

Site-Selective Modification of Hyperreactive Cysteines of Ryanodine 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 found to selectively and dose-dependently interact with a class of hyperreactive sulfhydryl groups localized on ryanodine-sensitive Ca^{2+} channels [ryanodine receptor (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 ($\text{IC}_{50} = 0.3$ and $1.8 \mu\text{M}$, respectively) localized on RyR and associated protein. The reduced forms of quinone, *tert*-butylhydroquinone, and 5-imino-

no-daunorubicin do not alter significantly the pattern or kinetics of CPM labeling up to $100 \mu\text{M}$, demonstrating that the quinone group is essential for modulating the state of hyperreactive SR thiols. Nanomolar NQ is shown to enhance the association of [^3H]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. These results provide evidence that nanomolar quinone selectively and reversibly alters the redox state of hyperreactive sulfhydryls localized in the RyR/ Ca^{2+} channel complex, resulting in enhanced channel activation. The Ca^{2+} -dependent cytotoxicities observed with reactive quinones formed at the microsomal surface by oxidative metabolism may be related to their ability to selectively modify hyperreactive thiols regulating normal functioning of microsomal Ca^{2+} release channels.

Quinone structures are ubiquitous in the human environment, having both natural and anthropogenic sources. Human exposure to quinones can occur clinically, e.g., the antineoplastic 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-

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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.

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 fluorogenic maleimide 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM; 0.01–1 pmol CPM/ μg 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 (Saito 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 $\mu\text{g}/\text{ml}$) diluted 100-fold in solution A consisting of 100 mM KCl and 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS; pH 7.0) just before initiating 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/ μg SR protein such that the SR thiol concentration greatly exceeded that of CPM. Unless otherwise noted, 50 $\mu\text{g}/\text{ml}$ 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 μM Ca^{2+} or quinone), was fit with single or multiexponentials, 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., $k = k_m [\text{SH}]_t$) (Liu et al., 1994).

SDS-polyacrylamide gel electrophoresis (PAGE). Native SR protein (10–20 reactions each at 50 $\mu\text{g}/\text{ml}$) 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/ μg 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 200,000g). The pellets were resuspended in a small volume of buffer and denatured with an equal volume of nonreducing sample buffer consisting of 48 mM NaH_2PO_4 , 170 mM Na_2HPO_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 μg 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 antipyrilazo III (APIII) or the fluorescent indicator fluo-3. SR membranes (50 $\mu\text{g}/\text{ml}$) 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 μM APIII or 0.5 μM fluo-3. A coupled enzyme (CE) system consisting of 1 mM MgATP, 10 $\mu\text{g}/\text{ml}$ 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 extracellular 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 [^3H]Ryanodine Binding and Data Analysis. Equilibrium and kinetic measurements of specific high-affinity [^3H]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 [^3H]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 of 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 [^3H]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 [^3H]ryanodine occupancy, B_{max} = maximum [^3H]ryanodine occupancy in the presence of quinone), with data between 10 to 90% of B_{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_{obs}) 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 CsCl, 50 μM CaCl_2 , and 10 mM HEPES (pH 7.4), whereas the *trans* side contained 100 mM CsCl, 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

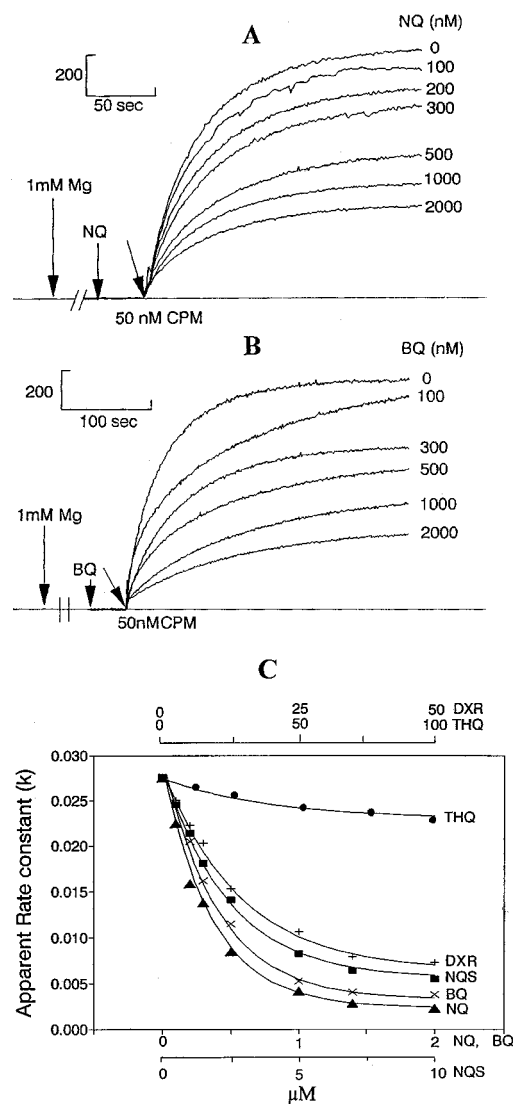


Fig. 1. Quinone compounds dose-dependently diminish the kinetics of CPM-thioether adduct formation with skeletal junctional SR membrane proteins. SR vesicles (50 $\mu\text{g}/\text{ml}$) were equilibrated at 37°C in solution A containing 100 mM KCl, 20 mM MOPS (pH 7.0) and 1 mM MgCl_2 for 3 min. The 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 (×), NQS (■), 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 Ca^{2+} , 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 $0.34 \pm 0.05 \mu\text{M}$ and $1.8 \pm 0.2 \mu\text{M}$, respectively, with a brief 30-s exposure before initiating adduct formation with CPM. 1,2-Naphthoquinone-4-sulfonic acid (NQS), $\text{IC}_{50} = 2.8 \pm 0.2 \mu\text{M}$, 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 ($\text{IC}_{50} = 16.3 \pm 0.8 \mu\text{M}$). Importantly, *tert*-butylhydroquinone (THQ), whose quinone moiety is fully reduced, lacks significant activity in the CPM assay at concentrations $\leq 100 \mu\text{M}$ 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 Ca^{2+} , 10 mM Mg^{2+}) 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 Sulfhydryls 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 Mg^{2+} , 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

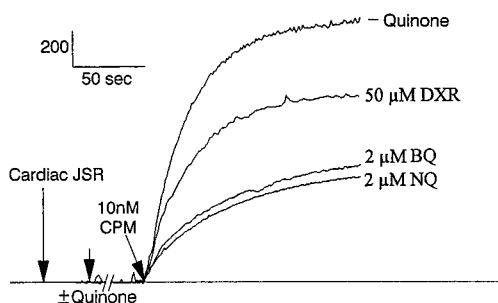


Fig. 2. NQ, BQ, and DXR dramatically reduce the rate of CPM-thioether adduct formation with cardiac junctional SR membrane proteins. Cardiac SR vesicles (50 $\mu\text{g}/\text{ml}$) were equilibrated at 37°C in solution A in the presence of 1 mM MgCl_2 for 3 min. Dimethyl sulfoxide (10 μl , trace labeled "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.

2–4 labeled NQ, BQ, and NQS, respectively). Digital imaging of the fluorescent bands on gels revealed a $>98\%$ decreased in the CPM fluorescence intensity associated with the RyR1 protomer and triadin in SR-pretreated with quinone compared to control SR treated with Mg^{2+} 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-

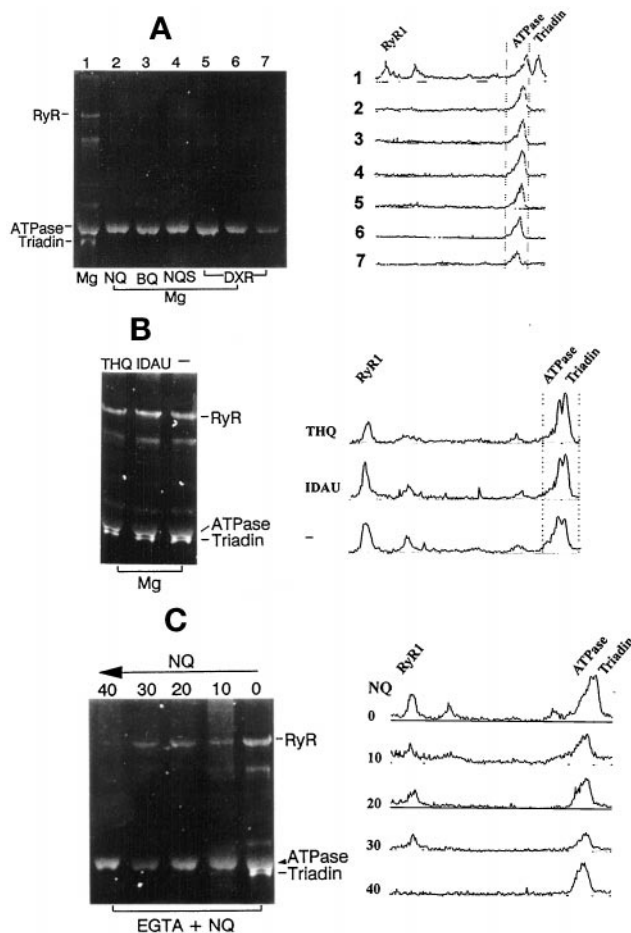


Fig. 3. Quinones selectively influence CPM labeling on RyR and triadin protomers in skeletal SR as revealed by CPM fluorescence on SDS-PAGE. SR protein (50 $\mu\text{g}/\text{ml}$; total 1 mg) in solution A was preincubated under varied conditions. A, in the presence of 1 mM MgCl_2 alone (lane 1), or 1 mM MgCl_2 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 μM DXR for 3, 10, and 30 min (lanes 5, 6, 7, respectively) at 37°C . Note the change in CPM labeling from RyR protomer ($M_r \sim 565,000$), a band at $M_r \sim 160,000$ (possibly a major proteolytic fragment of RyR) and triadin ($M_r \sim 5,000$) to the abundant Ca^{2+} -ATPase ($M_r \sim 110,000$). 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 Ca^{2+} Transport across Actively Loaded SR Vesicles. Figure 4A shows that NQ mobilizes Ca^{2+} 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 $\mu\text{g}/\text{ml}$ SR protein and transport buffer containing ATP and CE, the Ca^{2+} -sensitive dye APIII responded to addition of 100 μM Ca^{2+} with an abrupt rise in absorbance which was followed by a rapid decrease that stemmed from the uptake of Ca^{2+} into SR vesicles. Addition of 300 nM to 2 μM NQ induced a net efflux of Ca^{2+} 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 Ca^{2+} despite the presence of NQ. The threshold for NQ-induced Ca^{2+} 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 Ca^{2+} transport were observed with BQ (Fig. 4B). After the Ca^{2+} loading phase in which six additions of 20 μM CaCl_2 were made to the SR mixture, addition of BQ (300 nM to 2 μM) induced a dose-dependent release of accumulated Ca^{2+} 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 NQS were required to produce release rates comparable to NQ and BQ (not shown).

The hypothesis that quinones can alter Ca^{2+} transport across SR by a selective mechanism influencing hyperreactive channel sulfhydryl moieties within the RyR1 complex was further tested by measuring the rate of active Ca^{2+} accumulation in SR vesicles. Figure 5A reveals that Ca^{2+} uptake into the membrane vesicles was completely driven by SR/ER Ca^{2+} ATPase (SERCA) pump activity since active Ca^{2+} accumulation was eliminated by 1 μM thapsigargin (TG), a specific inhibitor of the Ca^{2+} (Mg^{2+})-ATPase (compare traces 1 and 3). NQ (2 μM) added 15 min before initiating

Ca^{2+} loading of SR significantly reduced the initial rate of Ca^{2+} 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 Ca^{2+} efflux experiments shown in Fig. 4 and confirmed that NQ at the highest concentration used in the present study reduced SR Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 sulfhydryls on the channel complex essentially mimics the actions of RR in restoring Ca^{2+} accumulation to control levels (Fig. 5B, compare trace 2 to trace

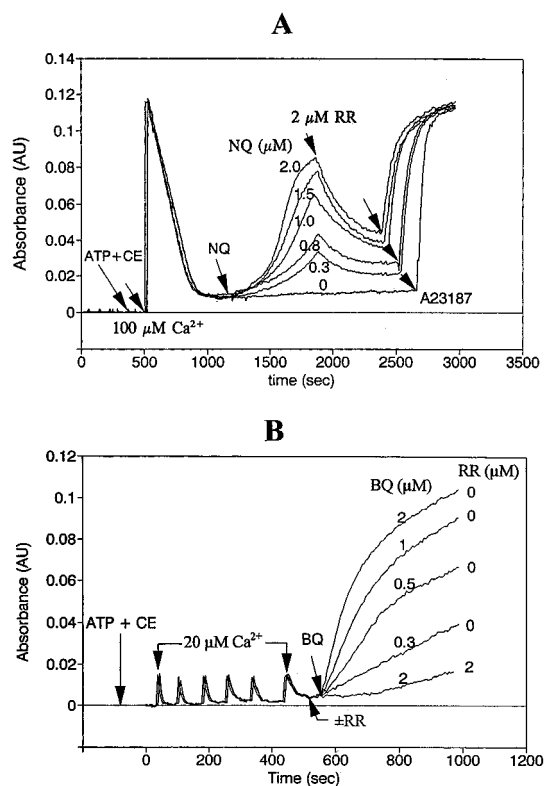


Fig. 4. NQ and BQ induce Ca^{2+} release from actively loaded skeletal SR vesicles in a dose-dependent and ruthenium-sensitive manner. SR vesicles (50 $\mu\text{g}/\text{ml}$) were added to Ca^{2+} transport assay buffer. MgATP (1 mM) and CE were added (first arrow). CaCl_2 (100 μM) was added to the mixture (second arrow) and absorbance was monitored as described in *Materials and Methods*. A, when Ca^{2+} uptake was complete, the indicated concentration of NQ was added (third arrow). Once release proceeded for 10 min, 2 μM RR (fourth arrow) was introduced to block RyR. Ionophore A23187 (2 μg) was added at the end of each assay to calibrate the signal and assure equal Ca^{2+} loading of the vesicles. B, Ca^{2+} uptake was carried out by six sequential additions of 20 μM CaCl_2 to the vesicle mixture. BQ potentially induced Ca^{2+} release from SR and its actions could be blocked by preincubation with RR (2 μM). These experiments were repeated three times with similar results.

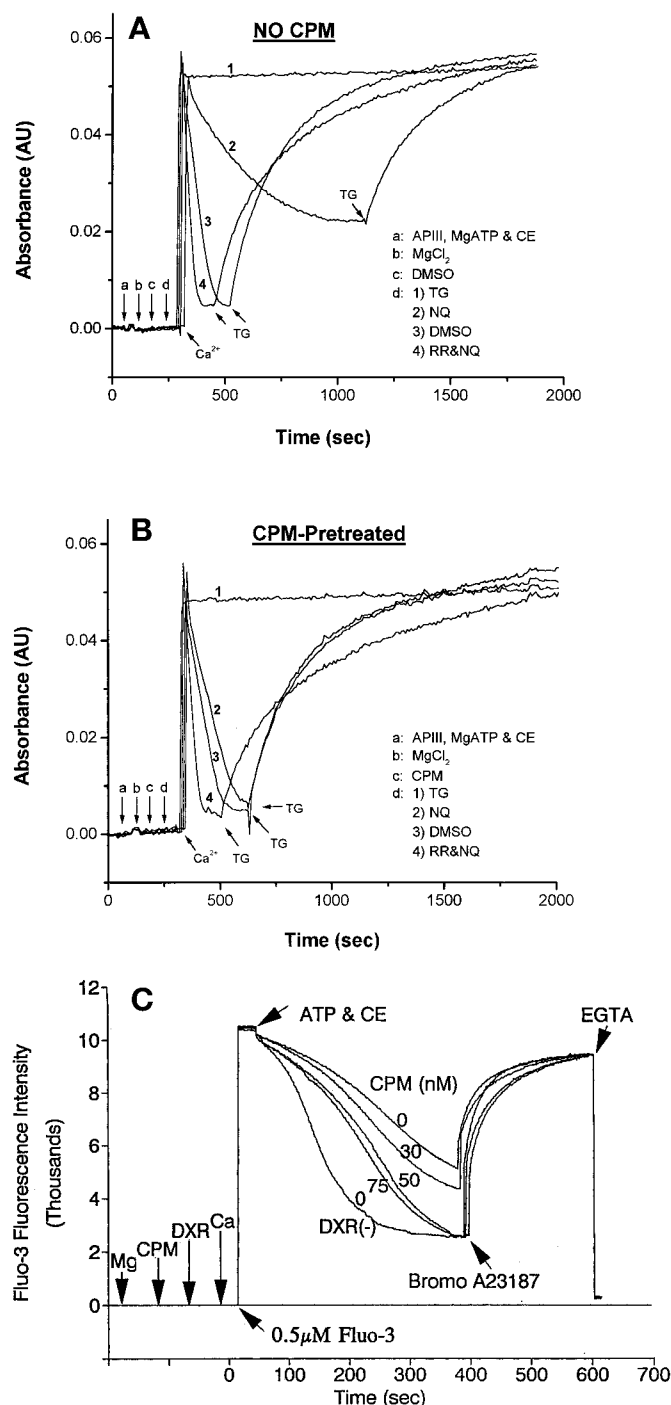


Fig. 5. Quinone-mediated reduction of active Ca^{2+} accumulation by SR is selectively eliminated by formation of CPM adducts with hyperreactive SR sulfhydryl moieties. SR membranes (50 $\mu\text{g}/\text{ml}$) were equilibrated in transport buffer at 37°C containing 1 mM MgCl_2 , 1.4 mM MgATP , and CE with either APIII (A and B), 2 μM fluo-3 (C) as a Ca^{2+} indicator. Reaction mixtures were then exposed for 1 min to either 1% dimethyl sulfoxide as vehicle control (A) or 50 nM CPM (B) and terminated by 50 μM reduced glutathione. One of the following was subsequently added to each reaction mixture: 1 μM TG (trace 1), 2 μM NQ (trace 2), 1% dimethyl sulfoxide (trace 3), or 10 μM RR and 2 μM NQ (trace 4). After 15 min of additional incubation, uptake was initiated by addition of Ca^{2+} (90 μM), and APIII absorbance was monitored as described in *Materials and Methods*. After the Ca^{2+} -loading phase was complete, TG (1 μM) was added to each cuvette to induce efflux of accumulated Ca^{2+} from the vesicles and to calibrate the dye. C, SR was pretreated in the presence of 1 mM MgCl_2 and the indicated concentration of CPM for 1 min. DXR (30 μM) or its solvent [trace labeled DXR (-)] was added into the reaction mixture and

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 and B, compare traces 2 to traces 3). 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 μM under identical condition did not significantly alter Ca^{2+} uptake rates (not shown).

DXR is an antineoplastic anthraquinone that has been shown to potentially 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 sulfhydryls 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 μM) extravesicular Ca^{2+} and 1 mM Mg^{2+} to inhibit the Ca^{2+} channel to selectively label hyperreactive sulfhydryl 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 μM 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 sulfhydryls 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 [^3H]ryanodine to SR membranes were examined under equilibrium and kinetic conditions. Figure 6A reveals that the ability of NQ to modify equilibrium binding of [^3H]ryanodine to SR (12.5 μg of protein) was highly dependent on concentration. Under assay conditions which were less than optimally favorable for the binding of [^3H]ryanodine (25 μM Ca^{2+} , 1 mM Mg^{2+}), nanomolar NQ enhanced occupancy of [^3H]ryanodine

100 μM CaCl_2 (Ca) (formed free Ca^{2+} $\sim$ 10 μM in the presence of pyrophosphate) and 0.5 μM fluo-3 were subsequently introduced. Fluorescence measurement of Ca^{2+} uptake was initiated by the addition of 1 mM MgATP and CE. At the end of each experiment, 1 μM Ca^{2+} ionophore 4-Br-A23187 was added to the cuvette to determine the amount of Ca^{2+} loaded into the vesicles, and 1 mM EGTA was added to chelate Ca^{2+} and to calibrate the dye. The experiments were repeated with three different independent preparations and similar results were obtained.

to SR membranes nearly 3-fold, with an $EC_{50} = 123$ nM (2.46 pmol NQ/ μ g SR; Fig. 6A). By contrast, low micromolar NQ inhibited the binding of [3 H]ryanodine to high-affinity sites with an $IC_{50} = 1.2$ μ M (24 pmol NQ/ μ g SR; Fig. 6A).

The concentration-dependent modulation observed with NQ in equilibrium-binding experiments were further studied by performing kinetic experiments. These experiments revealed that the actions of NQ are highly time-dependent. Figure 6B and Table 1 show that a low (500 nM) concentration of NQ enhances the initial observed rate (k_{obs}) of [3 H]RyR occupancy by 2.5-fold (k_{obs} increases from 0.0172 to 0.0430 min^{-1} , respectively) and increases occupancy >3-fold at 3 h (binding increases from 0.14 to 0.428 pmol/mg). Although 5 μ M NQ additionally enhances the apparent rate of association over control (from 0.0172 to 0.0559 min^{-1}), receptor occupancy is enhanced only 1.8-fold at optimal incubation time (~50–80 min). The latter is undoubtedly the result of the subsequent inhibitory phase of NQ on RyR function. Taken together, results from CPM-labeling kinetics, vesicle transport, and [3 H]ryanodine-binding studies suggest that NQ should initially activate the SR Ca^{2+} channel complex in a manner directly related to its concentration in the assay medium. Significant channel activation would be predicted to occur soon after addition of NQ with subsequent inhibition of channel gating only occurring with NQ concentration exceeding 1 μ M.

To directly address this hypothesis, the actions of NQ on single-channel gating kinetics were examined in bilayer lipid membrane (BLM) preparations. Figure 7A shows the typical effect of nanomolar NQ on a single SR Ca^{2+} channel incorporated into BLM. The channel shown is very active in the presence of 100 μ M Ca^{2+} *cis* ($P_o = 0.88$) and addition of 1 mM Mg^{2+} *cis* dramatically reduces P_o to 0.14. NQ (200 nM) results in a significant increase in channel activity (to $P_o = 0.22$) within 1 min, and channel activity progressively increases over a 40-min period (to $P_o = 0.73$). In six channels examined under these conditions, 200 nM NQ consistently activated Ca^{2+} channels in a time-dependent manner and was not observed to inhibit channel gating, even with records lasting >30 min (Fig. 7, A and C). The effects of nanomolar NQ toward enhancing channel activity was found to be readily reversible with perfusion of the *cis* chamber with buffer lacking NQ (Fig. 8). By contrast, 2 μ M NQ initially

enhanced channel-open probability from $P_o = 0.027$ (in the presence of 1 mM MgCl_2 *cis*) to $P_o = 0.75$ within 10 min (Fig. 7, B and D). Unlike nanomolar NQ, micromolar NQ produces a time-dependent inhibition of Ca^{2+} channel activity subsequent to the activation phase. Typically ($n = 12$ channels), 2 μ M NQ began to induce a decline in channel P_o between 10 and 13 min after its introduction to the *cis* chamber and led to full-channel inhibition within 30 min (Fig. 7, B and D). Furthermore, in 7 of the 12 channels studied, frequent transitions among subconductance states were observed during the inhibition phase (data not shown). Once the inhibitory actions of NQ were observed, perfusion of the *cis* chamber of the bilayer did not restore the channel behavior to that seen with control (Fig. 8).

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 [3 H]ryanodine-binding, macroscopic SR Ca^{2+} 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 sulfhydryl groups which are available for CPM labeling, (i.e., $k = K_m [\text{SH}]_t$) (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-

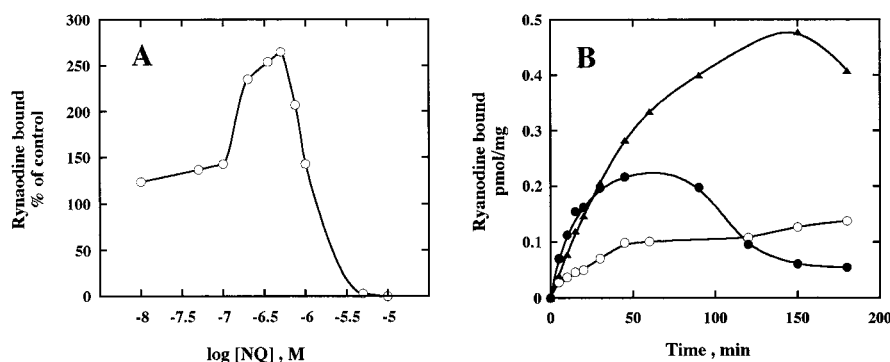


Fig. 6. Concentration- and time-dependent activation and inactivation of [3 H]ryanodine-binding activity of SR. SR membranes (50 μ g/ml) were incubated without or with NQ in a buffer containing HEPES (20 mM), pH 7.1, KCl (250 mM), NaCl (15 mM), CaCl_2 (25 μ M), MgCl_2 (1 mM), and [3 H]ryanodine (1 nM). A, equilibrium-binding experiments showing activation ($EC_{50} = 123$ nM) and inhibition ($IC_{50} = 1.2$ μ M) of the [3 H]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 μ M (●) 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-triad complex which renders a small number of cysteines 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 redox cyler than it is an arylator (Monks et al., 1992) and at low concentration (nanomolar) enhances channel activation in a manner indistinguishable from DXR, a pure redox cyler. Finally, this mechanism seems untenable when one considers that nucleophilic addition of CPM to hyperreactive thiols does not itself alter Ca^{2+} uptake but rather removes the ability of reactive quinones from affecting changes in Ca^{2+} uptake (Fig. 5).

In Fig. 9, scheme 3, agents that enhance channel-open

probability (Ca^{2+} , adenine nucleotides, caffeine, etc.) influence a conformational transition to the open state of the channel that masks the nucleophilic domain and dramatically reduces the reactivity of functionally critical cysteines. In scheme 3 (Fig. 9), the formation and elimination of a nucleophilic domain with native channel transitions in conformation corresponds to the appearance and disappearance of hyperreactive thiols detected by CPM fluorescence. Reactive quinones such as NQ, by virtue of their electrophilic redox potentials ($\text{NQ } E_{\text{redox}} = +36 \text{ mV}$; Clark, 1960) would be expected to perturb the redox microenvironment within the nucleophilic domain wherein hyperreactive thiols reside. The presence of low concentrations (nanomolar) of quinone would further enhance the nucleophilicity of hyperreactive thiols which could aid in promoting the deprotonation of R-SH to $\text{R-S}^- + \text{H}^+$. In the deprotonated state, the hyperreactive thiols may contribute significantly to decrease the stability of the closed state through disruption of key noncovalent interactions. Although the present study does not directly prove the mechanism proposed in scheme 3 of Fig. 9, several experimental observations are consistent with a redox-sensing model. The potency and rapidity with which channel activation occurs appears to follow the standard redox potential of the quinone. BQ ($E_{\text{redox}} +293 \text{ mV}$; Clark, 1960) and NQ ($E_{\text{redox}} +36 \text{ mV}$; Fig. 4) were found to be substantially more potent and rapid than anthraquinones (typical $E_{\text{redox}} < -150 \text{ mV}$) in releasing $\text{SR } \text{Ca}^{2+}$ (Abramson et al., 1988; 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).

Nanomolar NQ and BQ induce rapid and selective loss of hyperreactive thiol groups on RyR1 and triadin protomers, and the immediate functional consequence is enhanced channel activity and net $\text{SR } \text{Ca}^{2+}$ efflux. These results are in agreement with those of Aghdasi et al. (1997b) who found that channels incubated with a high concentration of NEM for increasing periods of time display three distinct phases of functional effects. However, the experimental design of Aghdasi et al. (1997b) did not account for the conformational state of the channel before addition of sulfhydryl reagent nor was the molar ratio of sulfhydryl reagent relative to SR protein adjusted to $<1 \text{ pmol}/\mu\text{g}$ protein. For these reasons, labeling was not limited to the most reactive channel thiols and comparisons about the functional consequence of sulfhydryl oxidation cannot be directly compared with the present study which addresses the functional ramifications of site-selective modification of the most reactive channel-associated thiols. The ability of NQ at a higher concentration to produce biphasic actions on both channel function and

TABLE 1

Effect of NQ on the association kinetic of [^3H]ryanodine with SR

SR membranes (50 $\mu\text{g}/\text{ml}$) 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 [^3H]ryanodine binding as a function of incubation time was fit with an exponential, from which equilibrium or maximal binding (B_{eq}), apparent association rate constant (k_{obs}), and apparent half-time ($T_{1/2}$) were calculated. The values are the average of two experiments, each performed in duplicate.

	Control	NQ, 0.5 μM	NQ, 5 μM
k_{obs} (min^{-1})	0.0172	0.0430	0.0559
$T_{1/2}$ (min)	40.3	16.1	12.4
B_{eq} (pmol/mg)	0.140	0.428	0.254

[^3H]ryanodine-binding kinetics support this interpretation since under these conditions it would be expected to 1) arylate protein thiols and 2) oxidize hyperreactive and less reactive but more abundant channel-associated thiols to disulfides. Experiments with additional quinone structures which exclusively arylate or redox cycle should clarify the relationship between chemical mechanism at the Ca^{2+} channel complex and functional response.

Micromolar NQ clearly shows biphasic actions on the binding of [^3H]ryanodine, first enhancing occupancy followed by inhibition (Fig. 6), whereas anthraquinones only enhance the binding of [^3H]ryanodine to SR across their dose-response range (1–200 μM) (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

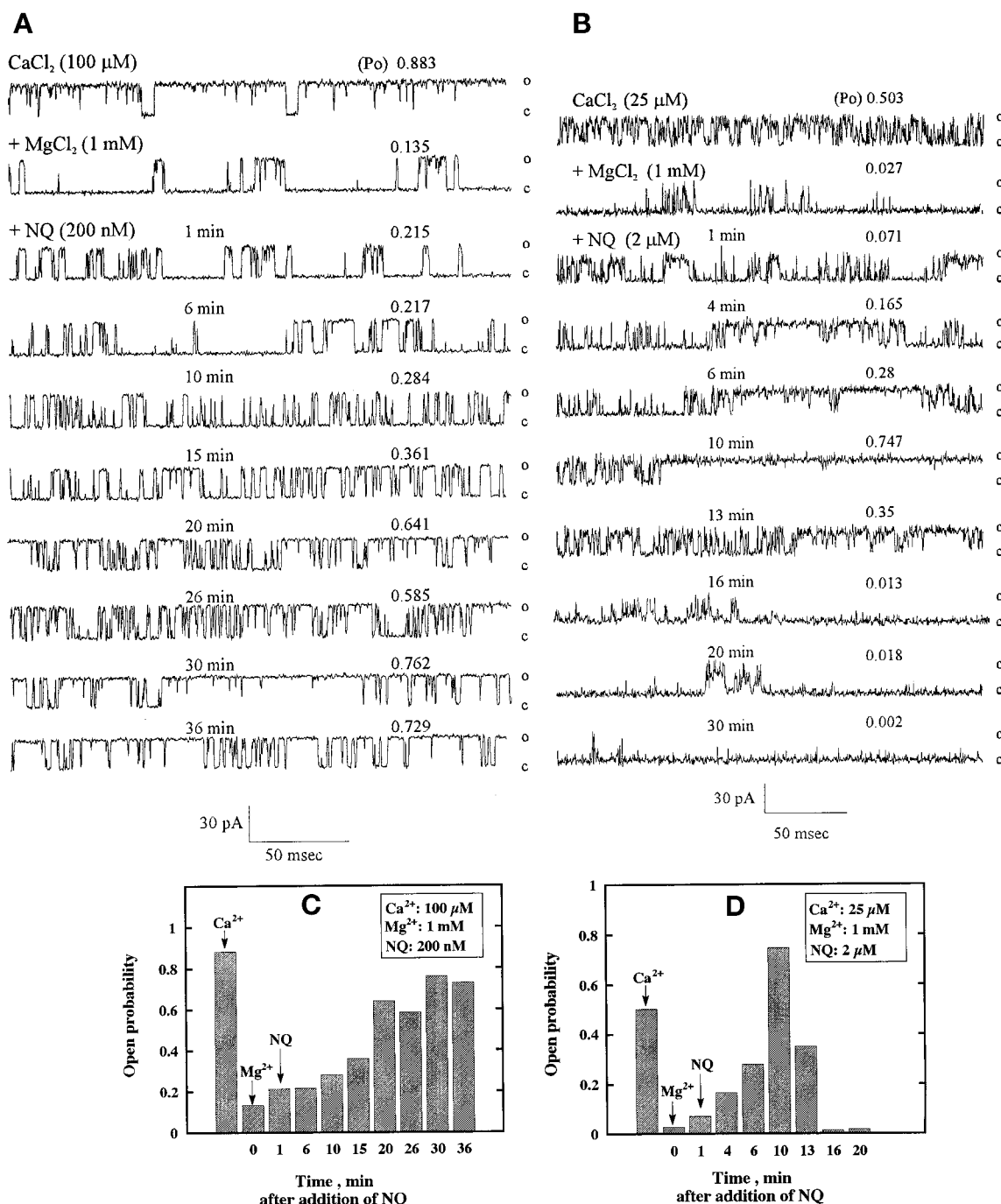


Fig. 7. Single-channel kinetics in BLMs. Single channel of RyR was reconstituted into planar lipid bilayer following addition of SR vesicles to the *cis* side of the bilayer. For all experiments, the *cis* chamber contained 500 mM CsCl, 50 μM CaCl₂, and 10 mM HEPES (pH 7.4), and *trans* contained 100 mM CsCl, 50 μM CaCl₂, and 10 mM HEPES (pH 7.4). After fusion of SR into the bilayer, the *cis* chamber was perfused with the same solution lacking Ca²⁺. The Ca²⁺ was then adjusted to a concentration indicated. After acquiring a period of control data without and with 1 mM MgCl₂, 200 nM (A) or 2 μM (B) NQ was added *cis*. Currents through the single channel were recorded at a holding potential of +30 mV for 30 to 40 min. The o and c indicate current levels of fully open and closed channels, respectively. Each experiment is a representative of 6 and 12 channels, respectively. C and D, a diary plot of results obtained from experiments at 200 nM and 2 μM NQ.

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

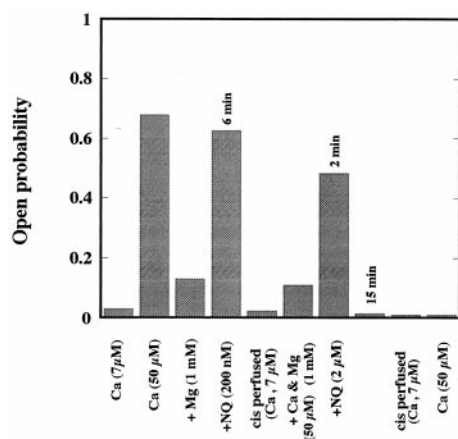


Fig. 8. Reversibility of the actions of NQ on single-channel gating kinetics. After the fusion of a RyR single channel into BLM, the channel activity was continuously recorded through sequential changes in *cis* solution as indicated below the graph. To test reversibility, *cis* perfusion was performed 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 μM 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.

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

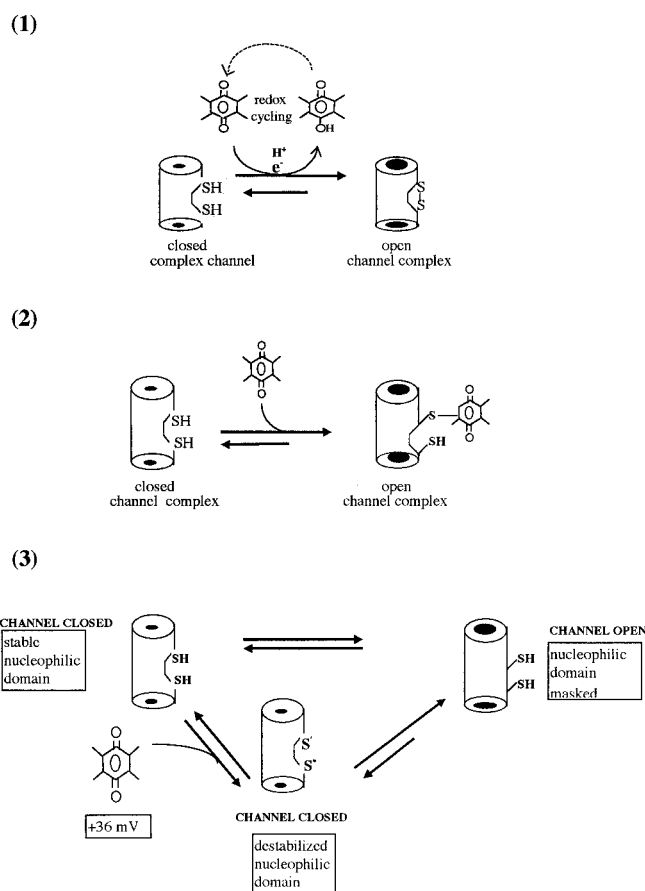


Fig. 9. Three possible mechanisms by which quinones modulate channel function. Scheme 1, redox cycling model for oxidation-induced channel activation by quinones. Scheme 2, nucleophilic arylation-induced channel activation by quinones. Scheme 3, redox-sensing model for quinone-induced channel activation. See text for details.

the major intracellular Ca^{2+} store. This mechanism may have both physiological and toxicological significance.

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References

- Abramson JJ, Buck E, Salama G, Casida JE and Pessah IN (1988) Mechanism of anthraquinone-induced calcium release from skeletal muscle sarcoplasmic reticulum. *J Biol Chem* **263**:18750–18758.
- Abramson JJ and Salama G (1989) Critical sulfhydryls regulate calcium release from sarcoplasmic reticulum. *J Bioenerg Biomem* **21**:283–294.
- Aghdasi B, Reid MB and Hamilton SL (1997a) Nitric oxide protects the skeletal muscle Ca^{2+} release channel from oxidation induced activation. *J Biol Chem* **272**:25462–25467.
- Aghdasi B, Zhang JZ, Wu YL, Reid MB and Hamilton SL (1997b) Multiple classes of sulfhydryls modulate the skeletal muscle Ca^{2+} release channel. *J Biol Chem* **272**:3739–3748.
- Berridge MJ (1987) Inositol trisphosphate and diacylglycerol: Two interacting second messengers. *Annu Rev Biochem* **56**:159–193.
- Bird GS, Burgess GM and Putney JW Jr (1993) Sulfhydryl reagents and cAMP-dependent kinase increase the sensitivity of the inositol 1,4,5-trisphosphate receptor in hepatocytes. *J Biol Chem* **268**:17917–17923.
- Bootman MD, Taylor CW and Berridge MJ (1992) The thiol reagent, thimerosal, evokes Ca^{2+} spikes in HeLa cells by sensitizing the inositol 1,4,5-trisphosphate receptor. *J Biol Chem* **267**:25113–25119.
- Buck ED and Pessah IN (1995) Mitoxantrone antagonism of doxorubicin at cardiac SR channels in the planar lipid bilayer. *Biophys J* **68**:A374.
- Clark WM (1960) *Oxidation Reduction Potentials of Organic Systems*, Williams & Wilkins, Baltimore, MD.
- Exton JH (1990) Signalling through phosphatidyl choline breakdown. *J Biol Chem* **265**:1–4.
- Farber JL (1990) The role of calcium in lethal cell injury. *Chem Res Toxicol* **3**:503–508.
- Frei B, Winterhalter KH and Richter C (1986) Menadione (2-methyl-1,4-naphthoquinone)-dependent chemistry, enzymatic redox cycling and calcium release by mitochondria. *Biochemistry* **25**:4438–4443.
- Herman M, Gore GJ, Nieminen AL, Kawanishi Y, Harman A and Lemasters JJ (1990) Ca^{2+} and pH in anoxic and toxic injury. *Crit Rev Toxicol* **21**:127–148.
- Hinson JA and Roberts DW (1992) Role of covalent and noncovalent interactions in cell toxicity: Effects on proteins. *Annu Rev Pharmacol Toxicol* **32**:471–510.
- Holmberg SR and Williams AJ (1990) Patterns of interaction between anthraquinone drugs and the calcium-release channel from cardiac sarcoplasmic reticulum. *Circ Res* **67**:272–283.
- Kaplan AI, Ferris CD, Voglmaier SM and Snyder SH (1994) Purified reconstituted inositol 1,4,5-trisphosphate receptors. Thiol reagents act directly on receptor protein. *J Biol Chem* **269**:28972–28978.
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature (Lond)* **227**:680–685.
- Lee KS, Frank S, Vanderklish P, Arai A and Lynch G (1991) Inhibition of proteolysis protects hippocampal neurons from ischemia. *Proc Natl Acad Sci USA* **88**:7233–7237.
- Liu G, Abramson JJ, Zable AC and Pessah IN (1994) Direct evidence for the existence and functional role of hyperreactive sulfhydryls on the RyR-triad complex selectively labeled by the coumarin maleimide 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin. *Mol Pharmacol* **45**:189–200.
- Liu G and Pessah IN (1994) Molecular interaction between ryanodine receptor and glycoprotein triadin involves redox cycling of functionally important hyperreactive sulfhydryls. *J Biol Chem* **269**:33028–33034.
- McConkey DJ, Hartzell P, Duddy SK, Hakansson H and Orrenius S (1988) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin kills immature thymocytes by Ca^{++} -mediated endonuclease activation. *Science (Wash DC)* **242**:156–159.
- Meissner G, Rousseau E and Lai FA (1989) Structural and functional correlation of the trypsin-digested Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum. *J Biol Chem* **264**:1715–1722.
- Monks TJ, Hanzlik RP, Cohen GM, Ross D and Graham DG (1992) Quinone chemistry and toxicity. *Toxicol Appl Pharmacol* **112**:2–16.
- Monks TJ and Lau SS (1992) Toxicology of quinone-thioethers. *Crit Rev Toxicol* **22**:243–270.
- Moore GA, Weis M, Orrenius S and O'Brien PJ (1988) Role of sulphydryl groups in benzoquinone-induced Ca^{2+} release by rat liver mitochondria. *Arch Biochem Biophys* **267**:539–550.
- Nicoterra P, Bellomo G and Orrenius S (1992) Calcium-mediated mechanisms in chemically induced cell death. *Annu Rev Pharmacol Toxicol* **32**:449–470.
- Nicoterra P, Hartzell P, Baldi C, Svensson SA, Bellomo G and Orrenius S (1986) Cystamine induces toxicity in hepatocytes through elevation of cytosolic Ca^{2+} and the stimulation of a non-lysosomal proteolytic system. *J Biol Chem* **261**:14628–14635.
- Olson RD and Mushlin PS (1990) Mechanisms of anthracycline cardiotoxicity: Are metabolites involved?, in *Cellular and Molecular Toxicology and In Vitro Toxicology* (Acosta D ed) pp 51–81, CRC Press, Boca Raton, FL.
- Ondrias K, Borgatta L, Kim DH and Ehrlich BE (1990) Biphasic effects of doxorubicin on the calcium release channel from sarcoplasmic reticulum of cardiac muscle. *Circ Res* **67**:1167–1174.
- Pessah IN, Durie E, Schiedt MJ and Zimanyi I (1990) Anthraquinone-sensitized Ca^{2+} release channel from rat cardiac sarcoplasmic reticulum: Possible receptor-mediated mechanism of doxorubicin cardiomyopathy. *Mol Pharmacol* **37**:503–514.
- Pessah IN, Molinski TF, Meloy TD, Wong P, Buck ED, Allen PD, Mohr FC and Mack MM (1997) Bastadins relate ryanodine-sensitive and ryanodine-insensitive "leak" Ca^{2+} efflux pathways in skeletal SR and BC₃H1 cells. *Am J Physiol Cell Physiol* **272**:C601–C614.
- Pessah IN, Stambuk RA and Casida JE (1987) Ca^{2+} -activated ryanodine binding: mechanisms of sensitivity and intensity modulation by Mg^{2+} , caffeine, and adenine nucleotides. *Mol Pharmacol* **31**:232–238.
- Quinn KE and Ehrlich BE (1997) Methanethiosulfonate derivatives inhibit current through the ryanodine receptor/channel. *J Gen Physiol* **109**:255–264.
- Reed DJ (1990) Review of the current status of calcium and thiols in cellular injury. *Chem Res Toxicol* **3**:495–502.
- Saito A, Seiler S, Chu A and Fleischer S (1984) Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. *J Cell Biol* **99**:875–885.
- Shulman H and Lou LL (1989) Multifunctional Ca^{++} /calmodulin-dependent protein kinase: domain structure and regulation. *Trends Biochem Sci* **14**:62–66.
- Sipple TO (1981) New fluorochromes for thiols: maleimide and iodoacetamide derivatives of 3-phenylcoumarin fluorophore. *J Histochem Cytochem* **29**:314–321.
- Xu L, Eu JP, Meissner G and Stamler JS (1998) Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science (Wash DC)* **279**:234–237.
- Zable AC, Favero TG and Abramson JJ (1997) Glutathione modulates ryanodine receptor from skeletal muscle sarcoplasmic reticulum. Evidence for redox regulation of the Ca^{2+} release mechanism. *J Biol Chem* **272**:7069–7077.

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