Site-Selective Modification of Hyperreactive Cysteines of Rybyodine Receptor Complex by Quinones

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ABSTRACT

Quinones undergo redox cycling and/or arylation reactions with key biomolecules involved with cellular Ca$^{2+}$ regulation. The present study utilizes nanomolar quantities of the fluorogenic maleimide 7-diethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin (CPM) to measure the reactivity of hyperreactive sulfhydryl moieties on sarcoplasmic reticulum (SR) membranes in the presence and absence of quinones by analyzing the kinetics of forming CPM-thioether adducts and localization of fluorescence by SDS-polyacrylamide gel electrophoresis. Doxorubicin, 1,4-naphthoquinone (NQ), and 1,4-benzoquinone (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-imida-

Quinone structures are ubiquitous in the human environment, having both natural and anthropogenic sources. Human exposure to quinones can occur clinically, e.g., the anti-neoplastic anthraquinones such as doxorubicin (DXR) (Olson and Mushlin, 1990) and by environmental exposure to diesel exhaust, cigarette smoke, and industrial particulate matter (Monks and Lau, 1992). In addition, a large number of environmental contaminants from industrial sources including carbamate pesticides, naphthalene, and polyaromatic hydrocarbons are metabolized via quinone intermediates. 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. The biological activity of quinones has been closely associated with changes in cellular Ca$^{2+}$ regulation in a number of cell types. However, there is a critical need to identify key Ca$^{2+}$ regulatory proteins that are the principle 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).

Ca$^{2+}$ channels localized to the sarcoplasmic reticulum (SR)/endoplasmic reticulum (ER) membrane including ryanodine receptors (RyRs) (Agdahsi et al., 1997a; Quinn and Ehrlich, 1997; Zable et al., 1997) and inositol 1,4,5-trisphosphate receptors (Bootman et al., 1992, Bird et al., 1993; Kaplin et al., 1994) have been shown to be extremely sensitive to oxidation-induced changes in function elicited by chemically diverse xenobiotic oxidizing agents. More re-

ABBREVIATIONS: BLM, bilayer lipid membrane; BQ, 1,4-benzoquinone; CE, coupling enzyme; CPM, 7-diethylamino-3-(4’-maleimidylphenyl)-4-methylcoumarin; DXR, doxorubicin; IDAU, 5-iminodaunorubicin; MOPS, 3-(N-morpholino)propanesulfonic acid; NQ, 1,4-naphthoquinone; NQS, 1,2-naphthoquinone-4-sulfonic acid; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; RR, ruthenium red; RyR1, skeletal isoform of ryanodine receptor; RyR2, cardiac form of ryanodine receptor; SERCA, SR/ER Ca$^{2+}$ ATPase; SR, sarcoplasmic reticulum, TG, thapsigargin; THQ, tert-butylhydroquinone.

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cently, nitric oxide has been demonstrated to activate cardiac RyRs by poly-S-nitrosylation (Xu et al., 1998), and nitric oxide seems to confer protection against oxidation-induced Ca\(^{2+}\) release (Aghdasi et al., 1997a). The mechanism by which diverse oxidizing agents alter Ca\(^{2+}\) channel activity has remained unclear. One possible mechanism underlying the high sensitivity of microsomal Ca\(^{2+}\) channels to oxidizing agents may involve the presence of a small number of extremely reactive (hyperreactive) sulfhydryl groups which are important for regulating aspects of function (Liu et al., 1994; Liu and Pessah, 1994). The existence of a class of hyperreactive sulfhydryl moieties associated with the RyR complex, which is several orders of magnitude more reactive than other SR protein thiols, was revealed by the ability of these sulfhydryls to rapidly and selectively form Michael adducts with a limiting concentration of the fluorescent maleimide 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM; 0.01–1 pmol CPM/mg of SR protein). A unique feature of channel-associated hyperreactive sulfhydryl moieties is that their hyperreactivity appears to be allosterically regulated by physiological ligands such as Ca\(^{2+}\) and Mg\(^{2+}\) and by pharmacological probes such as ryanodine, neomycin, and ruthenium red (RR). The RyR complex appears to possess a biochemical “sensor” which can monitor the local redox environment. Recent advances indicating microsomal Ca\(^{2+}\) channels are under strict redox control raises an important question as to whether redox active quinones can selectively target hyperreactive sulfhydryl moieties associated with ryanodine-sensitive Ca\(^{2+}\) channels (RyRs), thereby altering microsomal Ca\(^{2+}\) transport function. Fluxes of Ca\(^{2+}\) across SR/ER stores are essential for normal cellular signaling in healthy cells. The fact that oxidative metabolism of prooxidants to active quinone structures occurs principally by the cytochrome P-450 monooxygenases localized to the microsomal membrane raises the possibility that site-selective oxidation of ryanodine-sensitive calcium channels may be relevant to early mechanisms of oxidative damage. In the present article, fluorescent kinetic labeling experiments with discriminating concentrations of CPM and intact SR membranes are utilized to validate the hypothesis that the RyR complex is uniquely sensitive to local changes in redox environment induced by the presence of reactive quinones, thereby revealing an important mechanism by which quinones can alter cellular Ca\(^{2+}\) regulation.

**Materials and Methods**

**Preparation of SR Membranes.** SR membrane vesicles enriched in biochemical markers of the terminal cisternae were prepared from back and hind limb skeletal muscles of New Zealand White rabbits according to the method of Saito (Suito et al., 1984). Heavy SR from rat cardiac ventricles was prepared by sucrose-density gradient centrifugation, as described previously (Pessah et al., 1990). The preparations were stored in 10% sucrose, and 5 mM imidazole (pH 7.4) at ~80°C until needed.

**Kinetic Fluorescence Measurement of CPM-Thioether Adducts.** The nonfluorescent maleimide CPM (Molecular Probes, Eugene, OR) readily undergoes Michael addition with protein thiols producing an irreversible adduct with high fluorescent yield (Sipple, 1981). Studies aimed at quantifying the kinetics of forming CPM-thioether adducts were performed with SR protein (50 \(\mu\)g/ml) diluted 100-fold in solution A consisting of 100 mM KCl and 20 mM 3-(N-norpholino)propanesulfonic acid (MOPS; pH 7.0) just before initiation of an experiment. The measurement and analysis of the reaction kinetics of forming CPM-thioether adducts were performed according to the protocol of Liu et al. (1994) with minor modifications. All labeling studies utilized CPM at concentrations ranging between 0.2 and 1.0 pmol/\(\mu\)g SR protein) such that the SR thiol concentration greatly exceeded that of CPM. Unless otherwise noted, 50 \(\mu\)g/ml SR protein was exposed to 10 to 50 nM CPM. The vesicles were incubated with the test quinone in solution A for 5 min before the introduction of CPM by Hamilton syringe into a cuvette whose contents were stirred constantly at 37°C. The increase in fluorescence intensity was continuously monitored by a SML 8000 spectrofluorometer (SML Instruments Inc., Urbana, IL) interfaced with an IBM computer/recording system. Excitation and emission were set at 397 nm and 465 nm (width of slit = 4 nm), respectively. The rates of increasing fluorescence were sampled at 1 Hz and analyzed by nonlinear regression analysis (ENZFITTER, Elsevier BioSoft). Each of the agents used in the study (e.g., quinone, CaCl\(_2\), MgCl\(_2\)) were initially examined for autofluorescence or for their ability to quench CPM fluorescence in the presence of glutathione or SR vesicles (i.e., after CPM-thioether fluorescence had reached a maxima).

The time course of the increase in fluorescence intensity (F), obtained under conditions promoting channel closure (mM Mg\(^{2+}\) or with Ca\(^{2+}\) buffered to <100 nM by EGTA) or channel activation (in the presence of \(\mu\)M Ca\(^{2+}\) or quinone), was fit with single or multieponential, respectively, from which the corresponding time constants (\(k\)) or apparent half times (\(T_{1/2}\)) were calculated. The rate constant (\(k\)) was considered to be proportional to the number of free sulfhydryl groups available for CPM conjugation (i.e., \(k = k_m [SH]_r\)) (Liu et al., 1994).

**SDS-polyacrylamide gel electrophoresis (PAGE).** Native SR protein (10–20 reactions each at 50 \(\mu\)g/ml) was incubated with 1 mM Mg\(^{2+}\) or EGTA in the presence or absence of quinone compound at 37°C in solution A. After exposure of SR membranes to CPM (<1.0 pmol/\(\mu\)g protein) for 1 min, 2 mM N-ethylmaleimide (NEM) was added to quench the reaction. The CPM-labeled SR protein was combined and pelleted by centrifugation (90 min at 200,000\(\times\)g). The pellets were resuspended in a small volume of buffer and denatured with an equal volume of nonreducing sample buffer consisting of 48 mM Na\(_2\)HPO\(_4\), 170 mM Na\(_2\)HPO\(_4\) (pH 7.4), 6 M urea, 0.02% bromophenol blue, and 1% (w/v) SDS (final concentrations). The samples were incubated at 60°C for 10 min and 30 to 80 \(\mu\)g of protein was loaded onto a 3 to 10% gradient SDS-polyacrylamide gel (Laemmli, 1970) and electrophoresed at constant voltage (200 V). The fluorescent protein bands on PAGE gels were visualized at 360 nm excitation using a transilluminator and the fluorescence image photographed through a 450-nm cutoff filter. The fluorescence intensity of protein bands was digitized by a video analysis system (SPSS, Chicago, IL) and integrated by computer within the linear range of protein density.

**Ca\(^{2+}\) Flux Measurement.** Measurement of Ca\(^{2+}\) transport across SR membranes were performed using the absorbance dye antipyrilylazo III (APIII) or the fluorescent indicator fluo-3. SR membranes (50 \(\mu\)g/ml) were equilibrated at 37°C with transport buffer consisting of 92 mM KCl, 20 mM K-MOPS (pH 7.0), 7.5 mM Na-pyrophosphate, and 250 \(\mu\)M APIII or 0.5 \(\mu\)M fluo-3. A coupled enzymatic enzyme (CE) system consisting of 1 mM MgATP, 10 \(\mu\)g/ml creatine phosphokinase, and 5 mM phosphocreatine was present to regenerate ATP. Ca\(^{2+}\) fluxes were monitored by measuring APIII absorbance at 710 – 790 nm using a diode-array spectrophotometer (model 8452A; Hewlett Packard, Palo Alto, CA). Alternately, changes in fluo-3 fluorescence intensity were measured at 530 nm emission (510 nm excitation) at 37°C using a SML 8000 fluorometer. To measure the influence of quinones on Ca\(^{2+}\) efflux, SR was loaded either with six sequential additions of 20 nmol of CaCl\(_2\) allowing the extravesicular Ca\(^{2+}\) to return to baseline between additions, or one 100 nmol addition of CaCl\(_2\). Once the loading phase was complete, quinone or dihydroquinone was added to the cuvette to assess the influence on Ca\(^{2+}\) efflux. Alternately, quinone was added just before initiating SR.
Ca$^{2+}$ loading to assess influences on initial rates of uptake. In these experiments, some of the SR was incubated with 50 nM CPM for 1 min (terminated by 50 μM glutathione reduced form) at 37°C in the presence of 1 mM free Mg$^{2+}$ (to reduce channel-open probability) to selectively react with hyperreactive sulfhydryls to form thioether adducts. Raw data were collected digitally and analyzed by nonlinear regression analysis.

**Measurement of $[^{3}H]$Ryanodine Binding and Data Analysis.** Equilibrium and kinetic measurements of specific high-affinity $[^{3}H]$ryanodine binding were determined according to the method of Pessah et al. (1987). SR vesicles (50 μg protein/ml) were incubated with quinone (10 nM to 10 μM) in assay buffer containing HEPES (20 mM, pH 7.1), KCl (250 mM), NaCl (15 mM), CaCl$_2$ (25 μM), MgCl$_2$ (1 mM), and $[^{3}H]$ryanodine (1 nM). Equilibrium studies were performed by incubating the reaction at 37°C in the dark for 3 h, at which time the samples were filtered through GF/B glass-fiber filters and washed twice with ice-cold harvest buffer composed of 20 mM Tris-HCl, 250 mM KCl, 15 mM NaCl, and 50 μM CaCl$_2$ (pH 7.1). Apparent association kinetics were determined in the presence and absence quinone as described above except that reactions were quenched at times ranging between 5 min and 3 h. Each assay was performed in duplicate and repeated at least twice. Nonspecific binding was determined by incubating SR vesicles with the concentration of quinone that give maximum binding and 1000-fold excess unlabeled ryanodine.

The dose-response curves were plotted as specific binding of $[^{3}H]$ryanodine (pmol/mg protein) versus log concentration of the quinone. EC$_{50}$ and IC$_{50}$ values of the quinones were determined by logit-log analysis by plotting $[B/(B_{\text{max}} - B)]$ against log concentration of quinone (where B = specific $[^{3}H]$ryanodine occupancy, $B_{\text{max}}$ = maximum $[^{3}H]$ryanodine occupancy in the presence of quinone), with data between 10 to 90% of $B_{\text{max}}$. Association kinetics were analyzed excluding the inhibition phase (when present) by fitting to a single exponential and calculating the apparent association rate constant ($K_{\text{on}}$) and apparent half-time ($T_{1/2}$) (ENZFITTER, Elsevier Biosoft).

**Single-Channel Kinetics in Bilayer Lipid Membranes.** RyR channels were reconstituted into artificial planar lipid bilayer (5:3:2 phosphatidylethanolamine/phosphatidylserine/phosphatidylcholine, 60 mg/ml in decane) by introducing SR vesicles to the cis chamber. The cis chamber contained 0.7 ml of 500 mM CaCl$_2$, 50 μM CaCl$_2$, and 10 mM HEPES (pH 7.4), whereas the trans side contained 100 mM CaCl$_2$, 50 μM CaCl$_2$, and 10 mM HEPES (pH 7.4). Upon the fusion of SR vesicle into bilayer, the cis chamber was perfused with the identical solution, except lacking CaCl$_2$. Single-channel activity was measured at a holding potential of +30 mV (applied cis relative to the trans ground side) using a patch clamp amplifier (model 3900A, Dagan Co., Minneapolis, MN). The data was filtered at 1 kHz before acquisition at 10 kHz by a DigiData 1200 (Axon Inst., Foster City, CA). The data were analyzed using pClamp 6 (Axon Instruments, Burlingame, CA) without additional filtering.

**Results**

**Quinones Decrease Kinetics of Forming CPM-Thioether Adducts.** The presence of pharmacological or physiological agents that promote SR Ca$^{2+}$ channel closure have been shown to enhance significantly the rate by which CPM forms Michael adducts with hyperreactive sulfhydryl moieties localized on RyR1 (skeletal isoform of ryanodine receptor) and channel-associated proteins found within the triad junction (Liu et al., 1994; Liu and Pessah, 1994). Figure 1, A and B (traces labeled 0), show the rapid kinetics of adduct formation between 1 pmol CPM/μg skeletal junctional SR in the presence of 7 μM Ca$^{2+}$ and 1 mM Mg$^{2+}$ (calculated initial rate, $k = 0.0275 \pm 0.0035 \text{ s}^{-1}$; mean of 12 determinations). Under these conditions, the rate of CPM-thioether adduct formation was reduced in a dose-dependent manner by a 30-s pretreatment of SR membranes with 1,4-naphthoquinone (NQ) or 1,4-benzoquinone (BQ). The maximal concentration of NQ or BQ used in the present experiments (2 μM) decreased the initial rate of CPM labeling >10-fold ($k = 0.0023 \pm 0.0007$ with NQ, mean of four determinations), when compared with rates obtained in the absence of quinone. The presence of reactive quinone when channel closure is favored (in the presence of 1 mM Mg$^{2+}$) qualitatively and

![Image](https://example.com/image.png)

**Fig. 1.** Quinone compounds dose-dependently diminish the kinetics of CPM-thioether adduct formation with skeletal junctional SR membrane proteins. SR vesicles (50 μg/ml) were equilibrated at 37°C in solution A containing 100 mM KCl, 20 mM MOPS (pH 7.0) and 1 mM MgCl$_2$ for 3 min. The indicated concentration of NQ (A) or BQ (B) was added to the mixture and incubated for 30 s before CPM (50 nM, i.e., 1 pmol/μg protein) was introduced to the mixture to initiate fluorescent thiol labeling as described in Materials and Methods. For each control trace (labeled 0), 10 μl of solvent (dimethyl sulfoxide) was added instead of quinone. C, NQ (▲), BQ (▲), DXR (+), or THQ (●) was added and incubated for 30 s (3 min for DXR) before labeling with CPM. The experiments shown in A and B are representatives of at least three determinations and produced similar results. The data shown in C were fit with a single-or double-exponential model for the samples treated with 1 mM MgCl$_2$ or quinone, respectively. The apparent rate constants ($k$) were calculated from apparent half-time ($T_{1/2}$). Each datum point is the average from three experiments. The quinone compounds at concentrations used did not interfere with CPM fluorescence in the absence of SR membranes.
quantitatively mimics results obtained with a physiological channel activator, e.g., the presence of 100 µM Ca²⁺, in reducing CPM labeling kinetics (to $k = 0.0024 \text{ s}^{-1}$; Liu et al., 1994), but differs in the mechanism by which channel activation is obtained.

Figure 1C summarizes the rates of forming CPM-thioether adducts obtained with skeletal SR and several quinone structures. NQ and BQ were the most potent compounds tested and exhibited an $IC_{50}$ of 0.34 ± 0.05 µM and 1.8 ± 0.2 µM, respectively, with a brief 30-s exposure before initiating adduct formation with CPM. 1,2-Naphthoquinone-4-sulfonic acid (NQS), $IC_{50} = 2.8 ± 0.2$ µM, was 8.5-fold less potent than NQ under identical treatment conditions. By comparison to naphthoquinones, the anthraquinone DXR required incubations of ≥2 min with SR to significantly decrease the rate of formation of CPM-thioether adducts. With a 3-min pretreatment of SR, DXR was found to be nearly 50-fold less potent than NQ ($IC_{50} = 16.3 ± 0.8$ µM). Importantly, tert-butylhydroquinone (THQ), whose quinone moiety is fully reduced, lacks significant activity in the CPM assay at concentrations ≤100 µM with a 30-min treatment (Fig. 1C). Figure 2 shows that cardiac junctional SR enriched in RyR2 (cardiac form of ryanodine receptor) measured under conditions which favor channel closure (7 µM Ca²⁺, 10 mM Mg²⁺) exhibits rapid labeling kinetics in the absence of quinone. Like skeletal SR, cardiac SR is also highly sensitive to NQ, BQ, and DXR, which significantly slow the rate of forming CPM-thioether adducts with the same apparent rank order of potency.

**Quinones Alter Hyperreactive Sulphydryls on RyR1 and Triadin.** The identity of protein(s) labeled by CPM in the presence and absence of quinone was determined by visualizing fluorescent labeled bands after SDS-PAGE as described in Materials and Methods. Consistent with previous findings, SR labeled for 1 min in a medium containing 10 nM CPM and 1 mM Mg²⁺, but lacking quinone, revealed CPM fluorescence was predominantly localized to the RyR1 protomer of $M_r 565,000$, a major proteolytic fragment of RyR1 of $M_r 150,000$ (Meissner et al., 1989), and triadin of $M_r 95,000$ (Fig. 3A, lane 1 labeled Mg). A 30-s preincubation of SR with NQ (2 µM), BQ (2 µM), or NQS (10 µM) before labeling with CPM for 1 min revealed a selective loss of fluorescence associated with RyR1 and triadin protein bands (Fig. 3A, lanes 2–4 labeled NQ, BQ, and NQS, respectively). Digital imaging of the fluorescent bands on gels revealed a >98% decrease in the CPM fluorescence intensity associated with the RyR1 protomer and triadin in SR-pretreated with quinone compared to control SR treated with Mg²⁺ alone (Fig. 3A, left panel). However, no significant change in the pattern of CPM labeling was detected with SR pretreated with fully reduced THQ (50 µM for 30 min; Fig. 3B, lane labeled THQ) when compared with the control SR (lane labeled —). Consistent with the behavior of DXR in CPM kinetic labeling experiments, a higher concentration and longer pretreatment time were needed for anthraquinone to alter the pattern of fluo-

![Fig. 2](image)

Fig. 2. NQ, BQ, and DXR dramatically reduce the rate of CPM-thioether adduct formation with cardiac junctional SR membrane proteins. Cardiac SR vesicles (50 µg/ml) were equilibrated at 37°C in solution A in the presence of 1 mM MgCl₂ for 3 min. Dimethyl sulfoxide (10 µl, trace labeled "Quinone"), NQ (2 µM), or BQ (2 µM) was added to the mixture and incubated for 30 s. DXR was preincubated longer than NQ or BQ (3 min). CPM (10 nM, i.e., 0.2 pmol/µg protein) was introduced to the mixture and the increase in CPM fluorescence was monitored. The experiment was repeated with two independent preparations.

![Fig. 3](image)

Fig. 3. Quinones selectively influence CPM labeling on RyR and triadin protomers in skeletal SR as revealed by CPM fluorescence on SDS-PAGE. SR protein (50 µg/ml; total 1 mg) in solution A was preincubated under varied conditions. A, in the presence of 1 mM MgCl₂ alone (lane 1), or 1 mM MgCl₂ plus 2 µM NQ (lane 2), 2 µM BQ (lane 3), or 10 µM NQS (lane 4) for 30 s, respectively at 37°C; or 50 mM DXR for 3, 10, and 30 min (lanes 5, 6, 7, respectively) at 37°C. Note the change in CPM labeling from RyR protomer ($M_r 565,000$), a band at $M_r 160,000$ (possibly a major proteolytic fragment of RyR) and triadin ($M_r 95,000$) to the abundant Ca²⁺-ATPase ($M_r 110,000$). B, in the absence or presence of redox-inactive THQ (50 µM) or IDAU (50 µM) for 30 min or 16 h, respectively, does not alter the pattern of CPM fluorescence. C, SR was treated in the presence of 0.2 mM EGTA and 0, 10, 20, 30, or 40 pmol of NQ/µg of protein for 30 s at 37°C. In each case CPM (1 pmol/µg protein) was added to the mixture, incubated for 1 min, and the reaction was quenched with 2 mM NEM. The CPM-labeled protein was denatured in SDS sample buffer in the absence of reducing agents. Thirty micrograms of protein from each sample was loaded onto a 3 to 10% gradient gel and electrophoresed at 200 V. The fluorescent proteins on the gels were visualized on a transilluminator, photographed through a filter, and digitized as described in Materials and Methods.
rescent labeling on SDS-PAGE. The degree to which DXR (50 μM) decreased CPM labeling on RyR1 and triadin protomers by a detectable level was dependent on the length of time SR was exposed to the drug. SR protein pretreated with DXR for 3, 10, and 30 min largely eliminated detectable fluorescence associated with these bands (Fig. 3A, DXR lanes 5, 6, and 7, respectively). Importantly, 5-iminodaunorubicin (IDAU; 50 μM), which lacks redox activity, fails to alter the pattern of CPM labeling even with several hours of incubation (Fig. 3B, lane labeled IDAU).

Figure 3C shows that NQ dose-dependently decreased CPM labeling on RyR1 and triadin. The fluorescence associated with the RyR1 protomer at each NQ concentration was integrated and compared with that of control (Fig. 3C, lane labeled 0) in the presence of 0.2 mM EGTA to promote channel closure. SR protein treated with 10, 20, 30, and 40 pmol/μg protein (0.5, 1, 1.5, and 2 μM) of NQ for 30 s before labeling with CPM resulted in 53%, 48%, 30%, and 4% of the CPM fluorescence at the RyR1 protomer, respectively, relative to control (Fig. 3C, left panel, lanes labeled 10, 20, 30, and 40). Interestingly, CPM fluorescence associated with triadin dramatically declined near the limit of detection with the lowest concentration of NQ used in these experiments (0.5 μM NQ).

**Nanomolar Quinone Alters Ca²⁺ Transport across Actively Loaded SR Vesicles.** Figure 4A shows that NQ mobilizes Ca²⁺ from actively loaded SR in a dose-dependent manner that quantitatively parallels its ability to diminish labeling of hyperreactive SR thiols with CPM. In the presence of 50 μg/ml SR protein and transport buffer containing ATP and CE, the Ca²⁺-sensitive dye APIII responded to addition of 100 μM CaCl₂ with an abrupt rise in absorbance which was followed by a rapid decrease that stemmed from the uptake of Ca²⁺ into SR vesicles. Addition of 300 nM to 2 μM NQ induced a net efflux of Ca²⁺ from SR attributable to activation of the RyR1 complex. As expected, addition of 2 μM RR during the release phase blocks the channel and results in reaccumulation of Ca²⁺ despite the presence of NQ. The threshold for NQ-induced Ca²⁺ release ranged between 50 and 100 nM (n = 12 determinations). NQ was not found to interfere with the APIII dye signal at the concentrations used in these experiments by final addition of ionophore A23187 to calibrate the signal (Fig. 4A). Similar effects on Ca²⁺ transport were observed with BQ (Fig. 4B). After the Ca²⁺ loading phase in which six additions of 20 μM CaCl₂ were made to the SR mixture, addition of BQ (300 nM to 2 μM) induced a dose-dependent release of accumulated Ca²⁺ which could largely be inhibited by prior addition of 2 μM RR (Fig. 4B, lowest trace). Consistent with findings obtained from CPM-labeling kinetics, 5- to 6-fold higher concentrations of NQ were required to produce release rates comparable to NQ and BQ (not shown).

The hypothesis that quinones can alter Ca²⁺ transport across SR by a selective mechanism influencing hyperreactive channel sulphydryl moieties within the RyR1 complex was further tested by measuring the rate of active Ca²⁺ accumulation in SR vesicles. Figure 5A reveals that Ca²⁺ uptake into the membrane vesicles was completely driven by SR/ER Ca²⁺ ATPase (SERCA) pump activity since active Ca²⁺ accumulation was eliminated by 1 μM thapsigargin (TG), a specific inhibitor of the Ca²⁺(Mg²⁺)-ATPase (compare traces 1 and 3). NQ (2 μM) added 15 min before initiating Ca²⁺ loading of SR significantly reduced the initial rate of Ca²⁺ uptake, and this effect of NQ was inhibited by the presence of the RyR1 channel blocker RR (Fig. 5A, compare traces 2 and 4). These findings are consistent with results from Ca²⁺ efflux experiments shown in Fig. 4 and confirmed that NQ at the highest concentration used in the present study reduced SR Ca²⁺ buffering by selective activation of the RyR1 complex. If hyperreactive thiols associated with the RyR1 complex contribute an essential “redox-sensing” function to channel regulation, then formation of CPM-thioether adducts would be expected to selectively eliminate the inhibition of Ca²⁺ uptake by SR exposed to reactive quinones. Consistent with this hypothesis, SR pretreated for 1 min with 50 nM CPM had no observable influence on the rate of active Ca²⁺ accumulation (Fig. 5A and B, compare traces labeled 3), nor did it influence the ability of TG to inhibit SERCA pump activity (compare traces labeled 1). By contrast, SR vesicles pretreated with CPM selectively eliminated the actions of NQ (Fig. 5A and B, compare traces labeled 2). Thus, the formation of thioether adducts between CPM and hyperreactive sulphydryls on the channel complex essentially mimics the actions of RR in restoring Ca²⁺ accumulation to control levels (Fig. 5B, compare trace 2 to trace 4).
but are mediated by distinct mechanisms. RR interferes with NQ-induced changes in SR Ca\(^{2+}\) transport by blocking ion permeation through the pore, whereas formation of CPM adducts selectively abrogates sensitivity to redox active quinones (Fig. 5A, B, and 5C). A final addition of TG after uptake was complete revealed that SERCA pump inhibition unmasks a RR- and CPM-insensitive Ca\(^{2+}\) leak (Pessah et al., 1997) which releases all of the accumulated Ca\(^{2+}\) (Fig. 5A and B; where TG is indicated). Consistent with its inability to alter CPM labeling kinetics, addition of THQ as high as 5 \(\mu\)M under identical condition did not significantly alter Ca\(^{2+}\) uptake rates (not shown).

DXR is an antineoplastic anthraquinone that has been shown to potently activate RyR1 (Abramson et al., 1988) and the RyR2 (Pessah et al., 1990). Although the exact mechanism by which DXR activates the Ca\(^{2+}\) channel complex remains unclear, the active redox potential of its quinone appears to be essential for this activity since related structures lacking quinone moieties (e.g., IDA) lack activity. To further test whether hyperreactive sulhydryls of the RyR1 complex are important in the activity of DXR, parallel experiments examining Ca\(^{2+}\) uptake were performed with the Ca\(^{2+}\) sensitive dye fluo-3. SR was pretreated with CPM (30–75 nM) for 1 min under conditions of nominally free (7 \(\mu\)M) extravesicular Ca\(^{2+}\) and 1 mM Mg\(^{2+}\) to inhibit the Ca\(^{2+}\) channel to selectively label hyperreactive sulhydryl moieties associated with the channel complex (Fig. 5C). Ca\(^{2+}\), fluo-3, ATP, and CE were subsequently introduced to assess the ability of SR vesicles to accumulate Ca\(^{2+}\). Under these conditions, 30 \(\mu\)M redox-active DXR significantly reduced Ca\(^{2+}\) uptake rates of native SR (Fig. 5, compare traces labeled DXR(−) to that labeled 0 CPM). However, pretreatment of SR with CPM (traces labeled 30, 50, and 75 nM) revealed that formation of thioether adducts restored the rate of Ca\(^{2+}\) uptake toward that of control. Additions of ionophore 4-Br-23187 followed by 0.5 mM EGTA at the end of each experiment showed that the calibration of the dye remained unchanged and demonstrated that the reagents used did not interfere with the response of fluo-3. These results indicate that hyperreactive sulhydryls associated with the RyR1 complex contribute a redox-sensing function and that these effects are independent of the quinone or method used to make the measurement.

**Concentration- and Time-Dependent Mechanism by Which NQ Modifies RyR Function.** To further elucidate the mechanism underlying NQ-mediated effects on vesicular Ca\(^{2+}\) transport, the actions of NQ on the binding of \(^{3}\text{H}\)ryanodine to SR membranes were examined under equilibrium and kinetic conditions. Figure 6A reveals that the ability of NQ to modify equilibrium binding of \(^{3}\text{H}\)ryanodine to SR (12.5 \(\mu\)g of protein) was highly dependent on concentration. Under assay conditions which were less than optimally favorable for the binding of \(^{3}\text{H}\)ryanodine (25 \(\mu\text{M}\) Ca\(^{2+}\), 1 mM Mg\(^{2+}\)), nanomolar NQ enhanced occupancy of \(^{3}\text{H}\)ryanodine

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**Fig. 5.** Quinone-mediated reduction of active Ca\(^{2+}\) accumulation by SR is selectively eliminated by formation of CPM adducts with hyperreactive SR sulhydryl moieties. SR membranes (50 \(\mu\)g/ml) were equilibrated in transport buffer at 37°C containing 1 mM Mg\(\text{Cl}_2\), 1.4 mM MgATP, and CE with either APIIII (A and B) or fluo-3 (C) as a Ca\(^{2+}\) calibrate the dye. The experiments were repeated with three different preparations and similar results were obtained.
Materials and Methods. Data presented are the mean of two independent measurements, each performed in duplicate. Reactions were quenched by filtering samples through GF/B glass filters and washing the samples with ice-cold harvest buffer as described in 5. 0.22) within 1 min, and channel activity progressively in-

Discussion

The present results reveal that the RyR1 complex represents one of the most sensitive biological targets yet described for reactive quinones. Utilizing three different measures of channel function (analysis of [3H]ryanodine-binding, macroscopic SR Ca2+ transport, and single channels in BLM), nanomolar quinone is found to promote channel activation by a mechanism which modifies a very small number of hyperreactive cysteine residues localized primarily on the RyR and triadin. In this respect, the intact quinone moiety is essential for activity toward the channel since reduced forms such as THQ and IDAU have no significant effect on CPM-labeling kinetics, localization of fluorescence, or SR function (Pessah et al., 1990). This observation suggests that reactive quinones enhance channel-open state by a mechanism which alters the oxidation state of hyperreactive cysteines, a mechanism which is apparently conserved between skeletal and cardiac RyR isoforms. Previously, we showed that the rate constant (k) for CPM-thioether adduct formation is proportional to the number of free sulphydryl groups which are available for CPM labeling, (i.e., k = K_m [SH]) (Liu et al., 1994). The present results suggest that nanomolar naphtho-
or benzoquinone cause a quantitative diminution in the total number of hyperreactive thiol groups associated with SR membranes, as revealed by the dose-dependent slowing of CPM-labeling kinetics. Comparing Figs. 1 and 3 reveals that the slower kinetics of CPM labeling of SR induced by qui-

![Figure 6](https://example.com/figure6.png)

**Fig. 6.** Concentration- and time-dependent activation and inactivation of [3H]ryanodine-binding activity of SR. SR membranes (50 μg/ml) were incubated without or with NQ in a buffer containing HEPES (20 mM), pH 7.1, KCl (250 mM), NaCl (15 mM), CaCl2 (25 μM), MgCl2 (1 mM), and [3H]ryanodine (1 nM). A, equilibrium-binding experiments showing activation (EC_{50} = 123 nM) and inhibition (IC_{50} = 1.2 μM) of the [3H]ryanodine binding by NQ assayed at 37°C in the dark for 3 h. Control activity in the absence of NQ was 0.12 pmol/mg. Optimal activation was 0.30 pmol/mg. B, time-dependent binding assay was carried out in the absence (○) and presence of 500 nM (▲) or 5 μM (●) NQ for 5 to 180 min at 37°C in the dark. Reactions were quenched by filtering samples through GF/B glass filters and washing the samples with ice-cold harvest buffer as described in Materials and Methods. Data presented are the mean of two independent measurements, each performed in duplicate.
nones is associated with a selective disappearance of CPM labeling from channel-associated protein thiols. These data can be explained by one of three mechanisms (schemes 1–3, Fig. 9). Common to each mechanism is the presence of a nucleophilic domain within the RyR-triadin complex which renders a small number of cysteine hyperreactive (Fig. 9, shaded regions of schemes 1–3).

In Fig. 9, scheme 1, reactive quinones preferentially oxidize hyperreactive thiols to intramolecular or intermolecular disulfide bonds. Such a mechanism would be consistent with the hypothesis of oxidation-induced Ca\(^{2+}\) release as proposed by Abramson and Salama (1989) in which one or more intramolecular oxidations of critical thiols on the channel complex to disulfides (possibly as a result of redox cycling with quinone) are coupled to channel activation. Implicit in this mechanism is the requisite oxidation and reduction of critical thiols coincident with channel opening and closing. In scheme 1 (Fig. 9), naphtho- and anthraquinones accept one electron from hyperreactive thiols, thereby enhancing channel activation as a direct result of oxidizing “critical” channel thiols to disulfides. Whether oxidation/reduction of critical thiols is rapid enough to account for rapid channel transitions characteristic of RyR remains unproved. However, it is unlikely that the stimulatory actions of nanomolar quinone can be attributed to oxidation to intramolecular or intermolecular disulfides because: 1) the BQ semiquinone is extremely electrophilic, making it more likely that BQ will undergo arylation than redox-cycling reactions; 2) the in vitro conditions used in the present study lack reducing cofactor to drive redox cycling; and 3) channel activation induced by reactive quinones is readily reversible in the absence of reducing agent.

In Fig. 9, scheme 2, quinones undergo nucleophilic addition to hyperreactive thiols, resulting in an arylated channel complex. This mechanism implies that normal channel gating does not proceed with a requisite change in oxidation of critical receptor thiols to disulfides per se. Alternately, the formation of arylated thio-adducts induces allosterism which promotes channel activation. Again, this mechanism is less plausible considering the reversible nature of quinone-mediated channel activation. Furthermore, NQ is a better redoxycler than it is an arylator (Monks et al., 1992) and at low concentration (nanomolar) of quinone is associated hyperreactive thiols dramatically reduces the sensitivity of the channel to activation by Ca\(^{2+}\) (Abramson et al., 1988; and Pessah et al., 1990). Consistent with the model, NQ and BQ were also significantly more potent than DXR toward decreasing the rate of CPM labeling of hyperreactive thiols. The apparently higher potency of NQ compared to that of BQ in the CPM assay probably stems from the extreme nucleophilicity of the latter, which is expected to decrease the actual free concentration of quinone in aqueous solution. The concept of redox sensing by the Ca\(^{2+}\) channel complex is supported by the observation that pretreatment of SR with a concentration of CPM known to derivatize a large fraction of the channel-associated hyperreactive thiols dramatically reduces the sensitivity of the channel to activation by NQ and DXR. By destabilizing the closed state, the redox-sensing hypothesis (Fig. 9, scheme 3) could account for why anthraquinones can so effectively sensitize the channel to activation by Ca\(^{2+}\) (Abramson et al., 1988; and Pessah et al., 1990).

TABLE 1
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NQ, 0.5 μM</th>
<th>NQ, 5 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_{a} (\text{min}^{-1}))</td>
<td>0.0172</td>
<td>0.0430</td>
<td>0.0559</td>
</tr>
<tr>
<td>(T_{1/2} (\text{min}))</td>
<td>40.3</td>
<td>16.1</td>
<td>12.4</td>
</tr>
<tr>
<td>(E_{\text{redox}} (\text{pmol/mg}))</td>
<td>0.140</td>
<td>0.428</td>
<td>0.254</td>
</tr>
</tbody>
</table>

Effect of NQ on the association kinetic of \(^{1}H\)ryanodine with SR
SR membranes (50 μg/ml) were incubated in the absence or presence of NQ as described in Fig. 6B. Data were analyzed by nonlinear regression analysis (ENZFITTER, Elsevier Biosoft). Specific \(^{1}H\)ryanodine binding as a function of incubation time was fit with an exponential, from which equilibrium or maximal binding (\(E_{\text{redox}}\), apparent association rate constant (\(k_{a}\)), and apparent half-time (\(T_{1/2}\)) were calculated. The values are the average of two experiments, each performed in duplicate.
Micromolar NQ clearly shows biphasic actions on the binding of \(^{3}H\)ryanodine, first enhancing occupancy followed by inhibition (Fig. 6), whereas anthraquinones only enhance the binding of \(^{3}H\)ryanodine to SR across their dose-response range (1–200 \(\mu\)M) (Abramson et al., 1988; Pessah et al., 1990). Channel inactivation at high concentrations and longer exposure of the RyR complex to NQ appears to proceed by a mechanism different from that seen with nanomolar NQ. The
irreversible mechanism could stem from 1) oxidation of critical thiols or disulfides; 2) oxidation of another, less reactive, class of channel thiols to disulfides; or (3) arylation of the channel complex. In this respect, the actions of anthraquinones, which are poor arylators, have been shown to activate the gating of single RyR channels reconstituted in BLM in a persistent manner without a subsequent phase of inhibition (Holmberg and Williams, 1990; Buck and Pessah, 1995). Ondrias et al. (1990) have, however, reported that DXR exhibits biphasic actions in channels reconstituted from cardiac muscle. Despite the apparent discrepancy in the reported effects of DXR between laboratories (monophasic versus biphasic), it is unlikely anthraquinones promote channel inactivation. Indeed, radioligand-binding experiments with [3H]ryanodine and skeletal (Abramson et al., 1988) or cardiac (Pessah et al., 1990) SR demonstrated only DXR-induced activation of ligand binding, even after several hours of incubation in the presence of anthraquinone.

We provide the first direct evidence for a molecular mechanism by which quinones of toxicological concern selectively target a microsomal Ca\(^{2+}\) channel. Importantly, the present results raise the possibility that microsomal Ca\(^{2+}\) channels may actually utilize hyperreactive sulfhydryl chemistry in "sensing" localized changes in the redox environment. In this respect, the injurious effects of quinones have been attributed to their ability to 1) undergo redox cycling, thereby generating reactive oxygen species; and 2) directly arylate biological macromolecules (Monks et al., 1992). In both muscle and nonmuscle cells, the acute and chronic toxicity mediated by quinones or their precursor molecules are known to be closely associated with a rise in cellular Ca\(^{2+}\) that initiates functional and structural changes which eventually lead to cell death (Farber, 1990; Reed, 1990; Nicoterra et al., 1992). Increased intracellular Ca\(^{2+}\) is known to activate proteases (Nicoterra et al., 1986; Lee et al., 1991), endonucleases (McConkey et al., 1988), phospholipases C (Berridge et al., 1987) and A\(_2\) (Exton, 1990), and kinases (Shulman and Lou, 1989). Quinones that alter normal Ca\(^{2+}\) signaling can be expected to alter Ca\(^{2+}\)-dependent biochemical cascades responsible for maintenance of cellular homeostasis and function. The hypothesis that nonselective peroxidation of membrane lipids can fully account for the loss of ion barriers and the cytotoxicity of quinonoids has been questioned in recent years. Although disagreement exists concerning the sequence of events leading from quinone-mediated disruption of Ca\(^{2+}\) regulation to cell death (Herman et al., 1990), intense interest is now focused on the identity of specific cellular macromolecules which are primary targets of oxidative damage and on assessing their exact role in toxicity (Monks et al., 1992; Hinson and Roberts 1992). To date, most studies aimed at elucidating the molecular mechanisms underlying the cytotoxicity of anthraquinones (Olson and Mushlin, 1990), naphthoquinones (Frei et al., 1986), and benzoquinones (Moore et al., 1988) in a variety of cell types have examined loss of mitochondrial integrity. An added significance of the mechanism revealed in the present study is that RyRs represent a key Ca\(^{2+}\) regulatory channel that is widely expressed within microsomal membrane of a wide variety of cells where most quinone precursor molecules are metabolized to bioactive quinones by the cytochrome P-450 system. Colocalization of ryanodine-sensitive Ca\(^{2+}\) channels and cytochrome P-450 enzyme, which catalyze formation of quinone-containing compounds, could provide a fundamental mechanism by which localized oxidative stress is “sensed” by

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Fig. 8. Reversibility of the actions of NQ on single-channel gating kinetics. After the fusion of a RyR single channel into BLM, the channel activity was continuously recorded through sequential changes in cis solution as indicated below the graph. To test reversibility, cis perfusion was performed with 15 ml of identical buffer containing no NQ. The times denoted in the graph indicates the length of the time the channel was exposed to 200 nM NQ (6 min) or 2 \(\mu\)M NQ (2 min or 15 min) before perfusion. The channel-open probability was determined by measuring >20 s of recording at +30 mV holding potential. This is representative of three independent experiments with similar results.

Fig. 9. Three possible mechanisms by which quinones modulate channel function. Scheme 1, redox cycling model for oxidation-induced channel activation by quinones. Scheme 2, nucleophilic arylation-induced channel activation by quinones. Scheme 3, redox-sensing model for quinone-induced channel activation. See text for details.
the major intracellular Ca\(^{2+}\) store. This mechanism may have both physiological and toxicological significance.

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