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Functional Role of Hyperreactive Sulfhydryl Moieties Within the Ryanodine Receptor Complex

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ABSTRACT

Several laboratories using chemically heterogeneous sulfhydryl modifying agents have shown that sarcoplasmic reticulum (SR) Ca^{2+} channels known as ryanodine receptors (RyRs) are especially sensitive to modification of functionally important cysteine residues. The functional consequence of sulfhydryl modification of RyRs can include phases of activation and inhibition that are very much dependent on the concentration of the reagent used, the length of exposure, and the nature of the chemical reaction the reagent undertakes with sulfhydryl groups. Most challenging is understanding the relationship for how specific sulfhydryl moieties ascribe specific aspects of RyR function. Considering the structural complexity of the RyR complex with its associated proteins, this task is likely to be a formidable one. A small number of hyperreactive thiols have been shown to exist within the RyR complex. Their functional role does not appear to impact directly on channel gating. Rather hyperreactive cysteine (Cys) moieties may represent biochemical components of a redox sensor that conveys information about localized changes in redox potential produced by physiologic (*e.g.*, glutathione, nitric oxide) and pathophysiologic (quinones, reactive oxygen species) channel modulators to the Ca^{2+} release process. The molecular and functional details of such a redox sensor remains to be elucidated. Antiox. Redox Signal. 2, 17–25.

RECENT DEVELOPMENTS IN OXIDATION-INDUCED Ca^{2+} FLUXES IN SR

Abramson and Salama (Abramson *et al.*, 1983; Trimm *et al.*, 1986) first reported that heavy metals that can react with sulfhydryl groups cause a dramatic increase in the Ca^{2+} permeability of sarcoplasmic reticulum (SR). Because these reagents were found to also stimulate Ca^{2+}-ATPase (SERCA pump) activity, it was concluded that oxidation of one or more sulfhydryl groups to disulfides on the "Ca^{2+} release channel" underlies the rapid increase in calcium permeability of the SR vesicles derived from rabbit fast skeletal muscle. Shortly following this seminal observation, controversy emerged concerning which SR protein was in fact the critical target of oxidation-induced effects on SR Ca^{2+} sequestration, the SERCA pump, the Ca^{2+} release channel (ryanodine receptor, RyR), or both (Scherer and Deamer, 1986; Gould *et al.*, 1987; Brunder *et al.*, 1988; Abramson and Salama, 1989). Over the intervening years, this controversy seems to have been settled in favor of a selective mechanism involving the RyR complex. Data from several laboratories using chemically dissimilar oxidizing and sulfhydryl modifying compounds in conjunction with diverse methodological approaches have revealed that sulfhydryl reagents can (i) potently promote Ca^{2+} release

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from SR at concentrations that have no measurable effect on SERCA activity, and (ii) the release can be completely inhibited by known blockers of the RyR channel complex. Several classes of nonphysiologic organic compounds capable of modifying protein sulfhydryl groups have been utilized to alter Ca^{2+} release from skeletal or cardiac SR by selective modification of RyR1 or RyR2 function, respectively. The functional consequence of sulfhydryl modification of RyRs can include phases of activation and inhibition that are very much dependent on the concentration of the reagent used, the length of exposure, and the nature of the chemical reaction the reagent undertakes with sulfhydryl groups.

For example, aromatic disulfides such as 5,5′-dithiobis(2-nitro)benzoate (DTNB) and 2,2′-dithiodipyridine (DTDP) which undergo disulfide exchange with protein thiols have been shown to enhance channel activity at low (n*M*) concentration, whereas a higher (μM) concentration inhibited the channel complex (Pessah *et al.*, 1987; Zaidi *et al.*, 1989; Eager *et al.*, 1997). Similar biphasic actions on RyR function have also been reported with nanomolar to low micromolar redox active naphthoquinone or benzoquinone, which can potentially oxidize and/or arylate protein thiols (Feng *et al.*, 1999). By contrast, anthraquinones such as doxorubicin, which are pure redox cyclers, predominantly activate RyR channels (Zorzato *et al.*, 1985; Abramson *et al.*, 1988; Pessah *et al.*, 1990; Holmberg and Williams, 1990), although one report of biphasic channel effects was reported from measurements of single cardiac channels reconstituted in bilayers lipid membranes (Ondrias *et al.*, 1990). High micromolar to millimolar levels of the sulfhydryl oxidizing agent diamide were reported to activate only RyR1, whereas millimolar levels of the thiol alkylator *N*-ethylmaleimide exhibited three phases of channel modification, initially depressing, then activating, and finally depressing channel activity (Aghdasi *et al.*, 1997a). Several additional chemical classes of sulfhydryl oxidizing agents have been shown to enhance SR Ca^{2+} release selectively (or reduce Ca^{2+} sequestration) by selective activation of RyRs, including photo-oxidation of rose bengal (Stuart *et al.*, 1992; Xiong *et al.*, 1992), the porphyrin TMPPyP (Abramson *et al.*, 1993), alcian blue (Koshita *et al.*, 1993), thimerosal (Favero *et al.*, 1995; Eager and Dulhunty, 1999), and organomercurials (Suko and Hellmann, 1998). Interestingly, millimolar levels of the charged methanethiosulfonate $MTSEA^+$ modify the conduction properties of skeletal RyR1 by apparently alkylating Cys residues residing near the pore without activating the channel (Quinn and Ehrlich, 1997). Many of the aforementioned activating effects of sulfhydryl oxidizing agents can be prevented or reversed with reducing agents such as dithiothreitol (DTT) or glutathione, suggesting an oxidative mechanism.

Thus, results with sulfhydryl reagents have revealed that one or more classes of sulfhydryl groups residing on Cys residues of RyR1 and RyR2 channel complexes are important for native function and subject to chemical modification. Sensitivity to sulfhydryl oxidation appears to be a general property of SR/endoplasmic reticulum (ER) Ca^{2+} channels, including the inositol 1,4,5-trisphosphate receptor (IP_3R; Hilly *et al.*, 1993; Tanaka and Tashjian, 1994; Kaplan *et al.*, 1994) and the type 3 isoform of RyR (RyR3; Murayama *et al.*, 1999). Because of the initial suggestion by Abramson and Salama (1989) that sulfhydryl oxidation is a key step in channel activation, additional controversy has emerged regarding the physiologic relevance, if any, of oxidation-induced Ca^{2+} release. Do critical thiols oxidize in the normal process of channel gating? Alternatively, what allosteric modulatory function could sulfhydryl oxidation possibly contribute to SR/ER Ca^{2+} regulation and signaling? In this regard, sulfhydryl groups within RyR1 have been proposed to be important in defining the calmodulin binding site on RyR1 (Zhang *et al.*, 1999). It should not be surprising that a large protein complex whose tetrameric channel structure possesses nearly 400 cysteine (Cys) residues (99 per protomer in RyR1) is sensitive to functional modification by sulfhydryl oxidizing or reducing reagents, especially at the high concentrations typically used in most experiments. What has and will continue to prove challenging is understanding the relationship of how specific sulfhydryl moieties ascribe specific aspects of channel function. Considering the structural

complexity of RyR and its associated proteins, this task is likely to be a formidable one.

HYPERREACTIVE SULFHYDRYLS OF RyR COMPLEX

In 1993, Pessah and co-workers began studies aimed at determining if RyR complexes possessed a class of Cys moieties that could be distinguished on the basis of their chemical reactivity. The rationale for these studies was based on the fact that the pKa of the typical protein thiol dictates that it will be fully protonated (reduced) and have low chemical reactivity at physiologic pH (Jocelyn, 1972; Schöneich, 1995). In fact, disulfide bond formation (sulfhydryl oxidation), so critical to protein folding, typically takes place within the oxidizing environment of the SR/ER lumen, not in the reducing environment of the cytosol. Only when Cys residues reside within special microenvironments will protein thiol moieties be rendered reactive enough to take part actively in physiologic redox reactions. Examples come from the literature on enzyme-active sites such as glyceraldehyde-3-phosphate dehydrogenase (Habenicht, 1997), glutamine-dependent amidotransferases (Massière and Badet-Denisot, 1998), and glutathione reductases (Washburn and Wells, 1999).

Liu *et al.* (1994) devised an assay with the nonfluorescent maleimide, 7-diethyl amino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM), which undergoes Michael addition with protein thiols to produce a thioether adduct with high fluorescent yield (Fig. 1A). Using CPM at very low concentrations (1–50 n*M*, *i.e.*, 0.02–1.0 pmol/μg SR protein), the kinetics of labeling junctional and longitudinal SR protein from rabbit skeletal and rat cardiac muscle was examined in the presence of physiologically and pharmacologically relevant channel activators or inhibitors (Liu *et al.*, 1994). Under these conditions, where SR protein thiols greatly exceed the maleimide, the hypothesis that the RyR channel complex possesses a very small number of highly reactive (hyperreactive) thiol groups was tested. If this hypothesis proved incorrect, the anticipated (yet trivial) result would have been relatively slow formation of thioether adducts with the most abundant SERCA pump thiols, because SERCA pump protein accounts for 60–70% of the total SR protein. However, a very different result was observed. The CPM assay quantitatively revealed the existence of a very small number ($\leq$1 pmol/μg SR) of highly reactive Cys residues within junctional SR (JSR) membranes enriched in RyR1, traidin, and calsequestrin (Fig. 1B). Formation of CPM thioether adducts proceeded >10-fold faster in the presence of physiologic (*e.g.*, Mg^{2+}; Fig. 1C) or pharmacologic (Liu *et al.*, 1994) channel inhibitors when compared to rates in the presence of channel activators (*e.g.*, optimal Ca^{2+}; Fig. 1C). Furthermore, longitudinal SR membranes (LSR) lacking the channel complex only displayed slow labeling kinetics regardless of which channel modulator was present (Fig. 1, B and C, LSR). Fluorograms of JSR protein labeled for 1 min with 10 n*M* CPM and then size separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that in the presence of channel inhibitors CPM formed thioether adducts selectively with the RyR protomer and triadin (Fig. 1D; channel closed), whereas CPM labeling in the presence of channel activators form adducts with the abundant SERCA thiols (Fig. 1D; channel open). These results were rather remarkable because channel-associated proteins account for <5% of the total protein in these SR preparations; they attest to the hyperreactive nature of these thiol groups. Using CPM as a probe, Pessah and coworkers proceeded to show that RyR1 and triadin physically interact in the SR membrane and the apparent affinity of the interaction was enhanced with channel activation (Liu and Pessah, 1994). On the basis of these results, it was concluded that sulfhydryl oxidation contributes to stabilizing a RyR1/triadin complex during channel activation, possibly through intra- or intermolecular disulfide bonding. Additional support for a RyR1/triadin complex was provided by co-immunoprecipitation studies (Guo and Campbell, 1995) and the interaction appears to be functionally important (Groh *et al.*, 1999). Hamilton and co-workers (Aghdasi *et al.*, 1997a) failed to detect an RyR1/triadin complex that was stabilized by a thiol-disulfide-mediated interaction, whereas

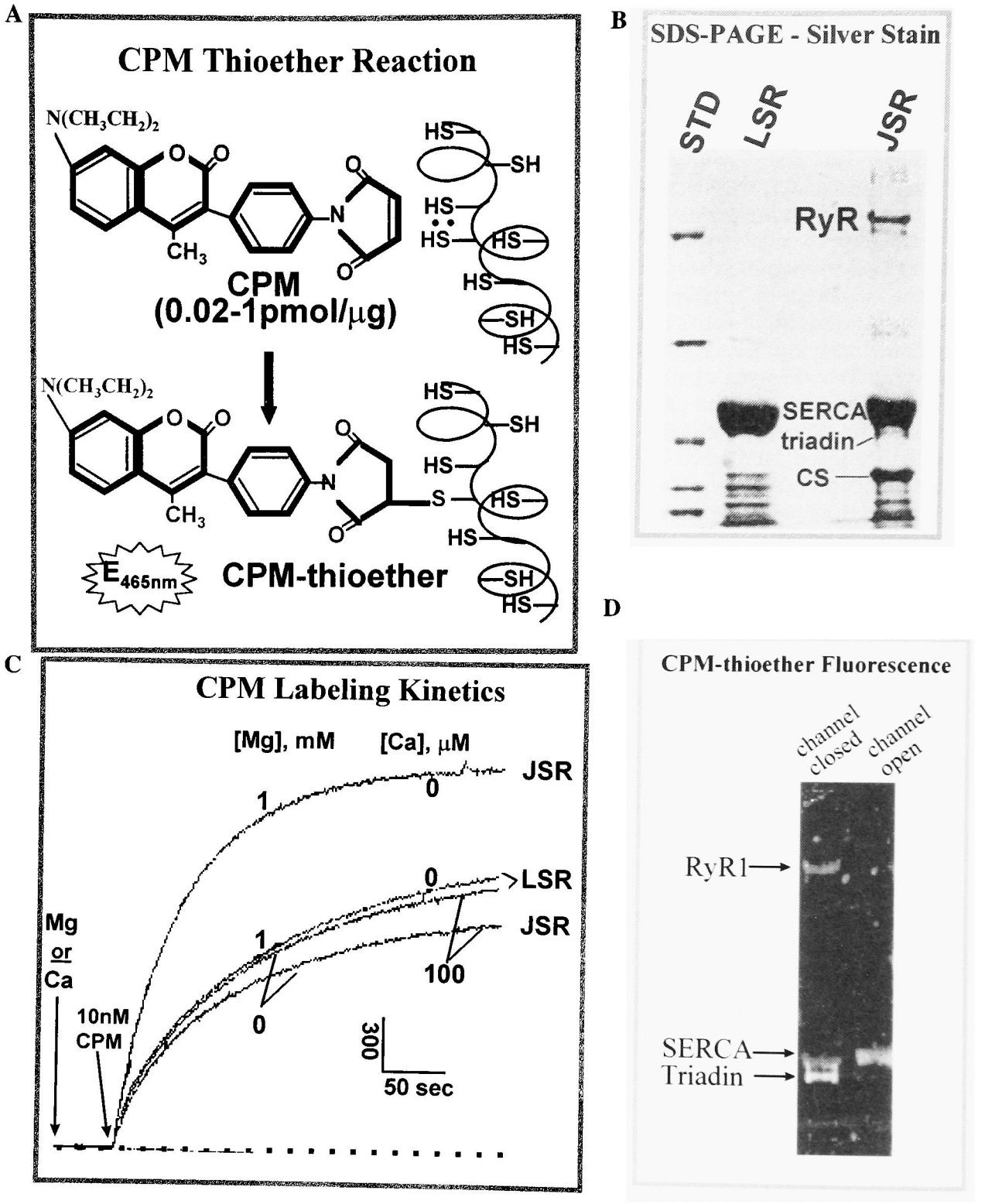

FIG. 1. A. Chemical reaction showing Michael addition between nonfluorescent CPM and protein thiols to form highly fluorescent CPM–thioether adducts. B. SDS-PAGE of light SR (LSR) and junctional SR (JSR). Although both LSR and JSR are enriched in SERCA pumps, only JSR possesses components of the Ca^{2+} release machinery (RyR, ryanodine receptor; triadin; CS, calsequestrin). **C.** Data traces showing the kinetics of forming CPM–thioether adducts with LSR and JSR. Note that JSR forms rapid adducts with CPM in the presence of the physiologic channel inhibitor Mg^{2+} but not in the presence of channel-activating Ca^{2+}. LSR exhibits only slow adduct formation regardless of which modulator is present. **D.** Fluorogram of SDS-PAGE separation of JSR labeled for 1 min with 10 n*M* CPM in the presence of either a channel inhibitor (1 m*M* Mg^{2+}) or channel activator (100 μM Ca^{2+}). Note that the rapid labeling observed in **C** corresponds to adduct formation primarily with RyR1 and triadin. By contrast, slow labeling in the presence of channel activator is exclusively on the abundant SERCA pump.

Ohkura and co-workers (Ohkura *et al.*, 1998) provided evidence for interactions between RyR1 and triadin that can be destabilized in the presence of the reducing agent dithiothreitol. More recently, Caswell and co-workers (Caswell *et al.*, 1999) identified a region of triadin spanning AA267 to AA280 that has high affinity for RyR1. This sequence is composed of a short relatively hydrophobic region containing one of two Cys in the triadin molecule that could be involved in stabilizing the triadin/RyR1 complex, especially in the open conformation of the channel as initially proposed by Pessah and co-workers (Liu *et al.*, 1994; Liu and Pessah, 1994).

The most likely explanation underlying the divergent results of Hamilton and co-workers (Aghdasi *et al.*, 1997a,b) is the fact that thiol-reactive reagents are unable to discriminate among different classes of sulfhydryl groups unless extreme care is taken to limit the extent of oxidation/reduction or alkyation/arylation reactions by defining the mole ratio of sulfhydryl reagent to RyR complex or SR protein. In this respect, the type of preparation used (SR bound vs. solubilized vs. purified receptor) undoubtedly has a significant impact on the pattern of sulfhydryl modification, even under defined conditions. Furthermore, the significant conformational transition observed with channel activation (Orlova *et al.*, 1996) must also be accounted for during any thiol-modifying protocol (Liu *et al.*, 1994).

PHYSIOLOGIC ROLE OF HYPERREACTIVE THIOLS: EVIDENCE FOR A REDOX SENSOR?

What possible role could hyperreactive sulfhydryl chemistry within the RyR1 complex contribute to physiologic control of Ca^{2+} release and signaling? To address this important issue, one must acknowledge that the cytosolic milieu of healthy muscle and nonmuscle cells possesses a reduction potential of approximately −230 mV (Hwang *et al.*, 1992). The major cytosolic redox buffers within mammalian cells are based on the relative concentration of reduced glutathione (GSH) and oxidized glutathione (GSSG) or NADH and NAD^+. Thus, the large junctional foot domain finds itself within a highly reduced microenvironment. There is convincing evidence that redox potential can dramatically influence RyR1 function from measurements made with both isolated SR and intact muscle fibers. For example, Koshita and co-workers found that Ca^{2+} release from SR could be induced by oxidizing compounds such as alcian blue and plumbagin and was partially blocked in the presence of GSH (Koshita *et al.*, 1993). More recently, Abramson and co-workers have reported that GSH reduces, whereas GSSG enhances, the activity of RyR1 (Zable *et al.*, 1997). In this regard, Marengo and co-workers (Marengo *et al.*, 1998) recently made an insightful observation that RyR1 and RyR2 channels reconstituted in BLM display different patterns of Ca^{2+} dependencies regardless of their origin and that the patterns could be deliberately altered with thiol-oxidizing reagents. Their results imply that in the reducing environment of the muscle cell, Cys residues critical to function are maintained in the reduced state and may account for low open probability (P_o) and low sensitivity to Ca^{2+} activation. However, the typical solutions used in the preparation of SR for *in vitro* study do not contain a redox buffer. It is likely that functionally important Cys within a large fraction of RyR complexes auto-oxidize, resulting in channels that have high P_o and heightened sensitivity to Ca^{2+} activation. How hyperreactive Cys moieties identified in the Pessah lab relate to redox control of RyR complexes and the nature of this control are unclear at the present time and are a major focus of current work. Initially, Pessah and co-workers reported that the influence of forming CPM–thioether adducts with junctional SR from skeletal muscle was to inactivate channels reconstituted in the planar bilayer gating and inhibit Ca^{2+} -induced Ca^{2+} release from SR vesicles (Liu *et al.*, 1994). However, these effects took several minutes to manifest and it is questionable whether formation of adducts with additional less reactive Cys residues was in fact responsible for channel inactivation. The involvement of less reactive Cys in channel inactivation is consistent with the latter phase of sulfhydryl modification reported with millimolar *N*-ethylmaleimide reported by Hamilton and

co-workers (Aghdasi *et al.*, 1997a). More recently, labeling experiments have been performed that limit CPM–adduct formation to ≤60 sec under conditions in which the channel P_o is very low to enhance specificity (Feng *et al.*, 1999). Under these conditions, SR Ca^{2+} transport was not inherently modified by the formation of CPM–RyR1 adducts. However, selective formation of CPM adducts with hyperreactive Cys moieties did eliminate the sensitivity of the channel complex to redox-active quinones, revealing a redox-sensing function (see below).

One possible physiologic role for hyperreactive sulfhydryl chemistry within the RyR complex may be to respond to redox-active signaling molecules such as nitric oxide (NO) (Aghdasi *et al.*, 1997b; Stoyanovsky *et al.*, 1997; Zahradníková *et al.*, 1997; Xu *et al.*, 1998), as has been observed for other ion channels. Thus, one plausible hypothesis is that hyperreactive thiols are an essential component of a redox-sensor that operates during normal intracellular signaling (for example, to sense NO) and may be involved in mediating changes in Ca^{2+} signaling during oxidative stress (see below). The general importance of redox status in regulating microsomal Ca^{2+} channels is further underscored by the finding that oxidized glutathione influences Ca^{2+} transport across the ER of hepatocytes by modulating the IP_3 receptor (Renard-Rooney *et al.*, 1995). In this respect, Pessah and co-workers are exploring if changes in the localized redox potential across the SR/ER compartment represent an important feedback mechanism that modulates SR/ER Ca^{2+} channel function, and thereby modifies microsomal Ca^{2+} fluxes in response to changing cellular redox state.

ROLE OF HYPERREACTIVE THIOLS IN OXIDATIVE STRESS

The RyR complex of skeletal and cardiac muscle appears to be exquisitely sensitive to mediators of oxidative stress, including hydrogen peroxide (Favero *et al.*, 1995; Suzuki *et al.*, 1998) and reactive oxygen species (Xiong *et al.*, 1992; Stuart *et al.*, 1992). Recently, we have examined the possible functional role of hyperreactive sulfhydryl moieties within the RyR complex in Ca^{2+} deregulation associated with redox active quinones (Feng *et al.*, 1999). Quinone structures are ubiquitous in the human environment, having both natural and anthropogenic sources. Quinones are of significant concern to human health because their intrinsic electrophilicity can induce various patterns of acute and chronic oxidative damage to biological tissues. Which critical biomolecules are the target of quinone redox cycling and/or arylation reactions and are primarily responsible for altered cellular Ca^{2+} regulation are largely unknown. Although the biological activity of quinones has been closely associated with changes in cellular Ca^{2+} regulation in a number of cell types, there is a critical need to identify key Ca^{2+} regulatory proteins that are the principal targets of quinone-mediated oxidative insult and to determine the exact role that these altered macromolecules play in cellular dysfunction and organ-selective toxicity (Monks *et al.*, 1992).

The fluorogenic maleimide CPM was used to measure the reactivity of hyperreactive sulfhydryl moieties on SR membranes in the presence and absence of quinones by analyzing the kinetics of forming CPM–thioether adducts and localization of fluorescence by SDS-PAGE. Doxorubicin, 1,4-naphthoquinone (NQ), and 1,4-benzoquinone (BQ) selectively and dose-dependently interact with a class of hyperreactive sulfhydryl groups localized on ryanodine-sensitive Ca^{2+} channels (RyR), and its associated protein, triadin, of skeletal-type channels. NQ and BQ are the most potent compounds tested for reducing the rate of CPM labeling of hyperreactive SR thiols (IC_{50} = 0.3 and 1.8 μM, respectively) localized on RyR and associated protein. The reduced forms of quinone, *tert*-butylhydroquinone and 5-iminodaunorubicin, did not significantly alter the pattern or kinetics of CPM labeling up to 100 μM, demonstrating that the quinone group is essential for modulating the state of hyperreactive SR thiols. Nanomolar NQ enhances the association of [^{3}H]ryanodine for its high-affinity binding site and directly enhance channel open probability in bilayer lipid membrane in a reversible manner. By contrast, micromolar NQ produces a time-dependent biphasic action

on channel function leading to irreversible channel inactivation. Most importantly, the sensitivity to redox-active quinones could be eliminated upon formation of thioether adducts between CPM and the most reactive thiols of the RyR channel complex. The Ca^{2+}-dependent cytotoxicities observed with reactive quinones formed at the microsomal surface by oxidative metabolism may be related to their ability selectively to modify hyperreactive thiols regulating normal functioning of microsomal Ca^{2+} release channels. Importantly, these results raise the possibility that microsomal Ca^{2+} channels may actually utilize hyperreactive sulfhydryl chemistry in "sensing" localized changes in redox environment.

CONCLUSIONS

A small number of hyperreactive thiols have been shown to exist within the RyR complex. Their functional role does not appear to impact directly on channel gating. Rather hyperreactive Cys moieties may represent biochemical components of a redox sensor that conveys information about localized changes in redox potential produced by physiologic (*e.g.*, glutathione, NO) and pathophysiologic (quinones, reactive oxygen species) channel modulators to the Ca^{2+} release process. The molecular and functional details of such a redox sensor remain to be elucidated.

ABBREVIATIONS

BQ, 1,4-Benzoquinone; CPM, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; Cys, cysteine; DTDP, 2,2′-dithiopyridine; DTNB, 5,5′-dithiobis(2-nitro)benzoate; DTT, dithiothreitol; ER, endoplasmic reticulum; GSH, reduced glutathione; GSSG, oxidized glutathione; IP_3R, inositol 1,4,5-triphosphate receptor; JSR, junctional sarcoplasmic reticulum; LSR, longitudinal sarcoplasmic reticulum; NO, nitric oxide; NQ, 1,4-naphthoquinone; P_o, open probability; RyR, ryanodine receptor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum.

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