Modification of ryanodine receptor/ Ca^{2+} release channel with dinitrofluorobenzene

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Modification of the ryanodine receptor (RyR)/ Ca^{2+} release channel with 2,4-dinitrofluorobenzene (DNFB) indicated that two classes of amino group interact with the reagent, as can be distinguished on the basis of their reactivity/accessibility and the effects on ryanodine binding and single channel activities. One group interacted very rapidly ($t_{1/2} < 30$ s) at 25 °C with low concentrations of DNFB [C_{50} (concentration of DNFB required for 50 % inhibition or stimulation of ryanodine binding) = 5 μM], and at pH values of 6.2 and higher. This interaction resulted in the marked stimulation of ryanodine binding and the complete inhibition of a single Ca^{2+} release channel incorporated into planar lipid bilayer. The second group is accessible at higher temperatures (37 °C); at pH values higher than 7.4 it reacted slowly ($t_{1/2} = 20$ min) with high concentrations of DNFB ($C_{50} = 70$ μM). This interaction led to the inhibition of ryanodine binding and single channel activity. Modification of RyR with DNFB under the stimulatory conditions resulted in 3.6-fold and 6-fold increases in ryanodine-binding and Ca^{2+} -binding affinities respectively. Modification with DNFB under the inhibitory conditions resulted in a decrease in the total ryanodine-binding sites. The exposure of the RyR single channel to DNFB under both inhibitory and stimulatory conditions led to the complete

closure of the channel. However, when modified under the stimulatory conditions, but not under the inhibitory ones, the DNFB-modified closed channel could be re-activated by sub-micromolar concentrations of ryanodine, in the presence of nanomolar concentrations of Ca^{2+} . The DNFB-modified ryanodine-activated RyR channel showed fast transitions between open, closed and several sub-conductance states, and was completely closed by Ruthenium Red. ATP re-activated the DNFB-modified closed channel or, if present during modification, prevented the inhibition of RyR channel activity by DNFB. Neither the stimulation nor the inhibition of ryanodine binding by modification with DNFB was affected by the presence of ATP. By using the photoreactive ATP analogue 3'-O-(4-benzoyl)benzoyl- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ we found that DNFB modification had no effect on the ATP-binding site of RyR. The results are discussed with regard to the involvement of amino group residues in channel gating, ryanodine association/dissociation and occlusion, and the relationship between the open/closed state of the RyR and its capacity to bind ryanodine.

Key words: sarcoplasmic reticulum, single channel.

INTRODUCTION

During excitation–contraction coupling, stored Ca^{2+} is released from the sarcoplasmic reticulum (SR) into the myoplasm via the ryanodine receptor (RyR)/ Ca^{2+} release channel located at the junctional face of the SR [1–6]. One major hypothesis for excitation–contraction coupling suggests that a dihydropyridine receptor, a voltage sensor molecule located on transverse tubules, undergoes conformational changes in response to depolarization [7,8]. These changes might be transmitted to the SR RyR/ Ca^{2+} release channel and could modulate its opening [7,8]. RyR is a homotetrameric complex composed of 565 kDa monomers; it exhibits intrinsic ryanodine-sensitive Ca^{2+} release channel activity and is modulated by Ca^{2+} , adenine nucleotides, calmodulin and Mg^{2+} (reviewed in [2–6]).

Chemical modification of proteins by various covalent probes has been widely used to establish the functional importance of amino acid residues in enzyme activity, and to elucidate the structure–function relations in various proteins [9]. Chemical modification of the RyR/ Ca^{2+} release channel, with various reagents directed towards amino acid side chains, have suggested the involvement of specific lysine, thiol, histidine and carboxy residues in channel activation or inactivation (reviewed in [10]).

FITC, which reacts specifically with the ϵ -amino groups of lysine residues, decreases ryanodine binding and markedly altered

single Ca^{2+} -release channel gating behaviour [11]. The fluorescent reagent NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) can react with the OH group of tyrosine, the SH group of cysteine and the $\epsilon\text{-NH}_2$ group of lysine [12]. The interaction of NBD-Cl with the $\epsilon\text{-NH}_2$ group of lysine in RyR results in the time-dependent activation and inactivation of ryanodine binding and single-channel activities, suggesting the involvement of lysine residues in the gating of the RyR channel [13]. DIDS (4,4'-diisothiocyanostilbene-2,2'-disulphonate), a known anion-channel blocker, was found to activate Ca^{2+} release in isolated SR vesicles and the RyR channel activity [14,15]. DIDS increased the open probability of cardiac Ca^{2+} release channel by inducing a long-lived open state, suggesting that DIDS modifies an amino group residing in the gating structure of the channel [14].

The involvement of SH groups in the modulation of the Ca^{2+} release channel has been proposed [16–19]. It has been postulated that the oxidation of SH groups to disulphides activates Ca^{2+} release, whereas reduction of the disulphides inhibits Ca^{2+} release [16]. Modulation of RyR activity by multiple classes of thiols has also been proposed [18]. It has recently been suggested that the RyR/ Ca^{2+} release channel is regulated by post-translational chemical modification(s) of sulphur atoms [19].

Dicyclohexylcarbodi-imide, a hydrophobic carboxy-modifying reagent, was found to inhibit ryanodine binding to RyR and to block channel opening completely [20]. It has been suggested that

Abbreviations used: Bz₂ATP, 3'-O-(4-benzoyl)benzoyl-ATP; DEPC, diethyl pyrocarbonate; DNFB, 2,4-dinitrofluorobenzene; DTT, dithiothreitol; PLB, planar lipid bilayer; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

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carboxy residues are linked to ryanodine binding directly or via the Ca^{2+} -binding sites [20,21].

Diethyl pyrocarbonate (DEPC), which reacts with histidine residues in proteins, was shown to induce the release of Ca^{2+} from SR vesicles [22]. Modification of RyR by DEPC inhibited ryanodine binding, markedly increased the permeability of SR vesicles to Ca^{2+} [23] and activated the RyR/ Ca^{2+} release channel incorporated into a planar lipid bilayer (PLB) at very low Ca^{2+} concentrations [10].

In the present study we used 2,4-dinitrofluorobenzene (DNFB), which reacts with amines to form dinitrophenyl derivatives, to modify the RyR activity. Depending on the modification conditions, DNFB was found either to inhibit or to stimulate ryanodine binding, to cause complete closure of the RyR single channel that can be re-activated by ATP and ryanodine.

EXPERIMENTAL

Materials

ATP, EGTA, Tris, CsCl, Hepes, Tricine, Mops, Mes, CHAPS, PMSF, DNFB, trypsin, soybean trypsin inhibitor and monoclonal anti-dinitrophenyl antibodies were obtained from Sigma. [α - ^{32}P]ATP and [^3H]ryanodine were purchased from New England Nuclear; unlabelled ryanodine was from Calbiochem. Molecular mass standard was from Bio-Rad. 3'-O-(4-Benzoyl)-benzoyl-ATP (Bz_2ATP) and [α - ^{32}P] Bz_2ATP were synthesized and purified as described previously [24]. Polyclonal antibodies against rabbit skeletal muscle RyR were raised in guinea-pigs [24].

Membranes and RyR preparations

SR membranes were prepared from rabbit fast-twitch skeletal muscle as described by MacLennan [25], except that 0.12 mM PMSF, 0.5 $\mu\text{g}/\text{ml}$ leupeptin and 0.4 mM benzamidine were added to all isolation solutions. The membranes were suspended to a final concentration of approx. 25 mg/ml protein in a buffer containing 0.25 M sucrose and 10 mM Tricine/NaOH, pH 7.4, and stored at -70°C . RyR was purified from unmodified and DNFB-modified SR by sucrose-gradient centrifugation [2]. Membrane-bound protein concentrations were determined by the method of Lowry et al. [26]; soluble purified RyR concentrations were measured by the method of Kaplan and Pedersen [27].

Modification with DNFB

SR (1.0 mg/ml) was incubated with DNFB under two different conditions: (1) stimulatory condition, in which the SR was incubated with DNFB for 15 min at 25°C in a solution containing 0.5 M CsCl and 10 mM Hepes, pH 7.6; (2) inhibitory condition, in which SR was incubated for 15 min at 37°C in 10 mM Tricine, pH 8.0. The reaction with DNFB was terminated by the addition of dithiothreitol (DTT) to a final concentration of 1–3 mM. Control and modified membranes were diluted 1:1 with 2-fold concentrated reaction mixture for ryanodine binding. DNFB was dissolved in DMSO; its final concentration in control and DNFB-modified samples was 1% (v/v). Modification of SR with DNFB was also performed under different conditions, as indicated in the figure and table legends.

Binding of [^3H]ryanodine

Unless specified otherwise, SR (0.5 mg/ml) was incubated for 1–2 hr at 37°C with 20 nM [^3H]ryanodine (20 Ci/nmol), in a solution containing 1.0 M NaCl and 20 mM Mops, pH 7.4, with

either 0.3 or 50 μM CaCl_2 . Membrane aliquots (50 μg) were filtered through Whatman GF/C filters, followed by washing three times with 5 ml each of ice-cold buffer A [0.2 M NaCl/10 mM Mops (pH 7.4)/50 μM CaCl_2]. Radioactivity in filters was determined by liquid scintillation. The binding of [^3H]ryanodine to purified RyR was assayed under the conditions described for membrane-associated RyR, except that, after 2 h of incubation at 30°C , the RyR-ryanodine complex was separated from free ryanodine by precipitation with poly(ethylene glycol) 600 in the presence of carried protein, followed by filtration through Whatman GF/B filters and three washes of 4 ml each with buffer A containing 10% (w/v) poly(ethylene glycol) solution. Non-specific binding of [^3H]ryanodine was determined in the presence of 20 μM unlabelled ryanodine.

Trypsin treatment

Unmodified or DNFB-modified SR membranes were treated with trypsin as described previously [28]. The membranes (5 mg/ml) in 10 mM Tricine (pH 8.0)/0.4 M NaCl were incubated at 24°C with bovine pancreatic trypsin (freshly prepared in cold 0.5 mM HCl and used immediately) at mass ratios of 1:700 to membrane protein. Trypsin digestion was stopped by the addition of a 10-fold molar excess of soybean trypsin inhibitor (type IS).

Immunoblotting and gel electrophoresis

Analysis of the protein profile by SDS/PAGE was performed by the method of Laemmli [29]. Gels were stained with Coomassie Blue or immunostained after their electrophoretic transfer to nitrocellulose membranes with 20 mM 3-(cyclohexylamino)-propane-1-sulphonic acid ('Caps') buffer, pH 10.5, containing 0.01% SDS. The membranes were blocked with 5% (w/v) non-fat dried milk and 0.1% (v/v) Tween-20 in Tris-buffered saline, incubated with anti-dinitrophenyl or anti-RyR antibodies (1:1500 and 1:500 respectively) and then with alkaline phosphatase conjugated to anti-mouse IgG antibodies or to protein A respectively as secondary antibodies.

Single channel reconstitution and analysis

Reconstitution experiments were performed as described previously [13,20]. PLBs were made with a 5:3 mixture of phosphatidylethanolamine and phosphatidylserine (Avanti Polar Lipids) at 50 mg/ml in decane. SR (1–3 μg) was added to the chamber defined as the *cis* side of the PLB set-up, which contained 250 mM CsCl, 50 μM CaCl_2 and 10 mM Hepes, pH 7.6. The *trans* chamber contained 50 mM CsCl, 50 μM CaCl_2 and 10 mM Hepes, pH 7.6. After a single step-like fusion event, the *cis* chamber was immediately perfused with identical buffer to remove excess SR. Currents were recorded under voltage-clamp with a Bilayer Clamp BC-525B amplifier (Warner Instrument Corp.). The currents were measured at +30 mV holding potential with respect to the *trans* side of the membrane (ground). The currents were low-pass filtered at 1 kHz (–3 dB) with a Bessel filter (Frequency Devices 902), processed with a VR-10B digital data recorder (Instrutech) and stored on VCR tape. Data were digitized with a Scientific Solutions analogue-to-digital converter (Labmaster TM-40) without additional filtering, and analysed with a pCLAMP (Axon Instruments). In a typical experiment, reagent was added to the *cis* or *trans* chamber and, after 1–2 min, single-channel fluctuations were recorded for at least 2 min.

RESULTS

DNFB modifications of RyR stimulates or inhibits ryanodine binding

Table 1 shows that the effect of DNFB modification of SR on ryanodine binding depends on the incubation conditions. We compared the effects of the modification of SR with DNFB on ryanodine binding under different conditions, including those used in the assay conditions of the single-channel activity of RyR reconstituted into PLB [CsCl, Hepes (pH 7.6) and different temperatures], and those used in our previous study [30] [Tricine (pH 8.0) and different temperatures] (Table 1). Incubation of SR with DNFB at 25 or 33 °C produced a stimulation (up to 3-fold) of ryanodine binding. In contrast, incubation at 37 °C led to an inhibition of ryanodine binding. Thus, for stimulatory effect, SR was incubated with DNFB at pH 7.6 and 25 °C, whereas for inhibitory effect the incubation was performed at pH 8.0 and 37 °C. The results in Table 1 indicate that the stimulatory effect of the modification of RyR with DNFB involves an increase in the Ca^{2+} binding affinity of RyR.

The concentration dependence of the modification of ryanodine binding with DNFB under stimulatory or inhibitory conditions is presented in Figure 1. Half-maximal stimulation of ryanodine binding was obtained at less than 5 μM DNFB; maximal stimulation (2.6-fold) occurred at approx. 50 μM DNFB (Figure 1). Inhibition of ryanodine binding was obtained with over 10-fold higher concentrations of DNFB, with half-maximal inhibition at 70 μM and complete inhibition at 200 μM DNFB. Similar results were obtained when ryanodine binding was assayed in the presence of either 0.3 μM (Figure 1) or 50 μM CaCl_2 (results not shown).

The pH dependence of DNFB modification of RyR on ryanodine binding under stimulatory and inhibitory conditions is presented in Figure 2(A). Whereas a stimulation of ryanodine binding by 2–3-fold was obtained by DNFB modification of RyR at relatively low pH values (between 6.2 and 7.0), an inhibitory effect was pronounced at alkaline pH. A 50% inhibition was obtained at pH 7.6, and increased with increasing incubation pH. The time courses of DNFB modification of

Figure 1 Effect on ryanodine-binding activity of the modification of SR with DNFB under two different conditions

SR membranes (1 mg/ml) were incubated for 15 min with the indicated concentrations of DNFB under condition I (stimulatory condition) (○) [0.5 M CsCl/10 mM Hepes (pH 7.6) at 25 °C] or condition II (inhibitory conditions) (●) (10 mM Tricine, pH 8.0, at 37 °C). The reaction was terminated by the addition of DTT to a final concentration of 2 mM. Membrane aliquots were assayed for ryanodine binding as described in the Experimental section, except that the Ca^{2+} concentration was 0.3 μM . The control activity (100%) was 1.97 ± 0.25 pmol of ryanodine/mg of protein. Error bars indicate S.E.M.

ryanodine binding under the inhibitory and stimulatory conditions show that the inhibitory effect occurred after several minutes of SR incubation with DNFB ($t_{1/2} = 20$ min); however, the stimulatory effect was apparent within several seconds of SR incubation with DNFB (Figures 2B and 2C). It should be noted that DNFB stimulated binding even in the presence of DTT, suggesting a more rapid interaction with the SR amino group(s) than with DTT (used to terminate the reaction). Therefore, in this case, DNFB modification was stopped by 10-fold dilution of the sample before ryanodine-binding activity was assayed. These results suggest that DNFB modifies two classes of amino group in the RyR and produces two different effects on ryanodine binding.

Table 2 shows that both the inhibitory and the stimulatory effects of DNFB modification of SR membranes on ryanodine binding were retained after solubilization with CHAPS and purification of RyR from the modified membranes. This suggests that the effect of DNFB on ryanodine binding is due to modification of the RyR molecule. Furthermore, DNFB-modified RyR cross-reacted with anti-dinitrophenyl antibodies, suggesting that RyR was labelled by DNFB (results not shown).

Table 1 Effect of modification of SR membranes by DNFB, under different conditions, on their ryanodine-binding capacity

SR membranes (1.0 mg/ml) were incubated with 100 μM DNFB under the indicated conditions. After 15 min incubation at the indicated temperature, the reaction was terminated by the addition of DTT to a final concentration of 1 mM. Control and DNFB-modified membranes were assayed for ryanodine binding as described in the Experimental section, except that the Ca^{2+} concentration was 0.3 or 50 μM . Control activities (100%) were 1.65 and 4.0 pmol of ryanodine bound/mg of protein for 0.3 and 50 μM free Ca^{2+} respectively. The results are representative of four similar experiments.

Preincubation conditions				Ryanodine bound (% of control)	
Buffer	pH	Temperature (°C)	[Ca^{2+}] (μM) ...	0.3	50
CsCl/Hepes	7.6	25*		285	131
		33		189	103
		37		139	72
Tricine	8.0	25		147	140
		33		120	89
		37†		36	20

* Stimulatory conditions used in the experiments.

† Inhibitory conditions used in the experiments.

Effect of DNFB modification of RyR on ryanodine-, Ca^{2+} - and ATP-binding sites

Figure 3 demonstrates the effect of DNFB modification of SR, under stimulatory and inhibitory conditions, on the ryanodine binding affinity of RyR. In DNFB-modified SR under stimulatory conditions (Figure 3A), when assayed at low Ca^{2+} concentrations, [^3H]ryanodine was bound to a single class of receptor site with an apparent dissociation constant (K_d) of 7.4 nM. This K_d is considerably lower than that of control membranes (26 nM)

Figure 2 pH- and time-dependence of modification of RyR activity by DNFB under stimulatory and inhibitory conditions

(A) SR was modified with DNFB (100 μ M) under stimulatory or inhibitory conditions as described in the legend to Figure 1, except that the pH was varied as indicated. The buffers used were 20 mM MES for pH 6.2 and 6.5, 20 mM Hepes for pH 7.0, and 20 mM Tricine for pH 7.6, 8.0 and 8.4. Control activities (100%) at the various pH values were between 0.6 and 2.2 and between 4.4 and 7.3 pmol of ryanodine/mg of protein for the stimulatory and inhibitory conditions, assayed in the presence of 0.3 and 50 μ M CaCl_2 respectively. Error bars indicate S.E.M. (B, C) SR (1.0 mg/ml) was incubated for the indicated durations without or with 50 μ M (○) or 200 μ M (●) DNFB under the stimulatory (B) or inhibitory (C) conditions respectively. In (B) the reaction was terminated by dilution 1:10 into the ryanodine-binding medium and DTT was added to a final concentration of 3 mM. In (C) the reaction was terminated at various times by the addition of DTT to a final concentration of 2 mM. Ryanodine binding was assayed in the presence of 0.3 or 50 μ M CaCl_2 for the stimulatory and inhibitory conditions respectively. Control activities (100%) were 1.1 pmol/mg protein in (B) (this is one of two similar experiments) and 3.8 pmol/mg protein in (C). Error bars indicate S.E.M.

Table 2 Retention of DNFB-modification effects on ryanodine binding by the purified RyR

SR was modified with DNFB (100 μ M) under stimulatory or inhibitory conditions, as described in the legend to Figure 1, except that the protein concentration was 2 mg/ml. Purification of RyR from control and DNFB-modified membranes was performed as described previously [2]. The CHAPS-solubilized membranes (0.4 mg/ml) and the purified protein (3 μ g/ml) were assayed for ryanodine binding in the presence of 2.0 mg/ml soybean lecithin, as described [44]. Control activities (100%) for stimulatory conditions were 2.3, 3 and 20 pmol of ryanodine bound/mg of protein, and for inhibitory conditions 5.5, 4.5 and 74 pmol of ryanodine bound/mg of protein, for SR, CHAPS-solubilized SR and purified RyR respectively. Results are representative of two similar experiments.

DNFB modification	^3H Ryanodine bound (% of control)		
	SR	Solubilized SR	Purified RyR
Stimulatory conditions	313	176	190
Inhibitory conditions	40	13	30

assayed under the same conditions. However, the total number of binding sites (B_{max}) was similar for control and DNFB-modified membranes. In SR modified with DNFB under the inhibitory conditions, B_{max} was decreased by 68 %, whereas K_d was altered only slightly (Figure 3B).

The time course of ^3H ryanodine binding by DNFB-modified SR under the stimulatory conditions shows that ryanodine binding increased by 3–4-fold within 12 min and reached a constant value after 40 min. In control SR, the binding increased slowly over 90 min, during which it was approx. 35 % of the binding of DNFB-modified SR (Figure 4A). Kinetic analysis of the data indicates that DNFB modification increased the observed association rate constant (K_{obs}) from 0.02 to 0.07 min^{-1} (Figure 4B). The calculated pseudo-first-order association rate constants (K_1) for control and DNFB-modified SR were 1.3×10^5 and $1.1 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$ respectively, indicating that DNFB modification increased the rate of ryanodine association with its binding site by approx. 8.5-fold. Dissociation of bound ryanodine at equilibrium, initiated by dilution 1:80, revealed a similar

dissociation in control and DNFB-modified SR ($K_{-1} = 0.015 \text{ min}^{-1}$; Figure 4C). K_d was not calculated from the association and dissociation rate constants because of differences in the assay conditions of ryanodine binding.

Comparison of the Ca^{2+} dependence of ^3H ryanodine binding to control and DNFB-modified SR under the stimulatory conditions shows that DNFB-modified membranes had an approx. 6-fold higher affinity for Ca^{2+} than did the unmodified RyR (half-maximal binding at 0.2 μM) (results not shown).

The effect of DNFB modification of SR on the ATP-binding site was tested by using Bz_2ATP , the photoreactive derivative of ATP. The labelling of RyR by $[\alpha\text{-}^{32}\text{P}]\text{Bz}_2\text{ATP}$ in control and in SR modified with DNFB indicated that DNFB modification under the inhibitory conditions, but not under the stimulatory ones, decreased the ATP binding affinity of the RyR to one-half. However, modification with DNFB had no effect on Bz_2ATP binding to RyR at Bz_2ATP concentrations above 4 μM (results not shown). The results in Table 3 show that the presence of ATP during DNFB modification affects neither the stimulation nor the inhibition of ryanodine binding by DNFB modification of SR. These results suggest that DNFB (at the concentrations used) does not interact directly with the ATP binding site of the RyR under either set of conditions.

Characterization of the DNFB-modified RyR channel

Figure 5(A) shows recordings of RyR single-channel activity incorporated into PLB, before (trace 1) and after the addition of 100 μM DNFB to the *cis* solution. DNFB closed the Ca^{2+} -activated channel within a few seconds of its addition (trace 2). This inactivation could be reversed by a low concentration (100 nM) of ryanodine and in the presence of low concentrations of Ca^{2+} (130 nM). Ryanodine re-activated (within 1 min) the DNFB-modified, closed channel to its maximal conductance state (trace 3). This DNFB- and ryanodine-modified channel was not converted either into locked fully open or locked at a sub-conductance state, but fluctuated between completely closed (trace 4), very briefly open (trace 5) and fully open (trace 6) states. However, at approx. 7 min after the reactivation by ryanodine, the channel converted into a sub-conductance state (trace 7). Higher concentrations of ryanodine (such as 1 μM) also

Figure 3 Modification of SR by DNFB under stimulatory and inhibitory conditions increases and decreases the ryanodine-binding affinity respectively

SR (1 mg/ml) was modified by 100 or 150 μM DNFB under stimulatory or inhibitory conditions respectively. [^3H]Ryanodine binding was assayed in the presence of 1.0 M NaCl and 0.3 μM CaCl_2 (**A**, top panel) or 50 μM CaCl_2 (**B**, top panel) as described in the legend to Figure 1, except that the [^3H]ryanodine concentration was varied as indicated. The K_d values calculated from the Scatchard plots (bottom panels of **A** and **B**) for stimulatory conditions are 26 and 7.4 nM for control and DNFB-modified membranes respectively; the calculated B_{max} values are 19.6 and 18.1 pmol/mg of protein for control and DNFB-modified membranes respectively. Under inhibitory conditions, the K_d values are 11.5 and 8.1 nM and the B_{max} values are 11.8 and 4.1 pmol/mg of protein for control and DNFB-modified membranes respectively. Symbols: ●, control SR; ○, DNFB-modified SR. This is a representative experiment from a total of three.

Figure 4 Effect of modification of ryanodine by DNFB on its association with, and dissociation from, RyR

SR was incubated without (●) or with (○) 200 μM DNFB under the stimulatory conditions described in the Experimental section. (**A**) The ryanodine-binding activity of control and DNFB-modified membranes was assayed for the indicated incubation durations (B_t). The maximal amount of ryanodine bound at the plateau (B_0) was 1.4 and 4 pmol/mg of protein for control and DNFB-modified membranes respectively. (**B**) Kinetic analysis of ryanodine binding. K_1 was calculated from K_{obs} as described previously [46], from the following equation:

$$K_{\text{obs}} = K_1[L][R]/B_0$$

where $[L]$ is the ryanodine concentration and $[R]$ is the RyR concentration equal to B_{max} (12.6 pmol/mg of protein). The calculated K_1 values are 1.33×10^5 and $1.1 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$ for control and DNFB-modified membranes respectively. This is one of two similar experiments. (**C**) Control and DNFB-modified membranes were incubated with 20 nM ryanodine in the presence of 0.3 μM CaCl_2 ; after 2 h, aliquots were assayed for bound ryanodine [B_0 values were between 0.56 and 7.3 and between 1.37 and 8.7 pmol/mg for control (●) and DNFB-modified (○) SR respectively]. Dissociation of bound ryanodine was initiated by dilution 1:80 with the binding medium (without ryanodine). The calculated K_{-1} is 0.020 min^{-1} . Error bars indicate S.E.M.

Table 3 Effect of ATP on the modification of RyR by DNFB

SR was incubated with DNFB under the stimulatory (100 μ M) or inhibitory (150 μ M) conditions in the absence or presence of ATP. Aliquots were assayed for ryanodine (20 nM) binding as described in the Experimental section, except that for the inhibitory conditions the NaCl concentration was 0.5 M. ATP was diluted 1:4 during the assay of ryanodine binding. Results are representative of two similar experiments.

Conditions	Addition to preincubation	Ryanodine bound (pmol/mg of protein)
Stimulatory	None (control)	3.6
	ATP (5 mM)	4.6
	DNFB	5.2
	DNFB + ATP	7.1
Inhibitory	None (control)	3.5
	ATP (2 mM)	4.2
	DNFB	0.4
	DNFB + ATP	0.8

re-activated the DNFB-closed channel, but the intervals between reactivation to fully open and conversion to sub-conductance state were much shorter (results not shown).

Another distinct finding about the ryanodine-re-activated DNFB-modified channel is the transitions between multiple conductance states (Figure 5B). After conversion of the DNFB-modified closed channel into the sub-conductance state by ryanodine, the channel alternated between a completely closed state (trace 8) and a full but sub-conductance open state (traces 10–12), and underwent fast transitions from the closed state to one-quarter and one-half conductance states, s_2 and s_1 , respectively (trace 9), as well as from s_1 to a state with conductance greater than that of s_1 (approx. three-quarters) (trace 10), to s_2 (trace 11) and to closed (c, trace 12) states.

It has been demonstrated that the ryanodine-modified channel locked at the sub-conductance state is insensitive to Ruthenium Red [31–35]. We found that the addition of Ruthenium Red converted the DNFB and ryanodine-modified channel to the closed state via fast transition between s_1 and s_2 conductance states, and within 30 s the channel was at the long-lived closed state (results not shown).

The DNFB-modified closed channel, but not the unmodified channel, was activated by low concentrations of ryanodine in the presence of low concentrations of Ca^{2+} (130 nM) (Figures 5A and 5C). Only when both free Ca^{2+} and ryanodine concentrations were increased by 5-fold and 10-fold respectively did ryanodine alter the RyR channel activity reconstituted from unmodified SR (Figure 5C). These results suggest that the DNFB modification of RyR enhanced the channel sensitivity to Ca^{2+} and ryanodine.

Figure 5(D) shows the effect of DNFB modification of RyR reconstituted into PLB under the inhibitory conditions (pH 8.0 and 37 °C) on its single-channel activity. DNFB irreversibly closed the RyR channel; it could not be re-activated by various concentrations of ryanodine and CaCl_2 (results not shown).

In the presence of ATP, the reconstituted RyR channel displayed high open probability ($P_o > 0.9$) (Figure 6A, trace 1). Neither the first nor the second addition of DNFB to the final concentrations of 0.2 or 0.4 mM respectively affected the channel activity (traces 2 and 3). Decreasing the Ca^{2+} concentration to 92 nM in the *cis* solution also had no significant effect on this ATP-activated RyR channel (trace 4). ATP not only prevented the RyR channel from inactivation with DNFB but also re-activated the DNFB-modified closed channel (Figure 6B). At a low free Ca^{2+} concentration (23.8 nM), the addition of ATP

(5 mM) to the DNFB-modified closed channel resulted in very brief opening events of the channel ($P_o < 0.1$) (trace 5). Increasing the free Ca^{2+} concentration to 73.8 nM led to the reactivation of the channel to the full conductance state (trace 6). A high Ca^{2+} concentration (8 mM), led to channel inactivation (trace 7), as expected for the unmodified channel.

Different RyR conformations are stabilized by modification with DNFB under the stimulatory and inhibitory conditions

The results in Figure 7 demonstrate the different tryptic maps of unmodified RyR and of RyR modified with DNFB, under the stimulatory and inhibitory conditions, as revealed with anti-RyR antibodies. The digestion profiles of control and DNFB-modified RyR under stimulatory conditions were similar, but that of DNFB-modified under inhibitory conditions was different, having more RyR fragments in the high-molecular-mass range. Several tryptic fragments were found to be common to control and DNFB-modified proteins. However, the 230, 160 and 115 kDa fragments were obtained from the unmodified and DNFB-modified RyR under the stimulatory conditions but not under the inhibitory ones. The 144 and 130 kDa fragments were obtained only in RyR modified with DNFB under the inhibitory conditions. These results show that limited tryptic digestion of membrane-bound RyR yields different peptide maps for control and DNFB-modified RyR under inhibitory conditions, suggesting that a distinct conformation of RyR is stabilized by this modification with DNFB.

DISCUSSION

This study presented the effects of modification of the RyR/ Ca^{2+} channel with DNFB, as reflected in ryanodine-binding and single-channel activities. According to the incubation conditions, the modification of SR with DNFB has either a stimulatory or an inhibitory effect on the binding of [^3H]ryanodine to RyR. Under the stimulatory conditions, modification with DNFB results in 6-fold and 3.6-fold increases in the Ca^{2+} -binding and ryanodine-binding affinities respectively. The single-channel activity of RyR reconstituted into PLB is completely inhibited by modification with DNFB under both conditions. Only when modified under stimulatory conditions did ryanodine and ATP activate the DNFB-closed channel in the presence of sub-micromolar concentrations of Ca^{2+} . These results provide insight into the involvement of lysine residues in the gating mechanism of the RyR channel, the association/dissociation of ryanodine from its binding sites, and the relationship between the channel opening state and its ability to bind ryanodine.

Two classes of amino group in the RyR are modified by DNFB

The interaction of DNFB with the RyR, as reflected in its ryanodine-binding and single-channel activities, clearly indicates that two different classes of amino group in the RyR are modified by DNFB, with regard to their accessibility/reactivity and to the effects produced: stimulation or inhibition. The different effects of DNFB on ryanodine binding under the two modification conditions suggest that distinct interaction sites for DNFB are accessible at different conformational states of the RyR. The conformational state stabilized at 37 °C and at alkaline pH has one or more accessible amino groups that interact with DNFB and lead to the inhibition of ryanodine binding and the activation of the RyR channel by Ca^{2+} and ryanodine (Table 1; Figures 1–3 and 5D). These amino groups are not as reactive as that involved

Figure 5 Modification by DNFB of single RyR/ Ca^{2+} -release channel activity reconstituted in PLBs, and its interaction with ryanodine

Reconstitution of a single Ca^{2+} -release channel from SR vesicles into a PLB and recordings (at +30 mV potential) were performed as described in the Experimental section. (**A, B**) To the incorporated Ca^{2+} -release channel (trace 1), DNFB was added to the *cis* chamber to a final concentration of 100 μM (traces 2–12). At 10 min after inactivation of the channel, ryanodine was added to the *cis* solution to a final concentration of 100 nM (trace 3). In the *trans* solution the free Ca^{2+} concentration was 50 μM ; in the *cis* solution it was adjusted to 0.13 μM by the addition of EGTA. (**C**) Activity of unmodified channel. Ryanodine and free Ca^{2+} concentrations at each stage of channel recordings are shown above each current trace. The letters o, s and c at the right side of the broken lines indicate full-conductance, sub-conductance and zero-conductance levels of the channel respectively; and s₁ and s₂ represent one-half and one-quarter of the channel full conductance of the control respectively. (**D**) Recordings were made as above, except that the buffer used was 10 mM Tricine (pH 8.0)/250 mM CsCl and the temperature was 37 $^{\circ}\text{C}$ to yield inhibitory conditions. After the appearance of RyR single-channel activity, DNFB (200 μM) was added to the *cis* solution; after 10 min the channel activity was measured. Results are representative of three (**A–C**) or two (**D**) similar experiments.

A. ATP prevents DNFB inhibition of RyR

B. ATP reactivates DNFB-modified RyR

Figure 6 Effect of ATP on the modification of the RyR channel by DNFB

Reconstitution and recordings of RyR single-channel activity were made as described in Figure 5. The final concentrations of ATP, free Ca^{2+} and DNFB in the *cis* solution at different stages of recording are shown above the channel current traces. The letters o and c at the right of the broken lines indicate the current levels of the fully opened and completely closed channels respectively. (A) ATP prevents the inactivation of the RyR channel by DNFB; (B) effect of ATP on the DNFB-modified RyR channel in the presence of different Ca^{2+} concentrations. Results are representative of two similar experiments.

in the stimulation of ryanodine binding as reflected in the concentration-dependence of the effects [a C_{50} (concentration of DNFB required for 50% inhibition or stimulation of ryanodine binding) of 70 μM compared with 5 μM], the modification time (a $t_{1/2}$ of 20 min compared with less than 30 s), and the pH dependence (pH > 7.5 compared with 6.2). The stabilization of two different RyR conformations by DNFB modification under the stimulatory and inhibitory conditions is also reflected in the different tryptic maps of the two modified proteins (Figure 7).

The ϵ -amino group of lysine side chains exhibits an average pK_a of 10–10.5. The pH dependence of the stimulation of ryanodine binding suggests that DNFB binds to the lysine ϵ -amino group with a pK_a of less than 7.0 (Figure 2A). This low pK_a value is indicative of a reactive group that might be involved in excitation–contraction coupling by being sensitive to the movement of one or more charges in the dihydropyridine receptor

at the transverse-tubules membranes [11]. The other less reactive group, which led to the inhibition of ryanodine binding and the irreversible closing of the RyR channel, might be involved in channel closing after its opening.

Modification of the Ca^{2+} -, ATP- and ryanodine-binding sites of the RyR by DNFB

RyR activity is strictly Ca^{2+} -dependent, stimulated by micromolar Ca^{2+} concentrations and inhibited by millimolar concentrations [3–6]. Our results show that DNFB modification, under the stimulatory conditions, increases the Ca^{2+} - and ryanodine-binding affinities of the RyR as revealed both in ryanodine-binding and single-channel activities. Modification of the RyR under the inhibitory conditions led to a decrease in the total ryanodine binding sites. This might have resulted from the

Figure 7 Different conformational states of RyR are stabilized by modification with DNFB at the stimulatory and inhibitory conditions as reflected in their tryptic maps

SR was modified with 50 or 150 μM DNFB under the stimulatory and inhibitory conditions respectively. After centrifugation at 44 000 g for 30 min at 4 $^{\circ}\text{C}$, the membranes were suspended (to 5 mg/ml) in 10 mM Tricine (pH 8.0)/0.4 M NaCl and subjected to trypsin treatment (see the Experimental section). At the indicated times, aliquots were subjected to SDS/PAGE [4–20% (w/v) gel] followed by staining with Coomassie Blue (A) or by immunostaining with anti-RyR antibodies (B). The open arrowheads indicate the protein bands present in control and DNFB-modified SR under stimulatory conditions, but not in SR modified with DNFB under inhibitory conditions. The filled arrowheads indicate the protein bands present only in the DNFB-modified SR under the inhibitory conditions. Results are representative of two similar experiments.

stabilization of the RyR in the closed state (Figure 5D), which could not bind ryanodine. Alternatively, DNFB, either directly or indirectly, modified the ryanodine-binding site. The presence of ATP during modification did not prevent the stimulation or inhibition of ryanodine binding by DNFB. Furthermore, ATP stimulated ryanodine binding to DNFB-modified RyR (Table 3). These results suggest that DNFB modification had no effect on the RyR ATP-binding site.

Modification of RyR single-channel activity by DNFB

The distinct findings from the DNFB modification of RyR single-channel activity are: (1) DNFB closes the reconstituted RyR channel immediately; (2) in a time-dependent manner, ryanodine activates the DNFB-modified closed channel to fully open followed by different sub-conductance states; (3) ATP reactivates the DNFB-modified closed RyR channel and prevents RyR channel inactivation by DNFB; (4) the DNFB-modified RyR closed channel has an enhanced binding affinity for ryanodine and Ca^{2+} ; (5) the DNFB-modified, ryanodine-re-activated RyR channel can be closed by Ruthenium Red. These results will be discussed below with regard to involvement of lysine residues in channel gating, ryanodine association, dissociation and occlusion, and the relationship between the RyR conformational state (open or closed) and its ability to bind ryanodine.

ATP re-activated the DNFB-closed channel

The DNFB-modified closed RyR channel can be re-activated by ATP (Figure 6A). Furthermore, the presence of ATP prevented the modification of the channel by DNFB (Figure 6B). The activation of the DNFB-modified closed channel by ATP is observed at very low concentrations of Ca^{2+} . The activation of RyR by ATP in the presence of very low concentrations of Ca^{2+} has been reported previously [36,37]. These results, like those with ryanodine binding, indicate that the ATP-binding site(s) of RyR are not modified by DNFB, and that ATP binding converts the DNFB-modified closed channel to an open channel. The

results suggest that modification with DNFB led to channel closure without affecting its modulation by ATP.

DNFB modifies both high-affinity and low-affinity ryanodine-binding sites

Ryanodine affects the channel by sequential activation and inactivation [2–6]. Ryanodine at nanomolar concentrations was shown to activate RyR single channels [38,39], whereas at 50 μM it converted the channel to a sub-conductance state and at 200 μM it induced channel closure [38]. However, ryanodine at 1 mM, which is considered to be only inhibitory to the channel, was shown to activate Ca^{2+} release from SR vesicles [40]. We have found that after DNFB modification of the RyR single channel and its conversion to the closed state, 0.1–2 μM ryanodine, in the presence of a low Ca^{2+} concentration (0.13 μM), reactivates the DNFB-modified closed channel to full conductance followed by transitions to multiple sub-conductance and closed states (Figures 5A and 5B), with no effect on the unmodified RyR channel (Figure 5C). The transition time of the DNFB-modified ryanodine-activated RyR channel from a full-conductance to a ryanodine-induced lower-conductance state is dependent on the ryanodine concentration, decreasing with increasing ryanodine concentration. The binding of ryanodine to the high-affinity site activates the DNFB-closed channel to a fully open state; binding to the second or third low-affinity site converts the channel to sub-conductance states, whereas the occupation of all four ryanodine binding sites in the tetrameric complex of RyR leads to channel closure. The findings that the sub-conductance states of the RyR channel are obtained in the presence of very low concentrations of ryanodine indicate that the modification of the RyR by DNFB increases the ryanodine-binding affinities of both high-affinity and low-affinity sites.

DNFB modification alters the association/dissociation of ryanodine with the RyR channel

Equilibrium and kinetic analyses of [^3H]ryanodine binding to RyR suggest a complex interaction between ryanodine and the

RyR subunits. A negative co-operativity has been proposed to describe the allosteric modulation of ryanodine binding to as many as four sites [41]. Ryanodine binding to lower-affinity sites induces subsequent conformational changes in the RyR, which convert it to the closed state [2–6]. Our results show that on several occasions the ryanodine-activated DNFB-modified RyR channel entered a closed state from either a full-conductance or a sub-conductance state (Figures 5A–5C). Appearance of the DNFB-modified RyR channel in the closed state in the presence of ryanodine reflects either the dissociation of ryanodine from the DNFB-modified channel or the binding of ryanodine to low-affinity sites. Ryanodine binding to the DNFB-modified closed channel activates it, whereas its dissociation from the binding site would convert it to the completely closed state. Furthermore, the DNFB-modified, ryanodine-re-activated channel has multiple sub-conductance states, in which transitions between sub-conductance states and closed state, and transitions from the one-half conductance state to the nearly full conductance state, are apparent (Figure 5B). The observations that the ryanodine-activated DNFB-modified RyR channel displays full, one-half, one-quarter and zero conductance, and the appearance of bi-directional transitions between open, closed and sub-conductance states suggest that ryanodine, with time, associates with and dissociates from both high-affinity and low-affinity binding sites of the RyR channel. Our findings that the ryanodine-activated DNFB-modified channel is sensitive to Ruthenium Red support our conclusion that ryanodine could dissociate from the channel. Because it has been shown that the sub-conductance state induced by ryanodine is insensitive to Ruthenium Red [31–35]. Uni-directional, but not bi-directional, transitions of the channel to sub-conductance states due to ryanodine modification of the native RyR channel have been resolved [38,42].

Relationship between the open or closed state of the RyR channel and its ability to bind ryanodine

DNFB-modified RyR binds Ca^{2+} and ryanodine with 6-fold and 3.6-fold higher affinities respectively. Although the DNFB-modified RyR channel is closed, the protein binds ryanodine with higher affinity, as reflected in the activation by nM ryanodine (Figures 5A–5C). These observations are not in accord with the currently accepted concept that ryanodine binds preferentially to the open conformational state of the channel [31,34]. However, the relationship between the open or closed state of the RyR channel and ryanodine binding is not as simple as has previously been suggested. Chemical modification by Rose Bengal [22,43,45], DEPC [10,22,23], thimerosal [16], FITC [11] and H_2O_2 [17] stimulated Ca^{2+} -release and single-channel activities but inhibited ryanodine binding (reviewed in [10]). These findings suggest that various modifications stabilize an open conformational state of the RyR that does not bind ryanodine. Alternatively, some modifications induce a closed channel that is able to bind ryanodine with a high affinity, thus indicating a more complex relationship between the opening state of RyR and its ability to bind ryanodine.

In conclusion, as revealed by modification with DNFB, two distinct lysine residues are involved in the RyR channel gating mechanism. These groups might have a physiological function in the modulation of the channel opening state by being sensors of the voltage-induced conformational changes in the dihydropyridine receptor at the transverse tubules.

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