQL), which is conserved in RyR1 and RyR2. The antibody specifically recognizes the 565 kDa RyR and the 410 kDa endogenous enzymatic fragment, both containing the peptide sequence FFPAGDCFRKQYEDQL. The antibody does not cross-react with other proteins that do not contain the peptide sequence, including RyR3. All other chemicals were of analytical grade.

Preparation of SR vesicles Rabbit skeletal muscle SR membrane fractions enriched in [^{3}H]ryanodine binding and Ca^{2+} release channel activities were prepared in the presence of protease inhibitors (100 nmol/L aprotinin, 1 μmol/L leupeptin, 1 μmol/L pepstatin, 1 mmol/L benzamidine and 0.2 mmol/L phenylmethylsulfonyl fluoride) as described previously[21].

Treatment of SR vesicles with H_2O_2 and purification of RyR1 complex SR vesicles were diluted with 0.1 mol/L KCl, 10% sucrose and 20 mmol/L Na-Pipes, pH 7.1 to a protein concentration of 5 mg/mL, then treated with 1–10 mmol/L H_2O_2 for 30 min at room temperature. The H_2O_2-treated SR vesicles were pelleted in a Beckman TL-100 ultracentrifuge at 110 000×*g* for 15 min in a TL-100.3 rotor and re-suspended in 1.0 mol/L NaCl, 100 μmol/L egtazic acid, 150 μmol/L $CaCl_2$, 0.2 mmol/L PMSF, 1 μmol/L leupeptin, and 20 mmol/L Na-Pipes, pH 7.1 (solubilization buffer). The SR vesicles were incubated with 2 nmol/L [^{3}H]ryanodine for 1 h to label the RyR1, then the SR vesicles were solubilized with detergent CHAPS. The RyR1 complex was purified by 5%–20% linear sucrose gradient centrifugation according to published procedure[22], except that dithiothreitol (DTT) was omitted from the gradient. The sedimentation position of RyR is defined by its sedimentation coefficient (30 S), which is determined by using a series of standard proteins with known sedimentation coefficient[22].

Immunoblotting analysis Samples were solubilized in non-reducing sample buffer, containing 62.5 mmol/L Tris-HCl, pH 6.8, 20% glycerol (*w*/*v*), 1.2% sodium dodecylsulfate (SDS), and 0.05% bromophenol blue, and loaded onto 2.5%–12.0% linear polyacrylamide sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and resolved by electrophoresis. After electrophoresis, the proteins were transferred overnight to a PVDF membrane. The transferred membrane was blocked in phosphate-buffered saline (PBS), containing 0.1% Tween-20 and 5% non-fat milk, with agitation for 1 h at room temperature, then incubated with primary antibodies for 2–3 h in blocking solution. The PVDF membrane was washed (3 times×5 min) in PBS, and incubated with peroxidase-coupled secondary antibody in blocking solution for 1 h, washed as described above, then developed with 0.6 mg/mL 3,3'-diaminobenzidine, 0.03% (*v*/*v*) H_2O_2 in PBS.

Electron microscopy Sample (5 μL) was applied to a glow-discharged, carbon-coated 400-mesh electron microscopy copper grid and allowed to absorb for 1 min. Excess sample was removed by touching the grid from the side with a piece of Whatman filtration paper. One drop (5 μL) of 1% uranyl acetate was applied to the grid. After 1 min, the grid was washed with 5 drops of 1% uranyl acetate. Excess solution was removed by touching the side of the grid with a piece of Whatman filtration paper, and the grid was air-dried.

Grids were examined in a Philips CM-12 electron microscope, operating at 100 kV. Pictures were recorded on Kodak S-19 films at a magnification of ×36 000 with exposure time of 1 s. Films were developed in an Ilford D19 developer for 12 min.

Results and discussion

Formation of high-molecular-weight complex SR membranes were treated with H_2O_2, which promotes ryanodine binding to SR membranes and Ca^{2+} release from SR vesicles[18]. Similar to diamide, one major target of H_2O_2 was the RyR1, as the intensity of the 565 kDa RyR1 peptide and its 410 kDa endogenous enzymatic fragment (fRyR) were dramatically reduced in H_2O_2-treated SR membranes (Figure 1A). In con-

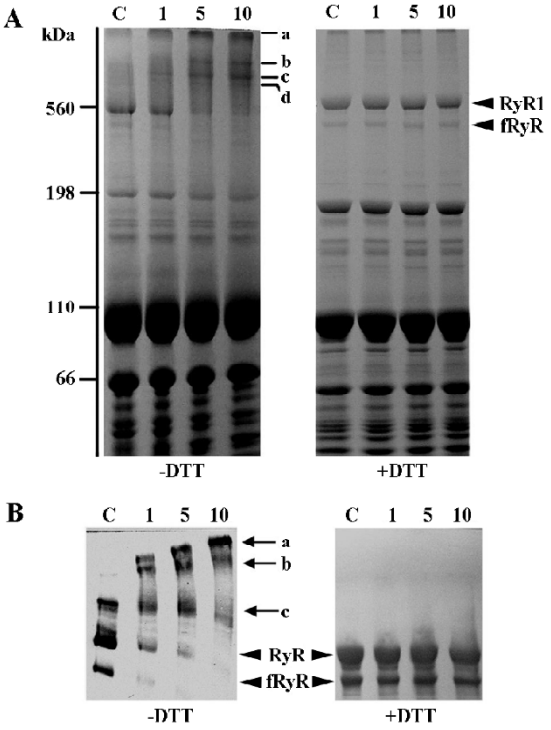

Figure 1. SDS-PAGE and Western blot analysis of H_2O_2-treated SR membranes. After H_2O_2 treatment, SR membrane proteins (30 μg) were resolved on a 2.5%–12% linear gradient polyacrylamide gel in non-reducing (-DTT) or reducing (+DTT, 10 mmol/L) conditions. Proteins were stained by Coomassie brilliant blue R250 (A) or transferred onto PVDF membrane and probed with anti-RyR antibody, Ab2160 (B). The positions of the 565 kDa RyR1 peptide, its 410 kDa endogenous enzymatic fragment (fRyR) and the HMWBs a–d are indicated; the H_2O_2 concentrations (mmol/L) are labeled above. A control SR membrane (C) without H_2O_2 treatment is included.

trast to diamide, in which only 2 high-molecular-weight bands (HMWBs) were observed[10,11], 4 major HMWBs appeared (Figure 1A, bands a–d). The HMWBs could be classified into 2 types. The first type (bands a–c) changed their intensities and progressively migrated towards the higher molecular weight range with increasing H_2O_2 concentrations. The second type (band d) appeared to have constant intensity throughout the various H_2O_2 concentrations used. To reveal whether all HMWBs were derived from RyR1, Western blot analysis was carried out. The data showed that the three first type HMWBs (bands a–c) were recognized by the anti-RyR antibody Ab2160, whereas the second type HMWB (band d) was not (Figure 1B, -DTT), indicating HMWBs a–c were derived from RyR1, but HMWB d was not. After incubation with DTT, a disulphide bond reducing agent, all HMWBs disappeared. In parallel, the intensity of the 565 kDa RyR1 peptide band and the 410 kDa endogenous enzymatic fragment recovered (Figure 1B, +DTT). These data suggested that, after H_2O_2 treatment, the RyR1 formed one (or several) high-molecular-weight complex (HMWC) through cross-linking using disulphide bonds. Notably, the first type HMWBs already existed as faint bands even in the control SR membrane (Figure 1B, control). This suggested that the RyR1 was very sensitive to reactive oxygen species and could pre-exist in a partially cross-linked state in the native SR membrane *in vivo*, or was oxidized during preparation of SR vesicles.

Molecular nature of the HMWC involving RyR1 It has been shown that the RyR1 is regulated by interaction with a variety of proteins[1–4]. Two of them, DHPR and triadin, have been suggested to directly interact with the RyR1[23–26]. It is possible that the HMWCs are adducts of RyR1 with these proteins. To reveal the molecular nature of the HMWCs involving RyR1, the SR membranes treated with H_2O_2 (10 mmol/L) were solubilized with detergent CHAPS, labeled with [^{3}H]ryanodine, and the sample was subjected to sucrose gradient sedimentation analysis. A comparison of [^{3}H]ryanodine sedimentation profiles in sucrose gradient between the H_2O_2-treated sample and the control sample revealed that the sedimentation profiles were virtually the same. In both cases, the RyR1 complexes sedimented normally as 30S particles (Figure 2, indicated by an arrow). This sedimentation profile is virtually the same in the H_2O_2 concentration used (data not shown). SDS-PAGE under non-reducing conditions revealed that the 30S fractions were comprised of three HMWBs equivalent to the first type of HMWBs (bands a–c) observed originally in H_2O_2-treated SR membranes (Figure 3A, -DTT). When the fractions were treated with DTT, however, all three HMWBs (bands a–c) disappeared. In parallel with this disappearance there was a recovery of the 565 kDa RyR1 peptide and the 410 kDa endogenous enzymatic fragment (Figure 3A, +DTT), suggesting that these three HMWBs were the cross-linked products of RyR1 subunits.

To further confirm that the HMWBs were indeed cross-linked products of RyR1 subunits, Western blot analysis of the sucrose gradient fractions was carried out under non-reducing conditions. The result showed that all three HMWBs were specifically recognized by the anti-RyR antibody Ab2160 (Figure 3B, -DTT). Western blot analysis using antibodies against DHPRα1 and triadin did not recognize any of these HMWBs. After the fractions were treated with DTT, the HMWBs disappeared; in parallel with this disappearance, the 565 kDa RyR1 peptide and the 410 kDa endogenous enzymatic fragment were detected by the same anti-RyR antibody, Ab2160 (Figure 3B, +DTT), whereas antibodies against DHPRα1 and triadin failed to detect any sig-

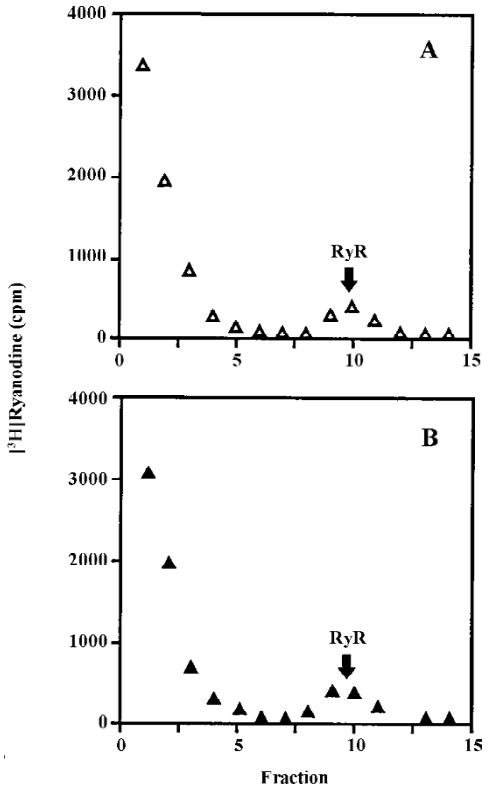

Figure 2. Sedimentation profiles of [^{3}H]ryanodine labeled, CHAPS solubilized SR membrane proteins in sucrose gradient. Three assays were done with similar results. The data shown is from a typical assay. (A) Control SR membrane; (B) H_2O_2-treated SR membrane. SR membranes treated with H_2O_2 (10 mmol/L, 30 min) were solubilized and labeled with [^{3}H]ryanodine. The proteins were resolved in a 5%–20% linear sucrose gradient as described in Materials and methods. Aliquots (50 μL) of each fraction were used to count radioactivity. The small peak (indicated by an arrow) represents the labeled RyR and the large peak are from free [^{3}H]ryanodine.

nal in the same fractions containing HMWBs. However, both DHPRα1 and triadin were indeed detected in the top fractions (fractions 3 for DHPRα1 and fraction 1 for triadin) of the sucrose gradient and both the H_2O_2-treated and control SR membranes (Figure 3B, ±DTT). These data suggest that, after treatment with H_2O_2, the RyR1 forms adducts in the SR membrane through cross-linked disulphide bonds, and the cross-linking takes place between subunits of the RyR1 tetrameric molecule. DHPRα1 and triadin, which have been suggested to directly interact with the RyR1, do not form adducts with RyR1 through disulphide bonds.

Morphological changes of the RyR1 molecule It has been shown that H_2O_2 promotes ryanodine binding to SR membranes, enhances Ca^{2+} release from SR vesicles, and increases channel open probability[18–20]. Hamilton and coworkers suggested that cross-linking between RyR subunits might be the molecular mechanism[10,11]. We revealed that H_2O_2 indeed induced cross-linking between RyR1 subunits. To see whether the chemical change induced structural change, we purified the RyR1 complex from H_2O_2-treated SR membranes and examined the morphology of RyR1 complex by electron microscopy. In contrast to the RyR1 particles purified from control SR membranes, which displayed a homogenous pinwheel-like appearance (Figure 4A), the RyR1 particles purified from H_2O_2-treated SR membranes displayed various appearances (Figure 4B), from pinwheel-like (Figure 4B, a) to square-like (Figure 4B, b) and round-shaped (Figure 4B, c) to windmill-like (Figure 4B, d). These data demonstrated that, after inter-subunit cross-linking, the structure of the RyR1 molecule changed. Consistent with the multiple cross-linked products observed by SDS-PAGE (Figures 1,3, bands a–c), different appearances were present. Despite their different appearances, the overall size of the particles was consistent with the size of the RyR1 molecule, 25–29 nm[27–30]. Intriguingly, the morphological changes were reversible: when disulphide bond reducing agent DTT was added to the RyR1 particles purified from H_2O_2-treated SR membranes, the particles regained their characteristic pinwheel-like appearance (Figure 4C), indicating that the effect of H_2O_2 on the morphology of RyR is reversible. This finding is consistent with the effect of H_2O_2 on the function of RyR, which is also reversible[18,19].

Sulfhydryl modifying agents have been shown to affect ryanodine binding to, and Ca^{2+} release from, SR vesicles. This led to suggestions that redox potential might be involved in the regulation of Ca^{2+} release from SR and excitation-contraction coupling in muscle[6–9]. The precise mechanism, however, might be different from agent to agent[14]. There are 3 possible explanations for the modulation of Ca^{2+} release from SR by H_2O_2: (1) oxidation of sulfhydryl might induce the RyR1 molecule to form adducts with other proteins, such as DHPRα1 and/or triadin, which have been shown to directly interact with RyR1[23–26]; (2) oxidation of sulfhydryl might induce the RyR1 molecule to form adducts with other RyR1 molecules, as the RyR1 oligomers are organized as ordered arrays in the SR membrane *in situ*[27,28] and physically coupled with each other[31,32]; and (3) oxidation of sulfhydryl might induce RyR1 to form a complex within the tetrameric molecule.

The data presented in this work indicate that possibility (1) is unlikely to be correct because, if it is correct, then RyR1–DHPRα1 and/or RyR1-triadin complexes will form in the SR membrane after treatment with H_2O_2. Neither SDS-PAGE nor Western blot analysis detected these complexes (Figure 3).

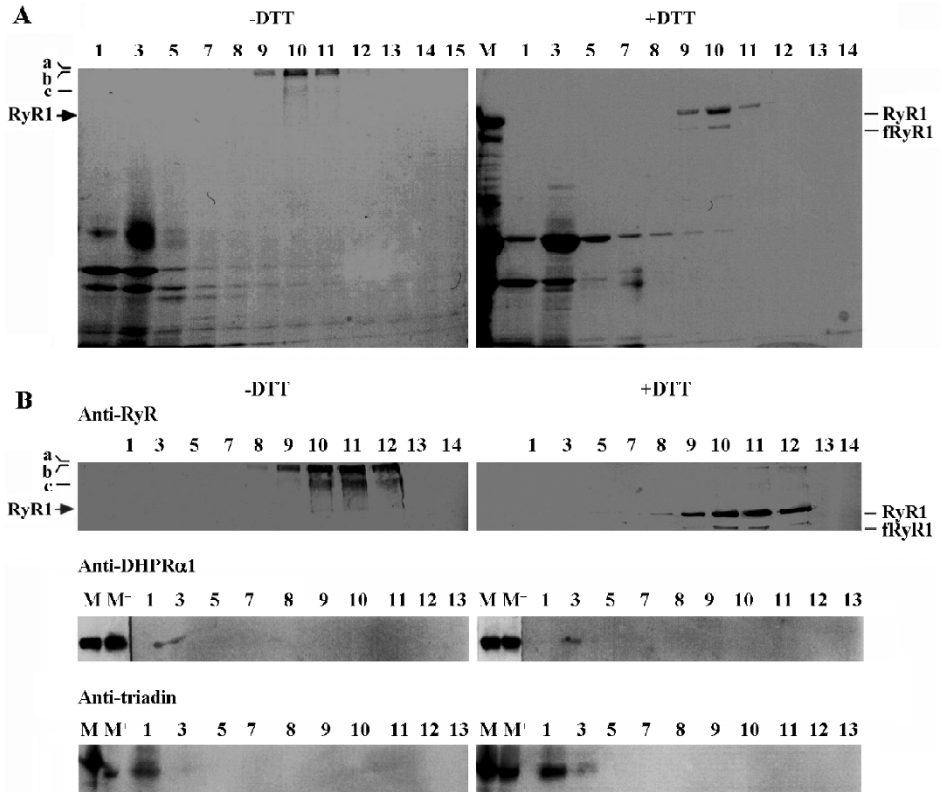

Figure 3. SDS-PAGE and Western blot analysis of HMWC involving RyR1. Proteins in each fraction obtained, as described in Figure 2, were resolved on 2.5%–12% linear gradient polyacrylamide gels at non-reducing (-DTT) or reducing (+DTT, 10 mmol/L) conditions then analyzed by Coomassie brilliant blue staining (A) and Western blotting (B). The positions of the 565 kDa RyR1 peptide, the 410 kDa endogenous enzymatic fragment (fRyR1), the HMWBs a-c are indicated, and the fraction numbers are labeled above. Also included in SDS-PAGE gels and Western blot analysis are control (M) and H_2O_2-treated SR membranes (M^+).

Possibility (2) is also ruled out for the following reasons: (1) if possibility (2) is correct, RyR1 will form giant RyR1 molecular complexes with single RyR1 molecules of multiple sizes, so the sedimentation coefficient will be a multiple of 30 S (such as 60 S, 90 S, or 120 S) that will sediment at marked different positions in 5%–20% linear sucrose gradient. The sedimentation profile of the RyR1 complex purified from H_2O_2-treated SR membranes is virtually the same as that of the RyR1 molecule purified from control SR membranes (Figures 2, 3), indicating the RyR1 complex formed after treatment with H_2O_2 is still 30S; (2) consistent with sedimentation analysis, electron microscopy of the RyR1 particles purified from H_2O_2-treated SR membranes revealed that the size of RyR1 particles is in the range of 25–29 nm (Figure 4), which is the typical size of a single RyR1 molecule[27–30].

It seems that the correct mechanism is possibility (3), and indeed all the data presented in this work conform to this possibility. Hamilton and coworkers proposed a similar mechanism of RyR modification by diamide[10,11]. They found that when SR membrane was treated with diamide, 2 HMWBs were detected in SDS-PAGE, which they assigned as the dimer of the 565 kDa RyR1 peptide and the dimer of the 410 kDa fragment of RyR1 peptide, according to their mobility[10,11]. From these data, they suggested that cross-linking of sulfhydryl between RyR1 subunits might play a role in the modulation of Ca^{2+} release from SR[8]. We demonstrated that H_2O_2, which is produced in living cells, produced similar effects. Our data are based on isolated RyR1 complex, which unambiguously identified the HMWBs as cross-linked RyR1 subunits (Figure 3). Furthermore, our data demonstrated that the cross-linking took place within the tetrameric RyR1 molecule, but not between the RyR1 oligomers (Figures 2, 4).

In conclusion, the data in this work revealed that, after oxidation by H_2O_2, disulphide bonds between subunits within the tetrameric RyR1 molecule formed. The inter-subunit cross-linking triggered structural changes that might alter ryanodine binding and channel activity.

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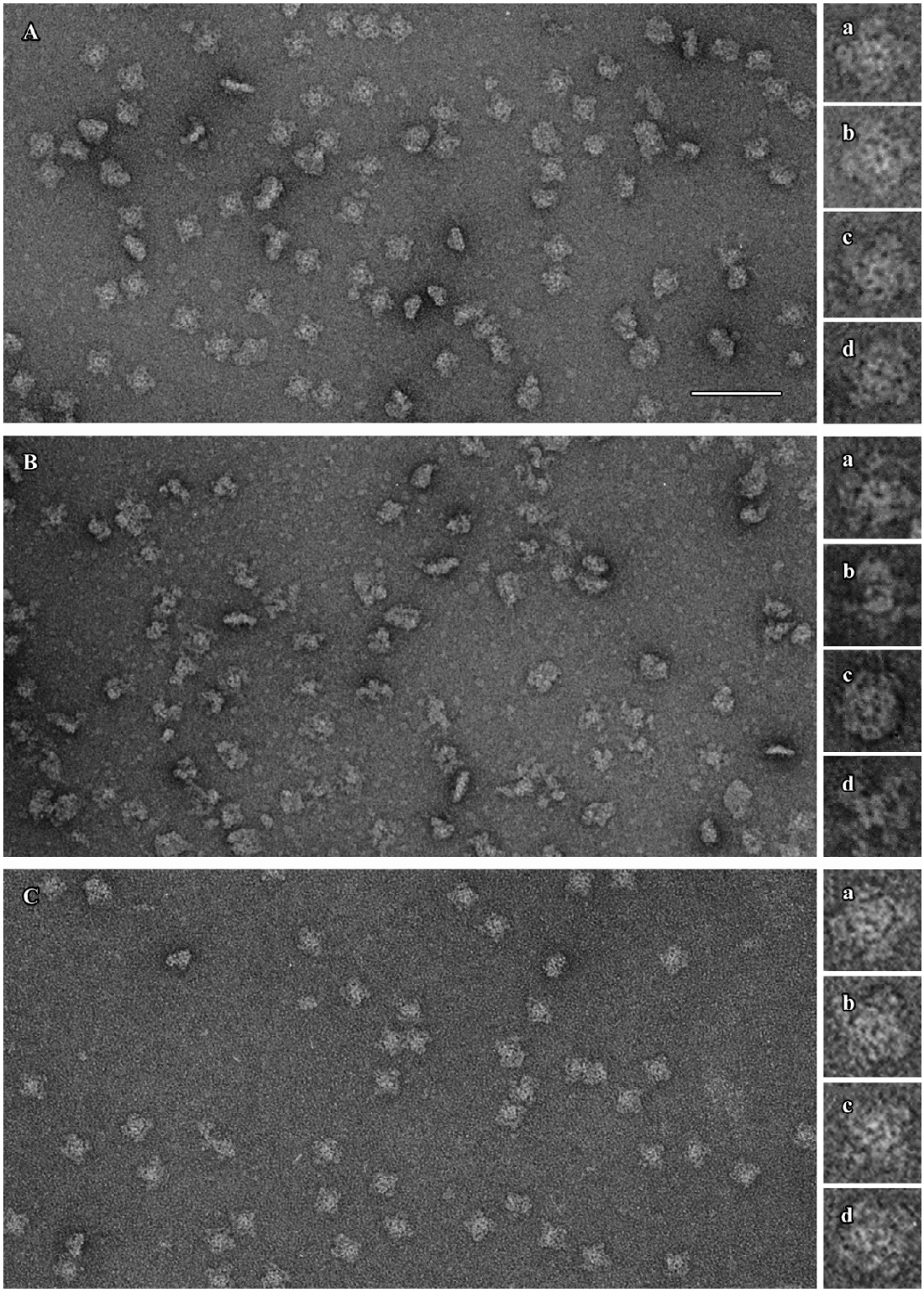

Figure 4. Electron microscopy of HMWC involving RyR1. SR vesicles were treated with 1 mmol/L H_2O_2 and RyR1 complexes were purified as described in Materials and methods. The purified RyR1 complexes were stained with uranyl acetate and examined by electron microscopy. (A) RyR1 particles purified from control SR membranes; (B) RyR1 particles purified from H_2O_2-treated SR membranes; (C) RyR1 particles purified from H_2O_2-treated SR membranes following treatment with 10 mmol/L DTT. Scale bar represents 100 nm. The corresponding galleries show the enlarged (magnification ×2.5) representative individual RyR1 particles.

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