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Oxidative Modification of Ion Channel Activity of Ryanodine Receptor

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ABSTRACT

The ryanodine receptor (RyR) is involved in the physiological Ca^{2+} release from the sarcoplasmic reticulum in both skeletal and cardiac muscles. The redox regulation is a plausible endogenous regulatory mechanism of the RyR. Sulfhydryl oxidation or *S*-nitrosylation of the cardiac RyR has been reported to activate the channel. Our laboratory demonstrated that hydroxyl radicals also activate the cardiac Ca^{2+}-release channel activity, likely through the modification of sulfhydryl groups of the RyR. Antiox. Redox Signal. 2, 35–40.

INTRODUCTION

THE RYANODINE RECEPTOR (RyR) is involved in the physiological Ca^{2+} release from the sarcoplasmic reticulum (SR) in both skeletal and cardiac muscles (Meissner, 1994). For better understanding of the physiological functions of the RyR, it is important to determine its regulatory mechanism. However, the physiological mechanisms of activation of the RyR are not well understood at the present time. The cytosolic Ca^{2+} concentration is a known determinant of RyR regulation. In addition to the cytosolic Ca^{2+}, several other endogenous regulatory factors have been suggested (Shoshan-Barmatz and Ashley, 1998); *e.g.*, Mg^{2+}, ATP, phosphorylation by protein kinase A, protein kinase C, or calmodulin-dependent protein kinase, cADP ribose, interaction with other small proteins, and redox state. In the present paper, we will focus on the redox regulation of the RyR activity.

Oxydative modification of membrane transporters

Various reactive oxygen species interact with ion transport pathways and induce impairment of plasma and internal membranes that control intracellular signal transduction (Kourie, 1998). Target molecules so far reported are: ion channels such as Ca^{2+} channels, K^+ channels, Na^+ channels, and Cl^- channels; ion pumps such as Ca^{2+} pumps, the Na^+ pump, and the H^+ pump; ion exchangers such as the Na^+/Ca^{2+} exchanger and Na^+/H^+ exchanger; ion cotransporters such as the K^+-Cl^-, Na^+-K^+-Cl^-, and P_i-Na^+ cotransporters (Kourie, 1998). The mechanism of the modification of ion transport pathways involves: (i) lipid peroxidation of membrane-constituting phospholipids, (ii) oxidation of relatively reactive sulfhydryl groups of the ion-transporting protein, and (iii) inhibition of membrane-bound regulatory enzymes and modification of intracellular ATP levels.

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In the case of SR, oxidative stresses have been shown to alter the intracellular Ca^{2+} concentration, mainly through a change in the permeability of the SR membranes (Okabe *et al.*, 1988). Among various ion-transporting machineries, the RyR is an important molecule whose activity could be modified by oxidative stress. Because the RyR is the integral membrane protein existing in SR membranes, the function of which is ion transport, a specialized assay system is required to investigate the regulation of its activity at the molecular level. The planar lipid bilayer technique is a unique system that enables us to measure the single ion channel activity of the ion channels located in internal membranes (Anzai *et al.*, 1994).

Activation of RyR by SH-oxidation

The RyR–triadin complex has hyperreactive sulfhydryl (SH) groups and the covalent modification of the SH groups with 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin inhibits both Ca^{2+}-induced Ca^{2+} release and the gating activity of single channels (Liu and Pessah, 1994). In addition, oxidation of the SH groups with 1,4-naphthoquinone-induced Ca^{2+} efflux from the SR (Liu *et al.*, 1994). These findings indicated the existence and functional role of hyperreactive cysteine residues on the RyR–triadin complex. Reduced glutathione (GSH) inhibited Ca^{2+}-stimulated ryanodine binding to the RyR of the skeletal muscle SR and the Ca^{2+} channel gating activity, whereas oxidized glutathione (GSSG) strongly stimulated the ryanodine binding and the Ca^{2+}-release channel activity (Zable *et al.*, 1997). Similar to GSH, SH-reducing agents, dithiothreitol (DTT), and β-mercaptoethanol also inhibited the ryanodine binding to the SR membranes. An SH-alkylating agent, *N*-ethylmaleimide, showed three distinct phases in its functional effects on the skeletal muscle Ca^{2+}-release channel reconstituted into planar bilayer membranes: inhibition, activation, and finally inhibition (Aghdasi *et al.*, 1997b). An oxidizing reagent, diamide, activated the Ca^{2+}-release channel, enhanced ryanodine binding, and cross-linked the subunits of the Ca^{2+}-release channel; these effects of diamide were reversed by DTT (Aghdasi *et al.*, 1997b). All these reports indicate that SH-oxidation activates the RyR channel (Fig. 1).

Most of the data so far reported used SR membrane vesicles as the material to incorporate the RyR and observe its activity in the planar bilayer membranes. To determine the exact site of oxidative modification, it is important to use experimental systems that are as simple as possible. Therefore, we used purified RyR protein instead of RyR-containing SR vesicles for the experiments (Anzai *et al.*, 1998). The heavy fraction of cardiac SR membrane vesicles was prepared from a porcine heart (Sitsapesan and Williams, 1990) and the cardiac RyR was purified and reconstituted into proteoliposomes with asolectin (Lindsay and Williams, 1991). Planar lipid bilayers were formed by the folding method (Anzai *et al.*, 1991) using phosphatidylethanolamine as the membrane-forming lipid. The RyR was incorporated into the planar bilayer by fusion of the reconstituted proteoliposomes to the planar bilayer membranes. K^+ currents instead of Ca^{2+} were used to detect the activity of RyRs because K^+ ions permeate the RyR if the Ca^{2+} concentration is low and K^+ currents are larger than Ca^{2+} currents (Lindsay and Williams, 1991).

The purified cardiac RyR incorporated into the planar bilayer membranes showed a single-channel activity with a conductance of 614 pS and 724 pS in symmetrical 200 m*M* and asymmetrical 900/200 m*M* (*cis/trans*) KCl, respectively, with the ion selectivity of P_K:P_{Cl} = 1:0.08 (Anzai *et al.*, 1998). ATP (1 m*M*) added in the *cis* compartment significantly increased the open probability (P_o) of the channel. Ryanodine (1 μ*M*) added in the *cis* compartment reduced the conductance to half of the original conductance, and it induced continuous channel opening. These characteristics assured the integrity of the sample. When 4,4′-dithiodipyridine (DTDP), an SH-oxidizing reagent, was added at 9.3 μ*M* to the cytoplasmic side of the RyR, the single-channel P_o increased significantly from 0.31 to 0.85. DTT at 3 m*M* reversed the effect of DTDP in accordance with the reports by Eager *et al.* (Eager *et al.*, 1997; Eager and Dulhunty, 1998, 1999). *N*-Succinimidyl 3-(2-pyridyldithio) propionate, another SH-oxidizing reagent, at 60 μ*M* also increased the P_o from 0.59 to 0.77. These results confirmed the

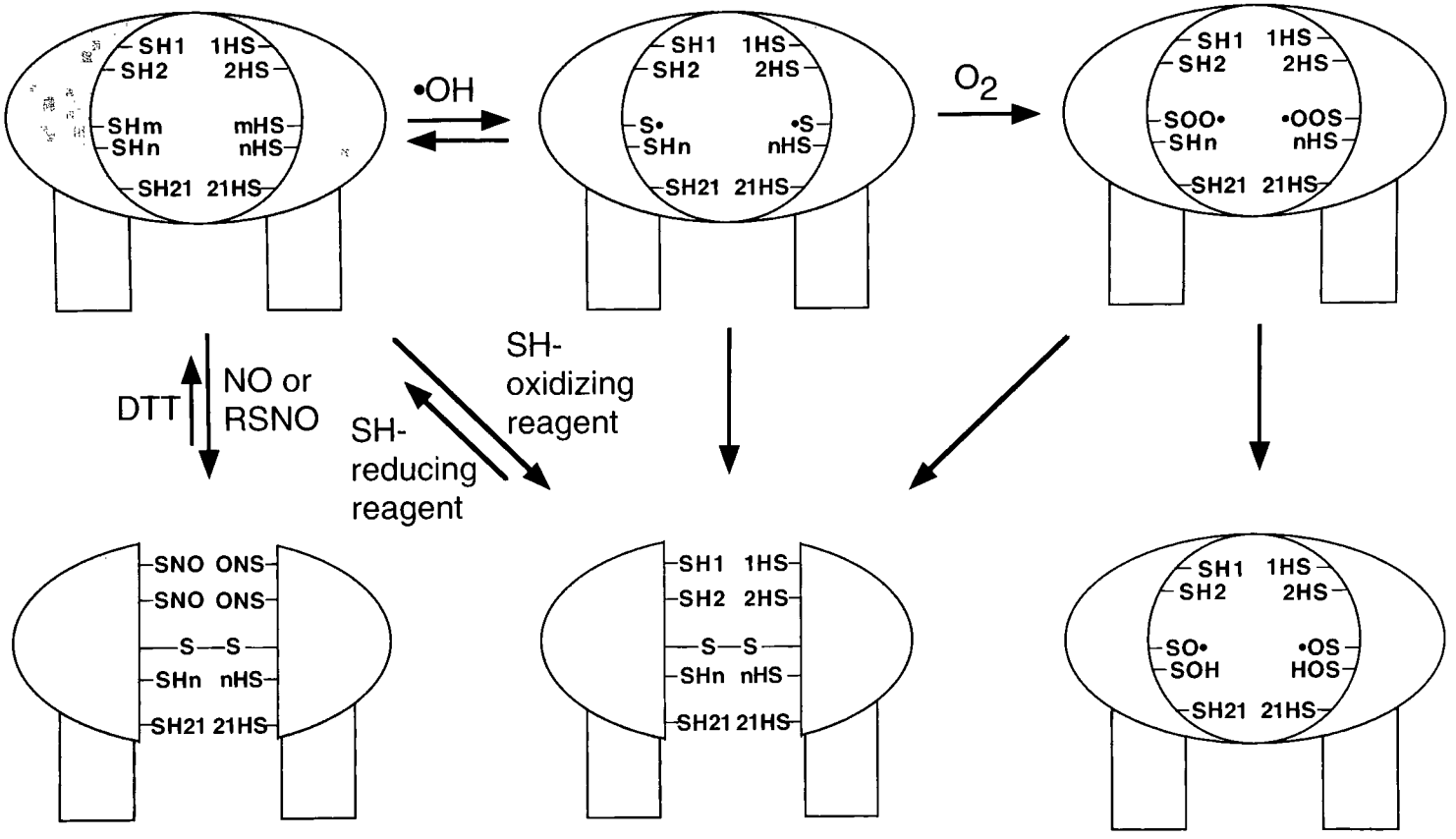

FIG. 1. Hypothetical scheme of the conformational change of the RyR channel associated with the plausible modification of reactive sulfhydryl groups. A subunit of the RyR channel has about 21 SH groups, some of which (SHm) have higher reactivity than others (SH1, SH2, . . .). SH-oxidizing agents oxidize several SH groups (SHm) to form disulfide bonds, a distinct class of which may result in an active conformation. NO or nitrosothiol can oxidize as well as nitrosylate SH groups. SH-reducing agents reverse the *S*-nitrosylation and oxidation of less than six SH groups per subunit (Xu *et al.*, 1998). Hydroxyl radicals may react with some SH groups and form thiyl radicals at first. This radical species is unstable and may be converted to disulfide bonds either directly or through formation of thiyl peroxyl radical. The thiyl peroxyl radical may also react with an adjacent SH group (SHn) to produce sulphinyl radical (Halliwell and Gutteridge, 1999). Two of four subunits of the RyR channel are drawn.

concept that the oxidation/reduction of some SH groups of the RyR channel regulates its activity.

Modification of RyR by ·OH and ·NO

Hydrogen peroxide (H_2O_2) is an endogenous oxidant produced mainly by the dismutation of superoxide inside cells. It was reported that H_2O_2 stimulated the ryanodine binding to the Ca^{2+}-release channel from skeletal muscle SR and also activated the channel gating (Favero *et al.*, 1995). The activation effect of H_2O_2 on sheep cardiac SR Ca^{2+}-release channel (3–5 m*M*, from the cytoplasmic side) was also reported (Boraso and Williams, 1994). Although these reports showed the involvement of H_2O_2 in the process of oxidative modification of the RyR, it is not clear if H_2O_2 itself reacted with the RyR or that some product of H_2O_2, probably the hydroxyl radical, is the true reactive substance. Ascorbate/iron was reported to activate Ca^{2+}-release channels of skeletal SR vesicles reconstituted in lipid bilayers (Stoyanovsky *et al.*, 1994). Because the ascorbate/iron redox couple is known to be an efficient generator of hydroxyl radicals and semidehydroascorbyl radicals, it is plausible that these radical species function physiologically as the oxidizer. Very small amounts of free iron or copper ion in its reduced form react with H_2O_2, and it produces hydroxyl radicals through Fenton reaction (Ozawa and Hanaki, 1991). Therefore, it is possible that a hydroxyl radical other than H_2O_2 directly affects the ion channel activity of the RyR.

The purified RyR was incorporated into planar lipid bilayer membranes, and the effects of the hydroxyl radicals on the channel activity were measured (Anzai *et al.*, 1998). Hydroxyl radicals were generated by the reaction of 1–10 m*M* H_2O_2 and 0.1 m*M* copper(II) complex of ethylenediamine ($Cu(en)_2$). The continuous generation of hydroxyl radical for more than 30 min was confirmed by the electron spin resonance (ESR) spin trapping method (Anzai *et al.*, 1999). The P_o of the RyR channel was increased from 0.27 to 0.94 after a lag time of about 2 min by the *cis*-application of hydroxyl radicals. The conductance, however, was not affected. The addition of H_2O_2 alone without $Cu(en)_2$ or $Cu(en)_2$ alone without H_2O_2 did not significantly increase the P_o. This observation is different from some other reports (Boraso and Williams, 1994; Favero *et al.*, 1995), where H_2O_2 alone activated the RyR channel. The purification and reconstitution of the RyR molecules in our study may remove some factor that is responsible for the H_2O_2-induced channel activation. The hydroxyl radicals generated by the reaction of H_2O_2 and $Cu(en)_2$ did not affect the permeability of pure lipid bilayers nor did they peroxidize the membrane lipids in this time range (Anzai *et al.*, 1999). Hence, the chemically generated hydroxyl radicals modify the RyR channel protein itself and increase the P_o of the channel without changing the conductance. The application of hydroxyl radicals or SH-oxidizing reagents from the cytoplasmic side increased the P_o of the RyR channel. Because the hydroxyl radical is a potent oxidant, it is possible from this correlation that the channel opening effect of the hydroxyl radical is derived from the oxidation of the same SH group that is oxidized by the SH-oxidizing reagents (Fig. 1), although another possibility that oxidation of different SH groups can activate the RyR channel (Eager and Dulhunty, 1999) is not ruled out.

Similar to the oxidation of the SH groups of the RyR, nitrosylation of several SH groups also increased the P_o of the channel, and the P_o depended on the number of the nitrosylated SH groups (Xu *et al.*, 1998) (Fig. 1). However, the effect of nitrosylation on the RyR channel activity is a little complex. Small amounts of nitric oxide (NO), which did not affect the skeletal muscle Ca^{2+}-release channel activity, blocked intersubunit cross-linking and prevented the activation of the channel by the disulfide-inducing agent, diamide (Aghdasi *et al.*, 1997a). These results indicate that NO at low concentrations modulates the RyR channel activity by preventing oxidation of essential SH groups, while higher concentrations of NO activate the channel.

Sidedness of SH modification

The RyR from rabbit cardiac muscle has about 5,000 amino acid residues, with 89 cysteines per molecule (Nakai *et al.*, 1990; Otsu *et al.*, 1990). Because the RyR channel is com-

posed of four subunits, the functional channel has 356 cysteine residues, of which about 84 residues are free, on the basis of a determination with a lipophilic fluorescent monobromobimane (Xu *et al.*, 1998). The results of planar bilayer experiments indicate that the SH groups affected by the hydroxyl radical, SH-oxidizing reagents, or S-nitroso compounds may be located on the cytoplasmic side of the RyR channel. However, in addition to the cytoplasmic SH groups, the activation of the RyR channel from frog muscle by the oxidation of SH groups on the luminal side by H_2O_2 treatment (Oba *et al.*, 1998) and the activation of the cardiac RyR by *trans* DTDP or thimerosal (Eager and Dulhunty, 1999) have been reported. Experiments with DTT in the *trans* chamber would be necessary to determine the location of the SH group modified by the agent that is membrane permeable.

CONCLUSION

In conclusion, the modification of SH groups of the RyR channel, such as by oxidation to disulfide and *S*-nitrosylation, induces an activated conformational state in the channel, which may be important for the physiological regulation of the RyR channel. In cardiac muscle cells, these oxidative modifications may increase the Ca^{2+} release from SR, which may cause harmful effects on the cardiac functions.

ABBREVIATIONS

$Cu(en)_2$, copper(II) complex of ethylenediamine; DTDP, 4,4'-dithiodipyridine; DTT, dithiothreitol; ESR, electron spin resonance; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; NO, nitric oxide; P_o, open probability; RyR, ryanodine receptor; SH, sulfhydryl; SR, sarcoplasmic reticulum.

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