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Cross-linking of the ryanodine receptor/Ca^{2+} release channel from skeletal muscle

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Abstract

The relationship between the tetrameric organization of the ryanodine receptor (RyR) and its activity in binding of ryanodine was approached through cross-linking studies using several bifunctional reagents, differing in their linear dimensions and flexibility, as well as in the reactivity of the active groups. Cross-linking with: 1,5-difluoro-2,4-dinitrobenzene (DFDNB); di(fluoro-3-nitrophenyl)sulfone (DFNPS), 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC); dimethyl suberimidate (DMS); ethylene glycol bis(succinimidylsuccinate) (EGS); and glutaraldehyde resulted in the disappearance of the, 470 kDa, RyR monomer protein band with concomitant appearance of additional bands of molecular masses higher than the monomer. At the relatively low concentrations of the reagents and the conditions used, RyR is the only cross-linked protein of SR membranes. The 'new' protein bands cross-react with antibodies against the RyR and correspond to dimers and tetramers of the RyR subunits while trimers were not detectable. DFDNB and DFNPS produced also a 560 kDa protein band which probably represents an intramolecular cross-linked monomer. The SDS-electrophoretic patterns of the cross-linked purified RyR resemble those of the membrane-bound receptor. Ryanodine binding to the high-affinity site was inhibited by modification of SR membranes with DFDNB and DFNPS, but not with DMS, EDC, EGS and glutaraldehyde, although RyR was completely cross-linked. The inhibition by DFDNB and DFNPS is due to modification of a specific lysyl residue which is also involved in the control of Ca^{2+} release. On the other hand, cross linking of the RyR with glutaraldehyde or EGS resulted in inhibition of ryanodine binding to the low-affinity, but not to the high-affinity binding sites. Thus, the cross-linking of two or more sites in each monomer (which lead to fixation of dimers or tetramers) did not prevent the conformational changes involved in the binding and occlusion of ryanodine at the high-affinity site, but inhibited its binding to the low-affinity sites.

Keywords: Sarcoplasmic reticulum; Ryanodine receptor; Cross-linking

1. Introduction

Depolarization of skeletal muscle cell membranes initiates a series of events, which result in the release of Ca^{2+} from the sarcoplasmic reticulum (SR) via Ca^{2+} regulated ion channels [1,2]. These channels are characterized by their ability to bind ryanodine [3,4], a plant toxic alkaloid. The purified ryanodine binding protein consists of high molecular weight polypeptides (~450 kDa), which are assembled into tetrameric complexes of apparent sedimentation coefficient of 30 S [5,6]. Quantitative analysis of [^{3}H]ryanodine binding to the purified 30 S receptor indicated a stoichiometry of one high affinity site per tetramer [7]. A tetrameric assembly of subunits for the ryanodine receptor was also suggested from ultra structural analysis using scanning transmission electron microscopy [7–9]. When incorporated into planar lipid bilayers, the purified protein exhibited a calcium conductance with pharmacological properties of the native SR Ca^{2+} release channel [9–11]. Structural analysis of the purified receptor suggests that it corresponds to the junctional feet structures which

Abbreviations: EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; Mops, 3-(*N*-morpholino)propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; DFNPS, di(4-fluoro-3-nitrophenyl)sulfone; DMS, dimethyl suberimidate; DNFB, 1-fluoro-2,4-dinitrobenzene; EGS, ethylene glycol bis(succinimidylsuccinate); DMSO, dimethyl sulfoxide; DTT, dithiothreitol; FITC, fluorescein 5'-isothiocyanate; RyR, ryanodine receptor.

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span the gap between SR junctional face membranes and the transverse tubule [12]. Cloning and sequence analysis of the cDNA [13–15] have completed the molecular picture of this membrane protein, and a molecular mass of 565 kDa was determined.

An important biophysical characteristic of the ryanodine receptor/Ca^{2+}- release channel is its organization into an oligomeric complex and the relationship between this organization and its activities both as a ryanodine binding protein and a Ca^{2+}-release channel. One approach to study these relationships is the use of chemical cross-linking.

The use of a cross-linker provided the possibility of investigating the arrangement of protein components in a complex, to determine the nearest neighbor relationships of membrane components and to study the structure-function relationships. In this paper, using several cross-linking reagents, the ryanodine receptor was cross-linked into dimers, tetramers and other intramolecular cross-linked species. This covalent cross-linking of the ryanodine receptor does not interfere with its ryanodine binding activity to the high-affinity site but prevents the binding to the low-affinity sites.

2. Experimental procedures

2.1. Materials

Glutaraldehyde, ATP, DFDNB, DFNPS, EGTA, Mops, Tricine, DMS and FDNB were obtained from Sigma. EDC and EGS were obtained from Pierce. [^{3}H]Ryanodine (60 Ci/mmol), and $^{45}CaCl_2$ were purchased from New England Nuclear. Unlabeled ryanodine was obtained from Calbiochem.

2.2. Membrane and purified ryanodine receptor preparations

Junctional SR membranes were prepared from rabbit fast-twitch skeletal muscle as described by Saito et al. [16] and the fraction R_4 was used in most of the experiments. The membranes were suspended to a final concentration of about 25 mg protein/ml in a buffer containing 0.25 M sucrose, 10 mM Tricine, pH 8.0 and 1 mM histidine, and stored at $-70°$ C. Protein concentration was determined by the method of Lowry et al. [17]. Ryanodine receptor was purified by the spermine-agarose method [18], and its protein concentration was determined as described previously [19].

2.3. Modification of SR membranes with the cross-linking reagents

Freshly prepared DFDNB, DFNPS and glutaraldehyde solutions were added to a mixture containing 20 mM Tricine, pH 8.0 and SR membranes (1 to 2 mg/ml). The reaction was carried out for 2 to 30 min at 37° C and terminated by the addition of DTT to a final concentration of 1 mM after which the samples were transferred to ice. The SR treatment with EDC or DMS was carried out for 30 min at 30° C in 50 mM imidazole, pH 7.0 or 20 mM Tricine, pH 8.6, respectively. The treatment with EGS (freshly prepared in DMSO) was carried out for 10 min at 24° C in 50 mM NaP_i, pH 7.2. The reaction was terminated by the addition of glycine to a final concentration of 40 mM. Aliquots were tested for ryanodine binding or processed for SDS-polyacrylamide gel electrophoresis as described below. SR vesicles were also modified with the reagents, under different conditions, as indicated in figure and table legends. DFDNB was prepared in 50% ethanol and DFNPS and FDNB were dissolved in DMSO. The final concentration of ethanol or DMSO in control and reagents containing samples was 2% (v/v) or less. DMS was dissolved in distilled water and the pH was adjusted to about 8.0.

2.4. [^{3}H]Ryanodine binding

Unless otherwise specified, control or modified SR membranes (final concentration of 0.5 mg/ml) were incubated for 1 to 2 h at 37° C with 20 nM [^{3}H]ryanodine in a standard binding solution containing 1.0 M NaCl, 20 mM Mops, pH 7.4 and 50 μM $CaCl_2$. Unbound ryanodine was separated from protein-bound ryanodine by filtration of protein aliquots (50 μg) through Whatman GF/C filters, followed by three washings with 5 ml of ice cold buffer containing 0.2 M NaCl, 5 mM Mops, pH 7.4 and 50 μM $CaCl_2$. The filters were dried and the retained radioactivity was determined by standard liquid scintillation counting techniques. Non specific binding was determined in the presence of 20 μM unlabeled ryanodine.

2.5. Gel electrophoresis

The modified and unmodified membranes were diluted with 1/3 volume of buffer containing 260 mM Tris-HCl, pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 4% (v/v) β-mercaptoethanol and incubated for 3 min at 100° C.

The analysis of protein profile by SDS-polyacrylamide slab gel electrophoresis was performed using Laemmli's [20] discontinuous buffer system in 1.5 mm thick slab gels with 3–13%, 3–6% or 2.6–6% acrylamide, using 3% or 2.6% stacking gel. Gels were stained with Coomassie brilliant blue. Molecular weight standards were: myosin, 200 000; β-galactosidase, 116 000; phosphorylase *b*, 97 400; bovine serum albumin, 66 200; and ovalbumin, 42 700 (Bio-Rad), and the phosphorylase *b* cross-linked (97 to 873 kDa, monomer to octamer, Sigma). Quantitative analysis of the protein bands was determined by densitometric scanning of the Coomassie stained gels, using a Molecular Dynamics computing densitometer and ImageQuant software provided by the manufacturer.

3. Results

Partial chemical cross-linking of polypeptides in oligomeric proteins results in the formation of dimers, trimers etc., which can be detected by SDS-PAGE [21,22]. However, intra-molecular cross-linking within the monomer was also observed in several proteins [23–25].

Fig. 1 shows the effect of preincubation of SR membranes with DFDNB, at relatively low concentration (0.5–50 μM), on the pattern of the SR polypeptides, as revealed by SDS-PAGE. As can be seen, the cross-linking of the SR membranes by DFDNB resulted in the disappearance of the 470 kDa protein band; ryanodine receptor monomer, with concomitant appearance of additional four bands of higher molecular masses than the monomer. At the relatively low concentrations of the reagent and the conditions used, ryanodine receptor is the only protein of the SR membranes that is cross-linked by DFDNB (Fig. 1A). For better demonstration of the cross-linking products (labeled a to d) the DFDNB-treated samples were also subjected to SDS-PAGE in linear gradient gels of relatively low concentrations of acrylamide; 3–6% (Fig. 1B) and 2.6–6% (Fig. 1C). An increase in the concentration of the cross-linking reagent leads to an increase in the number of bands, and in changes in their intensities (analyzed in Fig. 4). Like the RyR and its degradation product (350 kDa) the 'new' protein bands (a–d) cross react with polyclonal antibodies, prepared against the 470 kDa protein bands (RyR) cuts from gels and injected into guinea-pigs [39], suggesting that they are derived from the RyR (Fig. 2). No cross reactivity was obtained with antibodies prepared against the Ca^{2+}-ATPase (E52), sarcalumenin (G7 and G10, provided by Dr. D.H. MacLennan) or triadin (data not shown).

Due to the large molecular mass of these protein bands, precise molecular weights could not be accurately determined. Using the presently available molecular weight standards (see Experimental procedures), the cross-linking products (bands a to d) corresponded to relative molecular masses of 560, ~ 1000 and two bands of above 1000 kDa. This suggests that not all the bands represent covalent cross-linking between individual members of the ryanodine receptor complex. The 560 kDa band probably reflects an intramolecular (intersubunit) cross-linking which alters the mobility of the monomer (apparent M_r of 470000). However, we cannot rule out cross-linking between ryanodine receptor monomer and neighboring protein(s) suggested to be part of the foot structures [26,27]. The 1000 kDa band probably corresponds to the dimer, and the other two high molecular masses bands could be

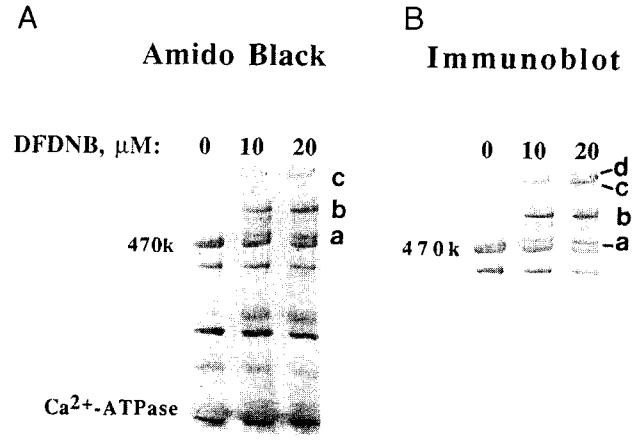

Fig. 2. Immunoenzymatic staining of the cross-linking products with anti-470 kDa antibodies. (A) Amido-black staining; (B) Immunoblot. SR membranes (1 mg/ml) were incubated without (control) or with 10 and 20 μM of DFDNB for 5 min as described in Fig. 1. Aliquots (20 μg) of the treated membranes were subjected to SDS-PAGE (2.6–6% acrylamide), followed by immunoblot analysis. Western blot analysis was carried out as described previously [38]. The separated proteins from SDS-PAGE were electrophoretically transferred onto Immobilon (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk and 0.1% Tween-20 in Tris-buffered saline, incubated with anti ryanodine receptor antibodies (1:500) and then with alkaline-phosphatase-conjugated protein A. The color was then developed (5 min) with 5-bromo-4-chloro-3-indolylphosphate and Nitro blue tetrazolium. Antibodies against the RyR were prepared as described previously [39]. Bands (a) to (d) are as in Fig. 3.

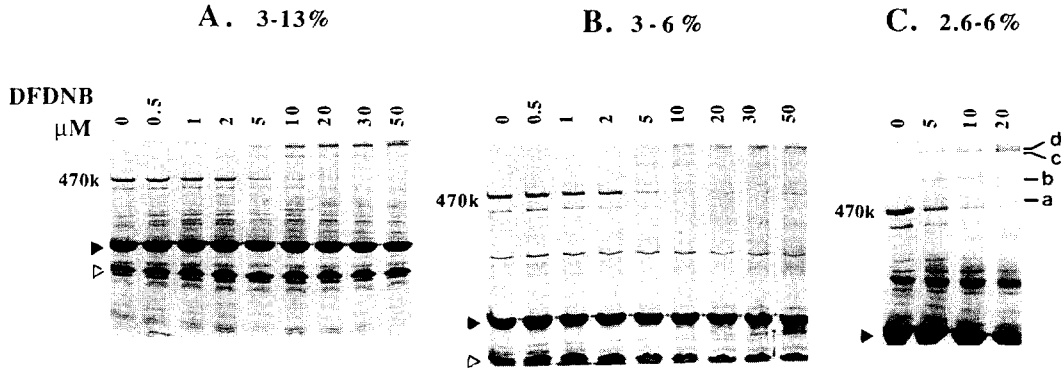

Fig. 1. SDS-PAGE profiles of SR membranes cross-linked with DFDNB. SR membranes (1.0 mg/ml) were incubated without or with the indicated concentration of DFDNB in 20 mM Tricine, pH 8.0. After 15 min at 37° C, DTT was added to a final concentration of 1 mM. Aliquots of the treated membranes (50 μg) were solubilized in SDS-buffer and subjected to 3–13% (A) or 3–6% (B) and 2.6–6% (C) SDS-PAGE as described under Experimental procedures. Full and open arrows indicate Ca^{2+}-ATPase and calsequestrin, respectively.

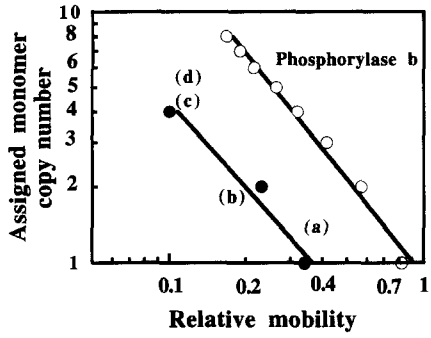

Fig. 3. Estimation of the apparent oligomeric state of the DFDNB cross-linking products in SDS-PAGE gels. SR membranes (1 mg/ml) were treated with DFDNB and subjected to SDS-PAGE. The mobilities of the Coomassie-blue stained bands observed in the SDS-PAGE gels were calculated relative to that of the Bromophenol blue tracking dye, and were plotted as a function of the logarithm of their assigned monomer copy numbers [22]. The monomer copy numbers assigned to the individual bands are: 470 kDa is the monomer, band (b) = dimer, and band (c) = tetramer. The positions of (a) and (d) are also indicated. A plot of the relative mobility of the phosphorylase *b* cross-linking products as a function of the logarithm of their monomer copy numbers is also presented. The results are the average of analysis of several gels.

trimers and tetramers. To test this proposal, a plot analysis of the logarithm of the assumed copy numbers versus the logarithm of the relative mobility of each of the bands [22] was carried out. The analysis of the RyR cross-linked products in several gels, and for comparison of a known cross-linked protein; phosphorylase *b* (Fig. 3) suggests the existence of a direct stoichiometric relationship between the monomer (470 kDa) and band b (dimers) and band c (tetramers). Band d may represent a pentamer, while the trimers are not detectable. Since it is well accepted that the ryanodine receptor is a tetramer [5–7,28], the apparent pentamer probably represents a tetramer with intrachain cross-linking.

Quantitative analysis of the 470 kDa band and of the cross-linking products (shown in Fig. 1C) is presented in Fig. 4, along with the effect of cross-linking on ryanodine binding. As can be seen, the treatment of SR membranes with DFDNB leads to a progressive disappearance of the ryanodine receptor monomer and to the loss of ryanodine binding activity. Half-maximal inhibition of ryanodine binding was observed at 50 μM DFDNB, while a 50% loss of the monomer was observed at 10 μM DFDNB, and complete effects with about 250 μM and 50 to 100 μM of DFDNB, respectively (Fig. 4A).

The relative proportions of the 560 kDa intramolecular cross-linked monomers (Mx), dimers (D) and tetramers (T) as a function of the DFDNB concentration is shown in Fig. 4B. The intramolecular cross-linked monomers are first to be formed, and are followed by the formation of the dimers and tetramers. This may suggest that the amino acid residues involved in the intramolecular cross-linking are more reactive than those involved in the formation of dimers and tetramers. Fig. 4B also shows that, as expected, the relative amount of the intramolecular cross-linked monomers and dimers is decreased upon tetramers formation. No linear relationship between the extent of inactivation of ryanodine binding and the disappearance of the monomer, or the appearance of either one of the cross-linking products was obtained (data not shown). This may suggest that the inhibition of ryanodine binding is not directly due to the cross-linking of the ryanodine receptor.

The time-and pH-dependence of DFDNB cross-linking of RyR together with its ryanodine binding activity is shown in Fig. 5. Quantitative analysis of the protein bands (as shown in Fig. 5A, inset), shows that increasing the time

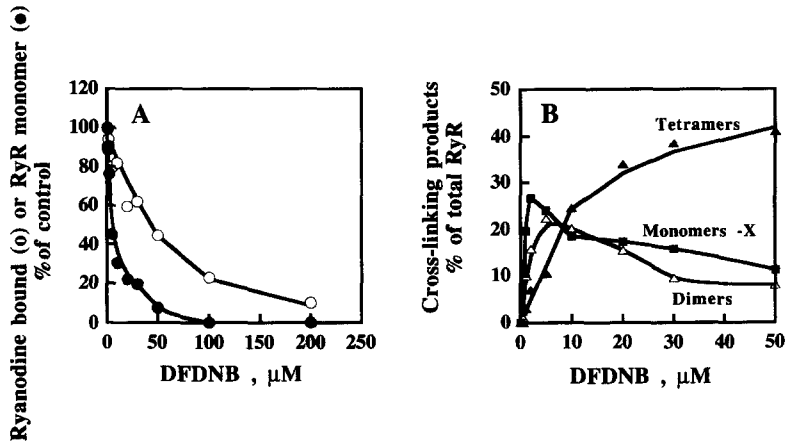

Fig. 4. Quantitative analysis of the ryanodine receptor cross-linking products and the relationship between them and the ryanodine binding inhibition. SR membranes (1.0 mg/ml) were treated with the indicated concentration of DFDNB as described in Fig. 1. Aliquots (50 μg) were subjected to SDS-PAGE (3–6% acrylamide) or were assayed for ryanodine binding (20 nM) (○) as described under Experimental procedures. Quantitative analysis of the ryanodine receptor monomers (●) in A and of the cross-linking products (△, ■, ▲) in B was carried out as described under Experimental procedures. In B, the amounts of the cross-linking products relative to that of the ryanodine receptor monomer are plotted. The intramolecular cross-linked tetramer (band d) is included in the amount of tetramers. The following symbols: ○, ■, △, ▲ indicate: monomers, intramolecular cross-linked monomers (Monomer-X), dimers and tetramers, respectively. Ryanodine binding activity (100%) = 8.2 pmol/mg protein.

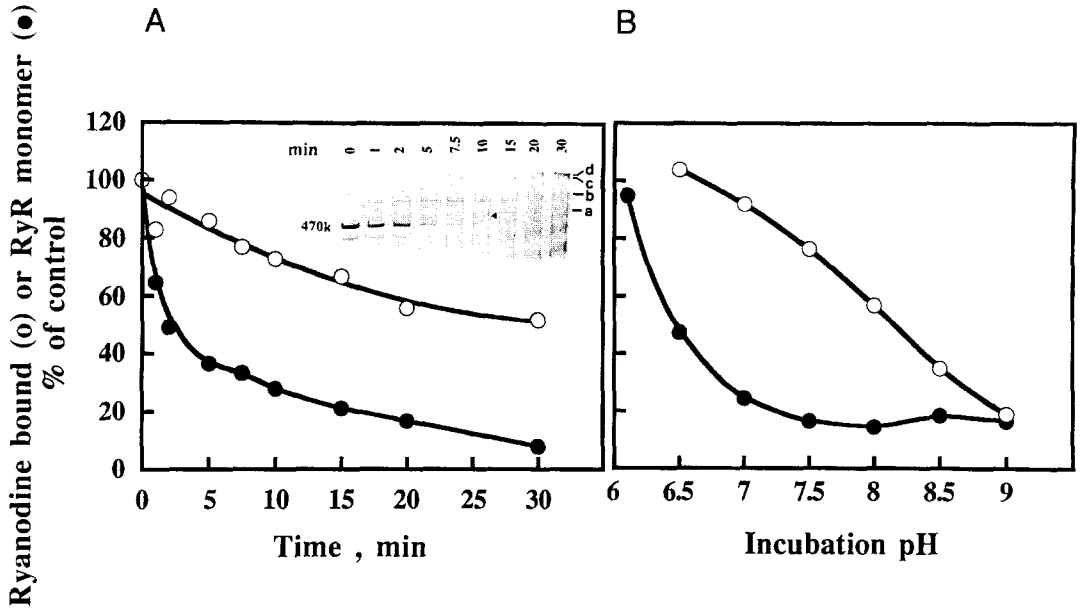

Fig. 5. Cross-linking of the ryanodine receptor with DFDNB as a function of incubation time or pH. (A) SR membranes (1.0 mg/ml) were incubated without or with 20 μM of DFDNB at pH 8.0 for the indicated time as in Fig. 1. Aliquots (50 μg) were subjected to SDS-PAGE (3 to 6%) (A, inset) or assayed for ryandine binding (20 nM). Quantitative analysis of the ryanodine receptor monomer was carried out as described under Experimental procedures. (B) SR membranes (1 mg/ml) were incubated with DFDNB (20 μM) at different pH values as in Fig. 1. The buffers used were 20 mM Mops for pH 6.5, 7.0 and 7.5 and 20 mM Tricine for pH 8.0, 8.5 and 9.0. Quantitative analysis of the monomer (●) and ryanodine binding (○) were carried out as described under Experimental procedures. Control activity (100%) was between 7 and 8.2 pmol ryanodine bound/mg protein.

of exposure to the reagent resulted in progressive inactivation of ryanodine binding ($t_{1/2} = 30$ min) and in the disappearance of the ryanodine receptor monomer ($t_{1/2} = 3$ min). The pH-dependence of the ryanodine binding inhibition and RyR monomer disappearance produced by the cross-linking with DFDNB is shown in Fig. 5B. Both the disappearance of the 470 kDa protein band and the inactivation of ryanodine binding increased with the increasing of pH in the DFDNB incubation medium. The results show a different pH dependency for the inactivation of the ryanodine binding and the disappearance of the ryanodine receptor monomer produced by DFDNB. The inactivation of ryanodine binding is consistent with reaction of an ionizable group with a pK_a of approx. 8.0, suggesting the involvement of lysyl residue in the inactivation of ryanodine binding. The results in Fig. 5 indicate that the inactivation of ryanodine binding by DFDNB is not directly related to the cross-linking of the ryanodine receptor.

The cross-linking of the ryanodine receptor by other

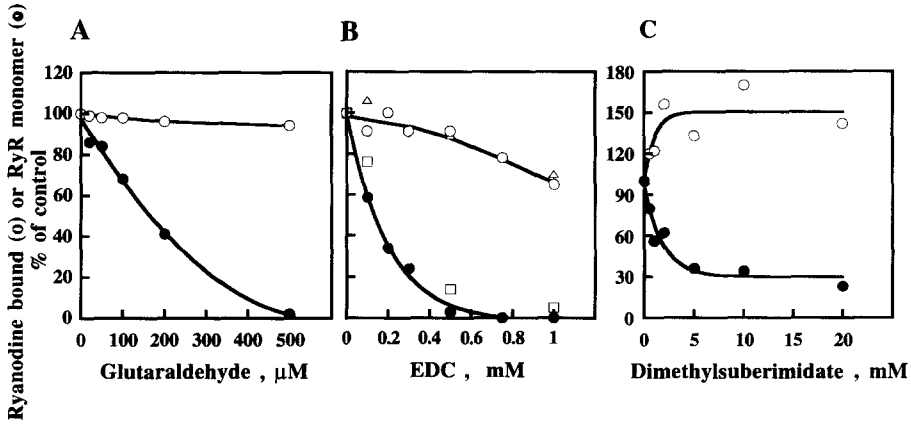

Fig. 6. Cross-linking of the ryanodine receptor with, glutaraldehyde, DMS and EDC. (A) SR membranes (1 mg/ml) were incubated for 15 min with the indicated concentration of glutaraldehyde (0.0002 to 0.005%) in 20 mM Tricine, pH 8.0 at 37° C. (B) SR membranes were incubated for 15 min with the indicated concentration of EDC in 50 mM imidazole, pH 7.0 at 30° C (○, ●) or 24° C (△, □). (C) SR membranes were incubated for 30 min with the indicated concentration of DMS in 20 mM Tricine, pH 8.6 at 30° C. Aliquots were assayed for ryanodine binding (20 nM) or subjected to SDS-PAGE (2.6–6%) and to quantitative analysis as described under Experimental procedures. Control activities (100%) were: 8.3, 5.3 and 2.9 pmol ryanodine bound/mg protein, for A, B and C, respectively.

bifunctional cross-linking reagents was studied. DFNPS has effective length of molecule of 9 Å compared to 5 Å for DFDNB, is also effective in producing the same cross-linking products and inactivation of ryanodine binding as DFDNB, although higher concentrations (20–300 μM) of the reagent are required. Here also there is no direct relationship between the inactivation of ryanodine binding and the disappearance of the 470 kDa (data not shown).

Another reagent shown to react with the purified ryanodine receptor and proposed to form dimers, trimers and tetramers is glutaraldehyde [28]. SDS-electrophoretic pattern of SR membranes treated with glutaraldehyde shows five 'new' protein bands (data not shown). According to the monomer copy number analysis [22], three of these protein bands corresponding to dimer, tetramer and pentamer which is probably intramolecular cross-linked tetramer. The other two bands, most likely, represent intramolecular cross-linking products with apparent molecular mass of 630 kDa and 705 kDa. The ryanodine binding activity of these glutaraldehyde treated membranes was not significantly affected, although the 470 kDa band completely disappeared (Fig. 6A). Also, both the ryanodine association with –and the dissociation from –its high-or low-affinity binding sites were not altered by the cross-linking of the ryanodine receptor with glutaraldehyde (data not shown). It should be mentioned that, in this work, we used glutaraldehyde concentrations about 100-times lower than those used for cross-linking of the purified ryanodine receptor [28] or the SR (Ca^{2+},Mg^{2+})-ATPase [24].

Cross-linking reactions were also catalyzed with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), a water-soluble carbodiimide that forms zero-length covalent bonds between carboxyl groups and nucleophilic groups, probably amino groups, in contact area between polypeptides or within it [30]. Fig. 6B shows the effect of EDC-modification of SR membranes on their ryanodine binding activity and on the cross-linking of the ryanodine receptor. EDC, at relatively low concentrations, induces cross-linking of the ryanodine receptor into several cross-linked species. This cross-linking does not impair the ryanodine binding activity.

Dimethyl suberimidate (DMS) reacts specifically with amino groups to produce amidine cross-links among the protomers of oligomeric proteins where the amido ester retains the same charge as the unreacted protein [29]. In contrast to DFDNB, DFNPS and glutaraldehyde, DMS at pH 8.0 and in concentrations up to 20 mM and incubation time up to 60 min at 37° C was ineffective in inhibiting ryanodine binding or producing cross-linking products. However, increasing the incubation pH to 8.6 resulted in cross-linking of the ryanodine receptor with about 30% stimulation of ryanodine binding (Fig. 6C).

Similar results as with glutaraldehyde, with respect to the cross linked products and the lack of effect on ryanodine binding to the high-affinity site, were obtained with another reagent, the lysyl-specific hydrophobic reagent; ethylene glycol bis(succinimidylsuccinate) (EGS) (see Fig. 10).

It seems important that the cross-linking of the ryanodine receptor takes place irrespectively of it being a membrane-bound or a purified soluble protein (Fig. 7). As shown DFDNB cross-linked the purified ryanodine receptor to form the intra-and inter-molecular cross-linking, and in parallel inhibited ryanodine binding (Fig. 7A). Glutaraldehyde cross-linked the purified receptor with no significant effect on ryanodine binding (Fig. 7B). A monomer copy numbers analysis [22] of the results indicates that the cross-linking products obtained with DFDNB and glu-

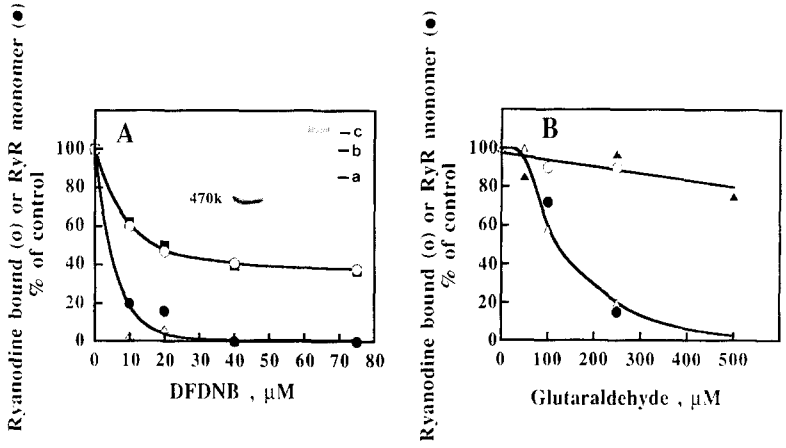

Fig. 7. Cross-linking of the purified ryanodine receptor with DFDNB and glutaraldehyde. Purified ryanodine receptor was first separated from DTT and sucrose by the chromatography-centrifugation method [37] using Sephadex G-50 pre-equilibrated with 0.2 M NaCl, 0.4% CHAPS and 20 mM Tricine, pH 8.0. Aliquots 4 to 5.4 μg were incubated with the indicated concentration of DFDNB (A) or with glutaraldehyde (B) as in Fig. 6. Then the protein samples were incubated for 10 min with phospholipids (2 mg/ml) and then assayed for ryanodine binding (20 nM) (○) as described previously [18] or subjected to SDS-PAGE (2.6–6%) and to quantitative analysis (●) as described under Experimental procedures. Control activity (100%) was: 101 pmol/mg protein. (■), (△) and (▲) indicate ryanodine binding and the relative amount of RyR, respectively, results from other experiments. Inset shows the SDS-PAGE profile of purified RyR incubated without or with 10 or 20 μM of DFDNB.

Table 1
Effect of prior chemical modification of SR membranes with DNFB or FITC on the cross-linking of the ryanodine receptor with DFDNB

Treatment I	Treatment II DFDNB (min)	Ryanodine binding (% of control)	Relative amount % of total RyR			
			M	Mx	D	T
(A) Control	0	100	100	0	0	0
	2	66	53	12	26	8
	6	33	8	9	19	36
	10	22	9	6	15	58
(B) FITC (100 μM)	0	46	100	0	0	0
	2	20	65	0	8	8
	6	7	18	0	10	31
	10	7	9	0	14	57
(C) DNFB (120 μM)	0	16	100	0	0	0
	2	12	83	0	0	0
	6	7	49	0	0	19
	10	4	41	0	0	9

SR membranes (1 mg/ml) were incubated with the indicated concentration of FITC or FDNB for 8 min or 15 min, respectively, in 20 mM Tricine, pH 8.0 (treatment I). Aliquots of the FITC-or FDNB-modified membranes were incubated with 20 μM DFDNB for the indicated time (treatment II). Control and modified membranes were assayed for ryanodine binding activity and analyzed by SDS-PAGE as described in Fig. 4. Control activity (100%) = 3.7 pmol/mg protein. M, Mx, D and T indicate: monomers, monomers cross-linked, dimers and tetramers, respectively.

taraldehyde are the same as those obtained with the membrane-bound receptor.

Comparison of the cross-linking of the RyR by the different bifunctional reagents as reflected by the appearance of the various cross-linking products and by their effects on ryanodine binding may suggest that the inhibition of ryanodine binding by DFDNB and DFNPS is due to the formation of the 560 kDa cross-linked product, or due to modification of an essential amino group(s) by the reagents. To modify amino groups with no cross-linking of the RyR, we used two monofunctional reagents that react with selectivity for amino groups. We have shown recently [31] that fluorescein 5′-isothiocyanate (FITC) a lysyl-specific reagent, interacts with the ryanodine receptor, inhibited ryanodine binding and promoted the appearance of an open Ca^{2+} release channel with sub conductance states. The effect of prior chemical modification of the ryanodine receptor with the monofunctional reagent, FITC, on the cross-linking of the 470 kDa by DFDNB is shown in Table 1. FITC modification diminished the formation of the 560 kDa cross-linked product. The appearance of the dimers and the other two high molecular masses cross-linking products was only slightly affected by this FITC modification. Table 1 also shows that prior modification of the ryanodine receptor with another monofunctional reagent; DNFB which inhibits ryanodine binding followed by treatment with DFDNB, resulted in a decrease in the disappearance of the ryanodine receptor monomer and in the appearance of the cross-linking products produced by cross-linking with DFDNB. These results suggest that the inhibition of ryanodine binding by DFDNB is due to modification of a lysyl residue(s), similarly to the effect of FITC [31]. This conclusion is supported by the observation that FITC inhibits ryanodine binding in SR membranes where the RyR is crossed-linked with glutaraldehyde (data not shown). These results suggest that in addition to its cross-linking activity, DFDNB acts similarly to FITC [31], modifies an amino group(s) essential for ryanodine binding.

Fig. 8 shows the effect of DFDNB modification of junctional SR membranes on their ryanodine binding affinity (K_d) and the total binding sites (B_{max}). K_d values of;

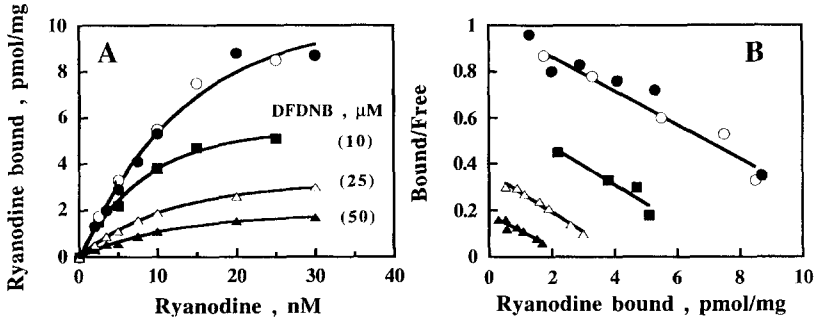

Fig. 8. The influence of DFDNB modification on the affinity and number of the high affinity ryanodine binding sites. HSR membranes were incubated without (○, ●) or with DFDNB: 10 (■), 25 (△) or 50 (▲) μM, as in Fig. 1, and then ryanodine binding was assayed as a function of its concentration as described in Experimental procedures. Saturation isotherms are shown in (A) and Scatchard plots in (B).

13.5, 12.5, 12, and 14 nM were obtained for control and membranes modified with 10, 25, and 50 μM of DFDNB, respectively. The total high-affinity binding sites, however, were decreased with increasing DFDNB concentration from 13.6 for control to 7.9, 4.3, and 2.6 pmol/mg protein for membranes modified with 10, 25, and 50 μM of DFDNB, respectively.

The results illustrated in Fig. 9 are consistent with the suggestion that in addition to its cross-linking activity, DFDNB similar to FITC [31], stimulates Ca^{2+} efflux from SR vesicles. Modification of SR membranes with DFDNB, but not with glutaraldehyde, resulted in a stimulation of Ca^{2+} efflux from SR vesicles passively loaded with $^{45}CaCl_2$. Fig. 9 also shows that DFDNB modification, which results in inhibition of ryanodine binding, diminished the activation of Ca^{2+} efflux by ryanodine. In contrast, glutaraldehyde which has no significant effect on ryanodine binding affects the ryanodine-induced Ca^{2+} efflux only slightly (Fig. 9B). Since the RyR was crossed linked almost completely (about 90%) by glutaraldehyde, these results suggest that the cross-linking of the RyR is not responsible for the activation of Ca^{2+} efflux produced by DFDNB. As suggested for the inhibition of ryanodine binding by DFDNB modification, the activation of Ca^{2+} efflux by this reagent is due to modification of a specific

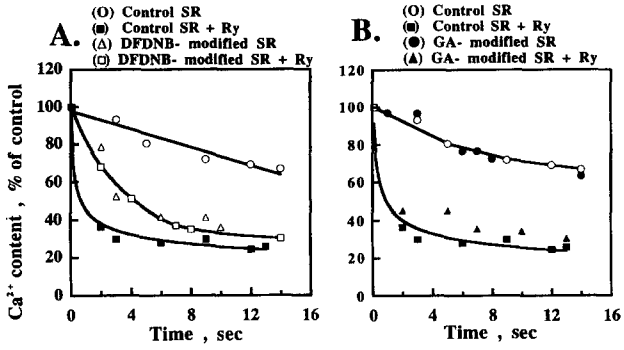

Fig. 9. Effect of cross-linking of RyR with DFDNB and glutaraldehyde on the membrane permeability for Ca^{2+} and on the activation of Ca^{2+} release by ryanodine. SR vesicles were incubated without (○) or with DFDNB (60 μM) (△, □) (A) or with glutaraldehyde (400 μM) (●, ▲) (B) for 15 min as described in Experimental procedures. Aliquots were assayed for ryanodine binding activity or subjected to SDS-PAGE. For passive loading with $^{45}CaCl_2$, the membranes were collected by centrifugation (40000 × g, 30 min) and resuspended (2 mg/ml) in a medium containing 0.3 M KCl, 20 mM Mops, pH 6.8 and 0.4 mM $CaCl_2$ (containing $^{45}CaCl_2$ $5 \cdot 10^4$ cpm/nmol), and incubated for 90 min at 24° C. Ryanodine to a final concentration of 0.5 μM was added to aliquots of the Ca^{2+} loaded vesicles of control (■), DFDNB (□) and glutaraldehyde-treated SR (▲), and then the membranes were incubated for additional 30 min. Ca^{2+} efflux assay was carried out as described previously [40], the loaded vesicles (20 μl) were placed on 0.45 μm nitrocellulose filters and the efflux was initiated by rinsing with different volumes of 0.3 M KCl, 20 mM Mops, pH 6.8 and 1 mM EGTA solution for the indicated time. The flow rate was 1 ml/s. Ryanodine binding activities were: 9.7, 3.5 and 9.4 pmol/mg protein for control, DFDNB-and glutaraldehyde-treated membranes, respectively. Ca^{2+} content of the loaded SR (100%) was: 9 nmol/mg protein.

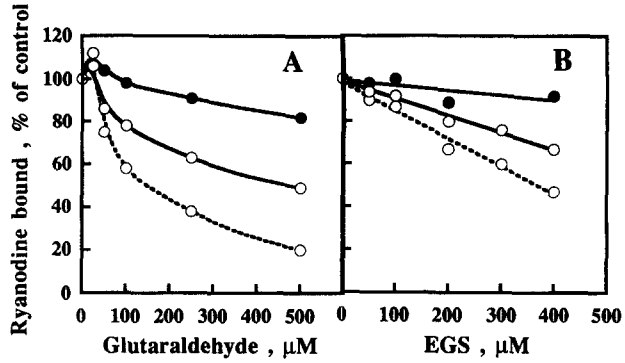

Fig. 10. Cross-linking of the ryanodine receptor with glutaraldehyde (A) and EGS (B) inhibits ryanodine binding to the low-but not to the high-affinity sites. (A) SR membranes were treated with the indicated concentrations of glutaraldehyde as in Fig. 6 or were incubated with the indicated concentration of EGS as described under Experimental procedures. Ryanodine binding to the unmodified and EGS-or glutaraldehyde-modified membranes were assayed as described under Experimental procedures in the presence of 20 nM (●) or 2 μM (○) of ryanodine. [^{3}H]Ryanodine (60 Ci/mmol) was diluted with unlabeled ryanodine 1:3 or 1:25 for 20 nM and 2 μM, respectively. Non-specific binding was determined in the presence of 500-fold excess of unlabeled ryanodine. Control activities (100%) were for A: 6.2 and 23 and for B: 4.3 and 20 pmol/mg protein for 20 nM and 2 μM of ryanodine, respectively. The dashed lines represent the results with 2 μM ryanodine after subtracting the binding to the high-affinity site. At the highest concentrations of glutaraldehyde or EGS used, RyR monomer was completely cross linked (not shown).

amino group(s) in the RyR. This is similar to previous results obtained with FITC modification [31].

Fig. 10 illustrates the inhibitory effect of cross-linking of the membrane-bound RyR with glutaraldehyde or EGS on its ryanodine binding to the low-affinity sites. Both reagents have no effect on ryanodine binding to the high-affinity site, but inhibit the binding to the low-affinity sites. Although the RyR monomer was completely disappeared (data not shown), ryanodine binding to the low-affinity site was only partially inhibited. The results from other experiments indicate that cross-linking of the RyR with glutaraldehyde has no significant effect on the K_d (16.5 ± 2.5 and 16.3 ± 3.7 nM for control and glutaraldehyde SR, respectively) and on the B_{max} (16.5 ± 0.5 and 13.7 ± 1.4 pmol/mg for control and glutaraldehyde SR, respectively) of the high-affinity ryanodine binding site, but this cross-linking decreased the binding of ryanodine to the low-affinity sites. Thus, the low-affinity but not the high-affinity ryanodine binding sites were altered by the cross-linking of the ryanodine receptor with glutaraldehyde and EGS.

4. Discussion

The relationship between the tetrameric organization of the ryanodine receptor and its ryanodine binding and Ca^{2+} release activities were approached through cross-linking

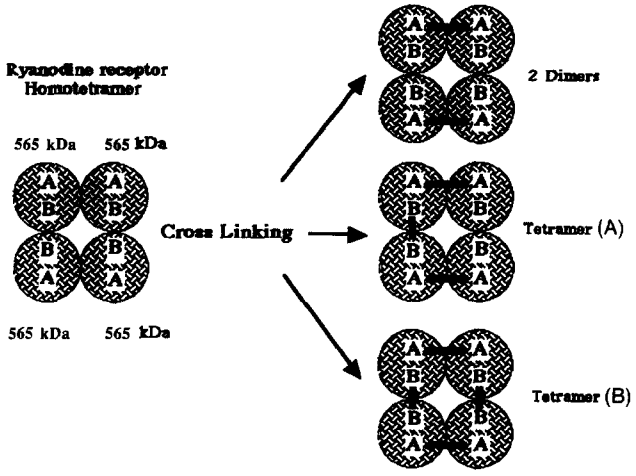

Fig. 11. Schematic model for the formation of dimers and tetramers but not trimers. A and B represent amino acid residues with different reactivity towards the cross-linking reagents.

studies using several bifunctional reagents. Under the conditions used here, treatment of SR membranes with several bifunctional cross-linking reagents resulted in cross-linking of the RyR, but of almost no other SR proteins. Cross-linking with DFDNB, DFNPS, DMS, EDC, EGS and glutaraldehyde resulted in the appearance of additional protein bands in the SDS-electrophoretograms, which corresponded approximately to the dimer and tetramer of the RyR subunits. These protein bands cross react with antibodies prepared against the 470 kDa protein band (RyR subunits) (Fig. 2), but not with antibodies against the Ca^{2+}-ATPase, sarcalumenin or triadin. However, two bands are visible at the position of the tetramer (Figs. 1 and 2) where the upper band, according to monomer copy numbers analysis [22] (Fig. 3), corresponds to pentamer. Since it is well accepted that the ryanodine receptor is a tetramer [5–7,28], this observation could be explained by assuming that the tetramer-forming protomers are cross-linked in two different ways (see Fig. 11 tetramer (A) and tetramer (B)), one of which migrates as a species with higher apparent molecular mass. It is also possible that within each molecular weight category, there is a variety of species with different numbers of intra-and interchain cross-links. An additional band with M_r of 560000 was obtained with DFDNB and DFNPS but not with EDC, DMS, EGS and glutaraldehyde. This band is probably formed by intrapeptide cross-linking, which alters the hydrodynamic properties of the monomer. A similar phenomenon has been shown for other proteins [23,24]. It is possible that more than one type of cross-linking is responsible for the formation of the 560 kDa. However, the generation of the 560 kDa cross-linking product could be also attributed to cross-linking of the RyR monomers (470 kDa) and an unidentified protein(s) (other than the Ca^{2+}-ATPase, sarcalumenine or triadin) associated with the 'foot' structure complex. Given the fact that the formation of dimers precedes that of tetramers (Figs. 4 and 5), and assuming that the reacting residues in the homotetramers are equally reactive towards the cross linkers, the absence of detectable trimers is expected. Indeed, occurrence of a single additional cross link between two dimers would lead to the formation of a tetramer (see Fig. 11).

The SDS-electrophoretic patterns of the purified cross-linked RyR resemble those of the membrane-bound receptor (Fig. 7). This is not surprising, since the major part of the ryanodine receptor molecule protrudes from the membrane [13], thus the accessibility of soluble and membrane bound RyR to cross-linking reagents in solution should be rather similar. The fact that the CHAPS-solubilized, purified ryanodine receptor is also cross-linked with DFDNB and glutaraldehyde supports the concept [5–7,28] that ryanodine receptor is an oligomer both in the solubilized and membrane bound forms, and that the soluble-purified-protein preserves the native structure of the complex.

Comparison of cross-linking using a zero length cross-linker; EDC, and DFDNB, whose approximate dimension between the two reactive groups is of 5 Å [21], indicates a tight contact between the subunits in the tetramer. Cross-linking is also observed with EGS, DFNPS and with DMS which have effective lengths of 6.4 Å, 9 Å and 11 Å, respectively [21]. The approximate distance between the glutaraldehyde reactive groups could not be estimated, because at neutral or slightly basic pH, the predominant reactive species of glutaraldehyde is the α,β-unsaturated aldehyde polymers, where the number of monomers is increased, as the pH is raised [34]. These results suggest that there is no critical distance between the ryanodine receptor reactive groups towards the bifunctional reagents, and that they are probably located in the protein, so they are accessible to both uncharged reagents (DFDNB, DFNPS), and to the charged DMS at alkaline pH (8.6). The finding that prior chemical modification with FITC, or FDNB which are specific for lysyl residues decreased the appearance of the cross-linked products (Table 1) implicates the involvement of lysyl residues in this cross-linking.

Our results show that ryanodine binding to the high-affinity site was not altered upon cross-linking of the ryanodine receptor with glutaraldehyde, DMS, EGS or EDC (Figs. 6, 7 and 10). No correlation was found between the disappearance of the RyR monomer or the appearance of its cross-linking products, and the inhibition of ryanodine binding produced by DFDNB and DFNPS (Figs. 4, 5 and 7). These observations suggest that cross-linking of the RyR to produce dimers or tetramers does not interfere with its ryanodine binding activity to the high-affinity site. On the other hand, the results may suggest that the 560 kDa cross-linked product produced by DFDNB and DFNPS is responsible for the inactivation of ryanodine binding. However, it is unlikely that the intramolecular cross-linking by itself causes the inactivation of ryanodine binding,

but rather the modification of a specific lysyl residue(s). We have recently shown that modification of SR membranes with FITC (modifies a lysyl residue(s)) resulted in inhibition of ryanodine binding and activation Ca^{2+}-release channels [31]. Similarly, DFDNB inhibits ryanodine binding and stimulates Ca^{2+} efflux from SR passively loaded with $^{45}CaCl_2$ (Fig. 9). Also, as with FITC [31], DFDNB modification decreases the total high-affinity ryanodine binding sites with no significant effect on the ryanodine binding affinity (Fig. 8).

Ryanodine receptor possesses, in addition to the high-affinity ryanodine binding site, 2 to 3 binding sites of lower affinity [28,32,33]. It has been suggested that the ryanodine receptor exhibits 'positive cooperativity' where binding at the low-affinity sites decreases the dissociation rate of ryanodine from the high-affinity site [28,32]. A model for the allosteric negative cooperativity between the four ryanodine binding sites in the homotetramer RyR was recently proposed [33]. The model suggests that the binding of ryanodine to one of the four sites (with high affinity) triggers the conversion of the other sites to low affinity(ies) binding sites. According to Monod et al. [35], this suggests that in order for RyR to manifest binding cooperativity, there must be two or more different states of the receptor with different affinities. Also, the protein must undergo a conformational change(s) in each subunit. In light of the allosteric interaction between the RyR subunits [28,32,33], the observation that 'fixation' of the RyR in irreversible tetrameric structure(s) do not affect its ryanodine binding to the high-affinity site is of interest. Ryanodine binding is Ca^{2+}-dependent and the complex [Ca^{2+}·RyR] is a very stable complex from which ryanodine is dissociated very slowly ($t_{1/2}$ of 14.4 h [36]). This would suggest that RyR undergoes conformational changes upon Ca^{2+} and/or ryanodine binding, where in the 'new' conformation, ryanodine is physically occluded and hindered from free diffusion from its binding site. Thus, the cross-linking of 2 or more residues in each monomer (which lead to fixation of dimers or tetramers) does not prevent the conformational changes involved in binding and occlusion of ryanodine at the high-affinity site. On the other hand, cross-linking of the RyR with glutaraldehyde or EGS results in a partial inhibition of ryanodine binding to the low-affinity binding sites (Fig. 10) due to a decrease in the number of the low-affinity sites.

In conclusion, we have described in this article covalent cross-linking of the RyR by six different bifunctional reagents, differing in their linear dimensions and flexibility, as well as in the reactivity of the active groups. A linkage between the RyR subunits to form irreversible dimers or tetramers does not affect its ryanodine binding to the high-affinity site, but inhibits the binding to the low-affinity sites. We anticipate that further investigation should provide important insights into the conformational changes involved in the Ca^{2+}-dependent ryanodine binding to both the high- and the low-affinity binding sites.

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