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Anthraquinone-Sensitized Ca^{2+} Release Channel from Rat Cardiac Sarcoplasmic Reticulum: Possible Receptor-Mediated Mechanism of Doxorubicin Cardiomyopathy

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SUMMARY

Rat cardiac membrane vesicles enriched in biochemical markers of the junctional region of sarcoplasmic reticulum (SR) and exhibiting ruthenium red-sensitive rapid Ca^{2+} release have been prepared. Doxorubicin and seven congeners are shown to enhance the binding of [^{3}H]ryanodine to the ryanodine receptor with a strong structural requirement. Doxorubicin enhances the binding of [^{3}H]ryanodine to SR membranes and soluble receptor preparations and induces Ca^{2+} release from SR vesicles in a highly Ca^{2+}-dependent manner, suggesting that anthraquinones promote the open state of the junctional Ca^{2+} release channel by increasing the affinity of the Ca^{2+} activator site for Ca^{2+}. Doxorubicin reduces the K_d of [^{3}H]ryanodine binding solely by enhancing the rate of association. Caffeine competes for the same site with anthraquinones, because the caffeine-activated binding of [^{3}H]ryanodine is inhibited by doxorubicin and vice versa. The acute effect of doxorubicin on the cardiac Ca^{2+} release channel is fully reversible; however, long term treatment (up to 24 hr) with doxorubicin increases the sensitivity of the preparation to subsequent acute challenge with doxorubicin. The thiol-reductive agent dithiothreitol enhances, whereas the reactive disulfide 4,4′-dithiodipyridine reduces, the doxorubicin-enhanced binding of [^{3}H]ryanodine. These results demonstrate that the acute and chronic cardiotoxicity of anthraquinones may be accounted for by a receptor-mediated mechanism. Our findings suggest that the chronic effects observed with the clinical use of anthraquinones may be the result of a receptor-mediated shift in the redox equilibrium of allosteric thiols at the ryanodine receptor complex, which in turn leads to long term sensitization of the Ca^{2+} release channel.

SR of cardiac and skeletal muscle makes an essential contribution to EC coupling by regulating the free Ca^{2+} levels in the myoplasm. Recent cloning and heterologous expression of the ryanodine receptor in hamster ovary cells (1) further supports the biochemical, electrophysiological (2–13), and electron microscopic (14–17) evidence that the [^{3}H]ryanodine receptor and the Ca^{2+} release channel are synonymous.

Because the ryanodine receptor is likely to be a critical component of EC coupling, its role as a proximal target in the etiology of genetic and drug-induced myopathies has begun to be actively addressed (18, 19). Anthraquinones such as DXR and daunorubicin are among the most widely used antineoplastic drugs (20, 21), but their clinical application is severely limited by a cumulative cardiotoxicity associated with their chronic use (22, 23). Anthraquinone-induced cardiomyopathy has been described by morphological (24) and biochemical (25) investigations, which show loss of myofibrils, vacuolization of SR, and swelling of mitochondria. One hypothesis describing the molecular mechanism of DXR cardiotoxicity involves quinone/semiquinone redox cycling with concomitant generation of superoxide and hydroxyl free radicals, which may cause peroxidation of cardiac membrane lipids and loss of permeability barriers to cations, especially Ca^{2+} (26–28).

Recent recognition that DXR and its metabolite doxorubicinol can directly alter the Ca^{2+} transport properties of skeletal (19, 29) and cardiac (30, 31) muscle SR raises the possibility of a receptor-mediated lesion that may occur early in the etiology of anthraquinone-induced cardiomyopathies. Olson *et al.* (31) have reported potent inhibition of several cation pumps, including the Ca^{2+}-dependent ATPase of canine cardiac SR, by doxorubicinol but not DXR, leading to the suggestion that the metabolite doxorubicinol is responsible for cardiotoxicity. More

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ABBREVIATIONS: SR, sarcoplasmic reticulum; B_{max}, maximum number of binding sites; DTT, dithiothreitol; DXR, doxorubicin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ED_{50}, concentration of compound resulting in 50% of the maximal activation; EGTA, [ethylene-bis-(oxyethylenenitrilo)]tetraacetic acid; EC, excitation-contraction; HEPES, *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid; K_d, dissociation constant; k_{+1}, association rate constant; k_{-1}, dissociation rate constant; n_H, Hill constant; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; MOPS, 3-[*N*-morpholino]propanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis-[2-ethanesulfonic acid].

recently, however, Abramson and co-workers (19) demonstrated that the potent effect of DXR and a number of analogs on the skeletal muscle SR Ca^{2+} release channel is mediated by a highly specific interaction with the ryanodine receptor. Micromolar anthraquinone appears to cause Ca^{2+} release from junctional SR by sensitizing the release channel to activation by Ca^{2+}, in a manner similar to that previously reported for caffeine (13).

The present work introduces an *in vitro* rat ventricle model for investigating the molecular mechanism by which DXR, doxorubicinol, and six congeners influence SR Ca^{2+} transport by directly correlating their kinetic interaction with the high affinity [^{3}H]ryanodine receptor and rapid Ca^{2+} release from SR vesicles. Two potent and distinct lesions at the ryanodine receptor are recognized; 1) binding of anthraquinone to sites competitive with caffeine, but distinct from the ryanodine binding sites, increases the affinity of the channel activator site for Ca^{2+} and, hence, enhances activation of the channel by low Ca^{2+} and 2) interaction of anthraquinones at their binding site influences the oxidation state of thiols at an allosteric site, which appears to be critical to normal channel protein function, and results in long term sensitization of the channel to subsequent anthraquinone exposure. The results presented strongly support an alternative, receptor-mediated hypothesis in the etiology of both short term and long term cardiotoxic lesions produced by anthraquinones.

Materials and Methods

Preparation of SR vesicles. Male and female Sprague-Dawley rats (200–250 g) were decapitated. The heart ventricles were quickly removed, trimmed of fat and connective tissue, and placed in ice-cold 0.3 M sucrose. Purified SR vesicles for binding studies were prepared by homogenizing 20–40 g of finely minced tissue in 7 volumes of ice-cold sucrose-HEPES buffer (0.3 M sucrose, 10 μg/ml leupeptin, 20 mM HEPES, 100 μM PMSF, pH 7.4) with a Waring blender at maximum speed for 1 min. The supernatant from an initial 20-min centrifugation at 8000 × *g* was filtered through cheesecloth and centrifuged at 61,400 × *g* for 90 min in a TFA 20.250 rotor (DuPont Instruments, Wilmington, DE). The second pellet was resuspended by Dounce homogenizer in 60 ml of sucrose-PIPES buffer (0.3 M sucrose, 0.4 M KCl, 0.1 mM $MgCl_2$, 150 μM $CaCl_2$, 100 μM EGTA, 5 mM PIPES, 10 μg/ml leupeptin, pH 6.8), equally distributed atop six discontinuous sucrose gradients (5 ml each of 27% and 32% and 6 ml each of 34%, 38%, and 45%, w/w, sucrose in PIPES buffer), and spun at 55,000 × *g* (20,000 rpm) in a SW 28 rotor (Beckman Instruments, Palo Alto, CA) for 3 hr. Two fractions having highly enriched ryanodine receptor binding capacity were collected individually from the 32–34% and 34–38% sucrose interfaces, diluted in 10 volumes of PIPES buffer lacking sucrose, and pelleted at 120,000 × *g* in a Beckman Ti 50.2 rotor for 60 min. The final pellets were resuspended in sucrose-HEPES buffer (20 mM HEPES, 0.3 M sucrose, pH 7.1) at approximately 4 mg/ml protein and aliquots were rapidly frozen in liquid N_2.

SR vesicles for transport assays were prepared as described by Harris and Doroshow (32), whereas purified junctional SR membranes from skeletal muscle were prepared by the method of Saito *et al.* (33). Protein concentrations were determined by the method of Lowry *et al.*. (34), after removal of HEPES buffer by precipitating protein with 2% perchloric acid, centrifuging at 30,000 × *g* and dissolving the pellet in 1 M NaOH.

SDS-polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed in 3–17% linear gradient polyacrylamide gels having a 3% stacking gel, in a Bio-Rad Mini-Protean Dual Slab Cell (Bio-Rad, Richmond, CA), with the buffer system of Laemmli (35). Gels were stained with Coomassie blue.

Solubilization of the ryanodine receptor. SR vesicles were resuspended at 5 mg of protein/ml of solubilization buffer, which consisted of 5 mg of CHAPS/mg of protein, 1 M NaCl, 10 μM PMSF, 10 μg/ml leupeptin, and 40 mM Tris maleate, pH 7.1, as previously described (4). Solubilization was achieved by shaking for 30 min at 5°, followed by centrifugation at 110,000 × *g* for 60 min to recover the soluble fraction.

Measurement of [^{3}H]ryanodine binding. Specific high affinity binding of [^{3}H]ryanodine was measured by the procedure developed by Pessah *et al.* (2). Briefly, SR vesicles (30 μg) were incubated in duplicate or triplicate at 37° for 2 hr with 0.5–5 nM [^{3}H]ryanodine, in the absence or presence of anthraquinones, in a final volume of 1 ml of 250 mM KCl, 15 mM NaCl, 140 μM $CaCl_2$, 1 mM $MgCl_2$, 0.05–0.24 mM EGTA as indicated, and 20 mM HEPES, pH 7.1. Mg^{2+} (1 mM) was present in all experiments (unless otherwise indicated) to mimic the physiologic intracellular concentration. The assays were terminated by addition of 2.5 ml of ice-cold wash medium, consisting of 20 mM Tris-HCl, 250 mM KCl, 15 mM NaCl, and 50 μM $CaCl_2$, pH 7.1, followed by rapid filtration through GF/B glass-fiber filters in a cell harvester (Brandel, Gaithersburg, MD); one additional 2.5-ml wash was used to rinse the filters. Radioactivity was measured by scintillation counting, with an efficiency of approximately 43%. Binding to soluble receptor was performed as for the membrane-bound receptor, with two exceptions. First, the assay buffer consisted of 1 M NaCl, 0.1% CHAPS, and 40 mM Tris maleate, pH 7.1. Second, binding was determined by filtration with filters presoaked in 5% polyethyleneimine for 15 min.

In all cases, nonspecific binding of [^{3}H]ryanodine was defined by the addition of a 100-fold excess of unlabeled ryanodine. The average value for nonspecific binding to the high affinity site amounted to 11% of the total binding of 1 nM [^{3}H]ryanodine. Filter binding of [^{3}H]ryanodine in the absence of vesicular preparation was negligible.

Free Ca^{2+} was adjusted with EGTA, based on stability constants published by Fabiato (36) and calculated using the SPECS computer program. The influence of caffeine on the ability of anthraquinone to activate [^{3}H]ryanodine binding and vice versa was measured under competitive conditions.

Measurement of the reversibility of the effect of DXR. SR vesicles were preincubated without and with 10 or 30 μM DXR, in 100 volumes of assay buffer containing 1 μM free Ca^{2+} (30 μg of SR protein/ml), for 0.5, 2, 5, and 24 hr at 0°, in the presence of 10 μg/ml aprotinin, 50 μg/ml pepstatin, and 50 μg/ml leupeptin. After the preincubation, samples were centrifuged at 30,000 × *g* for 15 min and each pellet was sequentially washed three times with assay buffer under identical conditions. The final pellet was resuspended in assay buffer and 30 μg of protein were assayed for [^{3}H]ryanodine (1 nM) binding, as described above.

Measurement of association/dissociation kinetics. The effect of DXR on the association kinetics of [^{3}H]ryanodine (1 nM) binding was measured by quenching the reaction by rapid filtration at times ranging from 10 to 360 min after the addition of SR vesicles. Dissociation of [^{3}H]ryanodine from the receptor equilibrium complex was determined by equilibrating 1 nM [^{3}H]ryanodine with membranes for 2 hr at 37°, adding 100-fold excess of assay buffer to the incubation mixture, and determining residual specific binding at subsequent times ranging from 10 min to 5 hr.

Spectrophotometric determination of Ca^{2+} release. Rapid Ca^{2+} release from SR vesicles was determined by the method of Palade (37). Briefly, 75 μg of SR protein were stirred at 35° in buffer solution, consisting of 95 mM KCl, 20 mM K-MOPS, 7.5 mM Na-pyrophosphate, 250 μM antipyrylazo III, 1 mM MgATP, 20 μg/ml creatine phosphokinase, and 5 mM phosphocreatine, pH 7.0, in a 1-ml final volume. Free Ca^{2+} concentration changes were monitored by measuring the absorbance at 710 nm and subtracting the absorbance at 790 nm, at 1-sec intervals, using a diode array spectrophotometer (Model 8452A; Hewlett Packard, Palo Alto, CA). The vesicles were actively filled with Ca^{2+}; preloading was complete after eight 10 μM $CaCl_2$ additions (unless stated otherwise). Ca^{2+}-activated Ca^{2+} release was induced by addition

of 20 μM $CaCl_2$ and was allowed to proceed for 3 min. Because the rate of Ca^{2+} release is dependent on the extravesicular Ca^{2+} concentration and the experimental circumstances used to trigger Ca^{2+} release, drug-induced releases were always initiated at the same level of free Ca^{2+} within one set of experiments, including the corresponding control. The ability of anthraquinone to further trigger rapid Ca^{2+} release was assessed exactly 3 min after initiation of Ca^{2+}-induced release of intravesicular Ca^{2+}. In other experiments, anthraquinone-induced release of accumulated Ca^{2+} was assessed only after the final preloading spike of $CaCl_2$ was fully taken up into the vesicles and the antipyrylazo III signal returned to the original baseline. The absorbance signals were calibrated by adding known amounts of $CaCl_2$ from a National Bureau of Standards standard to the complete transport mixture in the presence of 2 μg/ml A23187 to prevent Ca^{2+} accumulation.

Measurement of Ca^{2+} ($+Mg^{2+}$)-ATPase activity. Ca^{2+} ($+Mg^{2+}$)-ATPase activity was measured spectrophotometrically by enzymatically coupling ADP production to the oxidation of NADH with phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase (38, 39). The influence of anthraquinones on the Ca^{2+}-dependent ATPase component was assessed by initially blanking the spectrophotometer in the presence of anthraquinone before sequential addition of SR, ATP (total activity), and EGTA (Ca^{2+}-independent activity at 0.1 μM free Ca^{2+}).

Analysis of binding data. The effect of Ca^{2+} concentration on [^{3}H]ryanodine binding in the presence and absence of test compounds was analyzed by the method of Hill (40). A plot of $\log(B/B_{ns} - B)$ (where B is specific binding and B_{ns} is receptor occupancy at the near-saturating concentration of [^{3}H]ryanodine at the high affinity site, approximately 80% of the B_{max}) versus log [Ca^{2+}], with values from 10% to 90% of maximum receptor occupancy at B_{ns}, results in a straight line whose intercept with the abscissa is the measure of the apparent affinity of the activator site for Ca^{2+} ($K_{d_{Ca^{2+}}}$) and whose slope (n_H) reflects the degree of cooperativity for calcium activation of ryanodine binding sites. At least eight different concentrations of test compounds were used to assess their potency on the binding of [^{3}H]ryanodine. The ED_{50} values were computed by the ENZFITTER program. Equilibrium binding data from saturation analysis with varying concentrations of calcium and DXR were fitted to a one-site model, and the dissociation constant (K_d) and the maximal binding capacity (B_{max}) were determined by nonlinear regression analysis with the LIGAND program. The first-order association rate constant (k_{+1}) was calculated as described by Bennett (41) based on the dissociation rate constant (k_{-1}), which was calculated by least-squares linear regression analysis from $\ln(SB/SB_0) = k_{-1}t$, where SB is specific binding at time t and SB_0 is specific binding at time zero.

Chemicals. [^{3}H]Ryanodine (60 Ci/mmol) was obtained from New England Nuclear (Wilmington, DE). DXR and daunorubicin were purchased from Sigma (St. Louis, MO). Esorubicin, 3′-epiisodaunorubicin, 4-demethyl-6-deoxydaunorubicin, and 4-demethoxydoxorubicin were obtained from Farmitalia Carlo Erba (Milano, Italy), whereas doxorubicinol and 5-iminodaunorubicin were a generous gift from Dr. Michael Tracy of the Stanford Research Institute. All other chemicals were of the highest quality commercially available.

Results

Characterization of [^{3}H]ryanodine binding to rat cardiac SR vesicles. SDS-polyacrylamide gel electrophoresis of the SR microsomes and purified rat ventricle SR membranes is compared with skeletal muscle junctional SR vesicles in Fig. 1. Ventricle preparations exhibit biochemical markers of junctional SR, including the high molecular weight spanning protein of M_r 320,000, the Ca^{2+} pump protein of M_r 106,000, and calsequestrin of M_r 55,000 (Fig. 1). The 320,000 molecular weight for the rat cardiac ryanodine receptor is consistent with results obtained with canine ventricles under similar conditions (7). The cruder microsomal preparation (Fig. 1, *lane 3*) is richer in ATPase, whereas the sucrose gradient-purified preparation

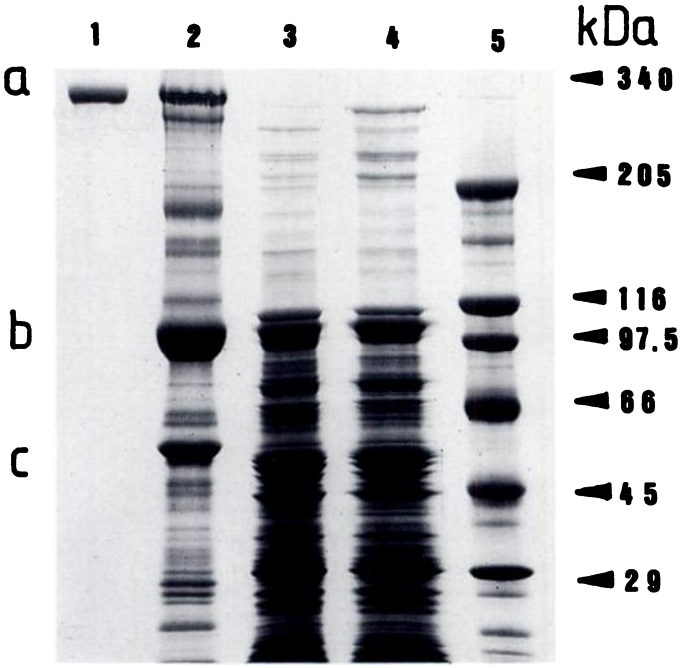


Fig. 1. Coomassie blue-stained SDS-polyacrylamide linear gradient (3–17%) gel electrophoresis of skeletal SR (*lane 2*, 10 μg), cardiac SR microsomes (*lane 3*, 40 μg), and purified cardiac SR (*lane 4*, 40 μg). A, Ryanodine receptor-complex; b, Ca^{2+} pump protein; c, calsequestrin. Standards shown in kDa are α-macroglobulin 340 (*lane 1*) and carbonic anhydrate (29), ovalbumin (45), bovine serum albumin (66), phosphorylase b (97.5), β-galactosidase (116), and myosin (205) (*lane 5*).

(Fig. 1, *lane 4*) is richer in the high molecular weight spanning protein, which constitutes the putative Ca^{2+} release channel.

Although both preparations exhibit multiple classes of [^{3}H]-ryanodine receptor sites, a specific marker of the ligand-gated calcium release channel, the sucrose gradient-purified membranes have 4-fold higher capacity to bind [^{3}H]ryanodine than the microsomal preparation (B_{max} = 7.5 versus 1.8 pmol/mg of protein, respectively) under saturating conditions for high and low affinity sites. For the purposes of this study, only interactions with the high affinity [^{3}H]ryanodine binding site are addressed.

Similar to properties exhibited by the skeletal muscle receptor previously reported (2, 3, 13), the binding of ryanodine to purified SR from rat ventricle is highly calcium dependent, with the $K_{d_{Ca^{2+}}}$ in the presence of 1 mM Mg^{2+} of 36.5 ± 9.2 μM (Fig. 2). At 50 μM Ca^{2+}, the Scatchard plots of [^{3}H]ryanodine binding isotherms are linear in the concentration range of 0.5–16 nM ryanodine, indicating a single class of high affinity receptor sites for ryanodine with a K_d of 1.79 ± 0.4 nM and a B_{max} of 1.94 ± 0.4 pmol/mg of protein. These values are the mean of several experiments done on preparations that expressed the highest density of ryanodine binding sites. The first-order rate constant for association (k_{+1}) of [^{3}H]ryanodine (1.3 nM) with its binding site in assay medium containing 50 μM Ca^{2+} in the absence of Mg^{2+} is 0.0048 nM^{-1} min^{-1} ($t_{1/2}$ = 48.7 min). The dissociation rate constant (k_{-1}) is 0.0080 min^{-1} ($t_{1/2}$ = 86.6 min) when determined by dilution in 100-fold excess assay buffer. The calculated K_d value based on the kinetic rate constants is 1.67 nM, which is not significantly different from the K_d of equilibrium experiments. The equilibrium and kinetic data at 30 μM Ca^{2+} are presented in Fig. 3. Solubilization of the ryanodine receptor with 5 mg of the zwitterionic detergent

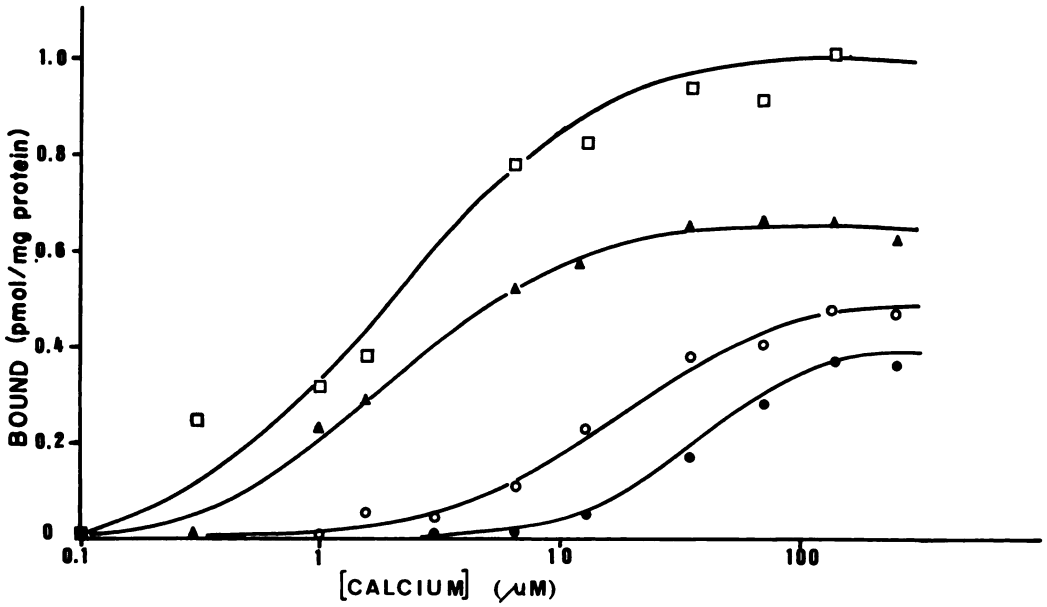


Fig. 2. Effect of DXR on the Ca^{2+}-dependent binding of [^{3}H]ryanodine. Binding of 5 nM [^{3}H]ryanodine was measured as described in Materials and Methods. Calcium concentrations were titrated with EGTA, based on the apparent affinity constant of 4.39×10^6 M^{-1} for Ca^{2+}-EGTA complex. Data shown are average of two independent experiments performed in duplicate. ●, Control; ○, 5 μM; ▲, 30 μM; □, 100 μM DXR. $K_{d_{Ca^{2+}}}$ in the absence and in the presence of 5, 30, and 100 μM DXR was 36.0 ± 9.0, 20.8 ± 3.8, 2.1 ± 0.01, and 1.8 ± 0.04 μM, respectively.

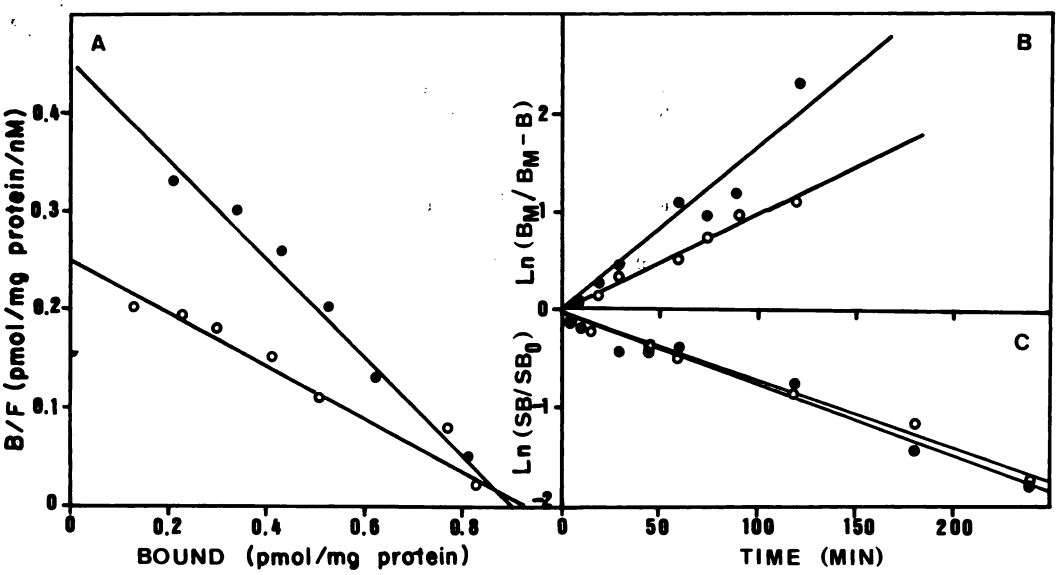


Fig. 3. Effect of 10 μM DXR on the kinetic and equilibrium constants of [^{3}H]ryanodine binding. Binding of 0.5–16 (A) and 1 (B and C) nM [^{3}H]-ryanodine was measured in the presence of 30 μM Ca^{2+} and 1 mM Mg^{2+}. Scatchard curves in the absence (○) and in the presence (●) of 10 μM DXR are shown in A. The K_d values are 3.7 and 2.0 nM, and the B_{max} values are 0.92 and 0.90 pmol/mg of protein in the absence and in the presence of DXR, respectively. *B/F*, Bound/Free. B, Time course of association of [^{3}H]ryanodine to its binding site in the absence (○) and in the presence (●) of 10 μM DXR; k_{obs} values are 0.00932 and 0.013 min^{-1}, respectively. B_M, Maximal number of binding sites; *B*, specific binding at time *t*. C, Time course of dissociation of 1 nM [^{3}H]ryanodine induced by 100-fold dilution of the assay medium in the absence (○) and in the presence (●) of 10 μM DXR; k_{-1} values are 0.00703 and 0.0077 min^{-1}, respectively. SB_0, Specific binding at time 0; *SB*, specific binding at time *t*. The calculated association rate constants are 0.0023 and 0.0053 nM^{-1} min^{-1} in the absence and in the presence of DXR. The calculated K_d values based on the association and dissociation constants are 3.05 and 1.45 nM in the absence and presence of DXR, which are not significantly different from the K_d values of equilibrium experiments. Data shown are from single experiments performed in triplicate, which were replicated twice.

CHAPS/mg of protein does not significantly alter the activation of the [^{3}H]ryanodine binding site by Ca^{2+}. For example, the binding of [^{3}H]ryanodine at 20 and 300 μM Ca^{2+} increases from 0.98 to 1.57 pmol/mg of protein. At 50 μM Ca^{2+}, the K_d is 1.23 nM and the B_{max} is 1.16 pmol/mg of soluble protein, which is similar to that of SR vesicles. The sucrose gradient-purified cardiac vesicles exhibit negligible ATP-stimulated Ca^{2+} uptake and, hence, are not suitable for rapid calcium release studies, most likely because of the high channel/ATPase ratio, which renders the vesicles too leaky to accumulate Ca^{2+}. Cardiac SR vesicles prepared according to the method of Harris and Doroshow (32) exhibit similar [^{3}H]ryanodine binding affinity (K_d = 3.3 nM) but reduced channel density (B_{max} = 0.51 pmol/mg of protein) and have significantly higher Ca^{2+}-dependent ATPase activity than sucrose gradient-purified membranes (2.06 ± 0.25 versus 0.79 ± 0.13 μmol of P_i/mg of protein/min, respectively), in agreement with the relative staining intensity of these proteins on SDS-polyacrylamide gels (Fig. 1). The cruder SR preparations accumulate Ca^{2+} to a maximum capacity ranging from 1.0 to 1.5 μmol/mg of protein (range of five experiments), which can be rapidly released via a ruthenium red-sensitive channel by a number of ligands including Ca^{2+}, anthraquinones, and caffeine (see below).

Mode of action of DXR at the high affinity [^{3}H]ryanodine receptor. DXR, a powerful antineoplastic agent with serious cardiotoxic side effects, has been found to enhance the specific high affinity binding of [^{3}H]ryanodine to the Ca^{2+} release channel of rat cardiac SR, with an ED_{50} of 31.7 ± 4.0 μM (mean ± SE, five experiments) and a Hill coefficient of 1.49 ± 0.12 when assayed at 1 μM Ca^{2+} (Fig. 4). DXR-enhanced binding of [^{3}H]ryanodine to the CHAPS/NaCl-solubilized receptor exhibits an ED_{50} of 8 μM at 20 μM Ca^{2+} (Fig. 4, *inset*), strongly suggesting that DXR interacts directly with the channel protein rather than with membrane lipids. Ca^{2+} concentrations for these experiments were chosen based on the observation that the activation of ryanodine binding by anthraquinones exhibits a sharp dependence on submicromolar Ca^{2+}. Optimal Ca^{2+} for ryanodine binding was 50 and 300 μM for the sucrose gradient preparation and for the solubilized receptor, respectively. Scatchard analysis of the binding of [^{3}H]ryanodine in the absence and presence of DXR shows a marked increase of the binding affinity and a negligible influence on the B_{max} (Fig. 3 and Table 1). DXR (10 μM) reduces the K_d nearly 2-fold at 30 μM Ca^{2+} (Fig. 3A). Fig. 3, B and C, demonstrates that enhancement of the binding affinity of ryanodine is due exclusively to an increase in the rate of association, because DXR has no influence on the dissociation rate of [^{3}H]ryanodine.

In the presence of 1 mM Mg^{2+}, DXR causes a dose-dependent decrease in the apparent dissociation constant of Ca^{2+} (Fig. 2), thereby increasing the affinity of Ca^{2+} for its binding site and enhancing Ca^{2+}-activated binding of [^{3}H]ryanodine. The K_d and B_{max} values of [^{3}H]ryanodine binding in the absence and presence of DXR at several calcium concentrations are summarized in Table 1. The lower the calcium concentration, the more pronounced is the effect of DXR on the K_d of ryanodine binding (Fig. 5).

Structure-activity relationships of DXR congeners. The potency with which eight anthraquinone analogs enhance Ca^{2+} activation of the [^{3}H]ryanodine binding site has been examined and their affinities are expressed relative to that of DXR (Table 2). DXR is the most potent compound. Changing the keto group on R_1 to an OH— (doxorubicinol) negligibly diminishes potency. Changing the OH— group to H— on R_1 (daunorubicin) or demethylation of R_4 with concomitant dehydroxylation of R_6 (4-demethyl-6-deoxydaunorubicin) reduces the potency by 33 and 38%, respectively. An epimer of daunorubicin, 3′-epiisodaunorubicin, has somewhat greater potency than daunorubicin itself. Substituting OH— to H— on R_3 (esorubicin) reduces potency by 44%. Changing the reactive 5-keto group to an imino group (5-iminodaunorubicin) or elimination of the 4-methoxy group (4-demethoxydoxorubicin; idarubicin) dramatically reduced the potency of these anthraquinones on the calcium release channel. All the anthraquinones tested have a Hill number greater than 1 for the binding of [^{3}H]ryanodine at 5 nM concentration (Table 2).

Daunorubicin enhances Ca^{2+}-induced release of Ca^{2+} from cardiac SR vesicles. SR from rat ventricles were preloaded with eight incremental additions of 10 μM $CaCl_2$ by

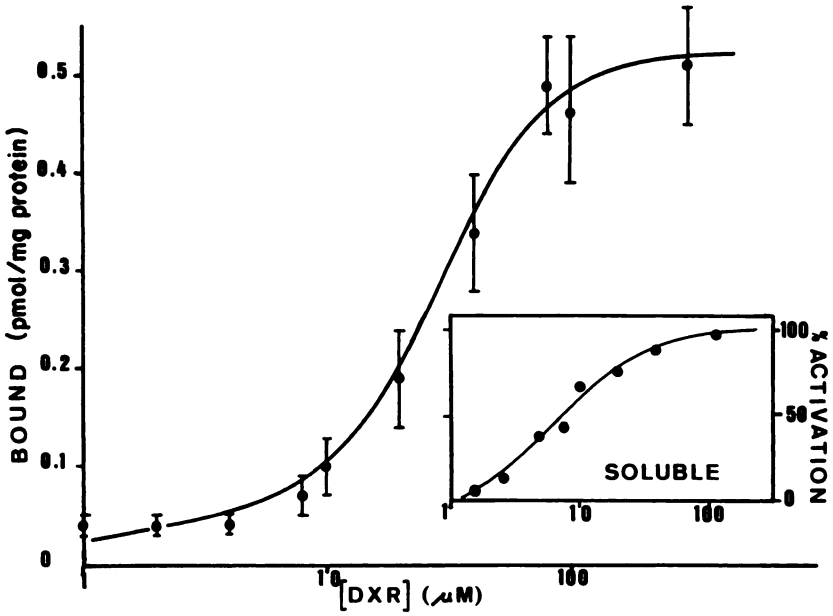


Fig. 4. Effect of DXR on the binding of [^{3}H] ryanodine. Binding of 5 nM [^{3}H]ryanodine was measured in the presence of several concentrations of DXR at 1 μM Ca^{2+} and in the presence of 1 mM Mg^{2+}. Data shown are averages of three independent experiments performed in duplicate. *Inset*, effect of DXR on the binding of 1 nM [^{3}H] ryanodine to solubilized receptors, which was performed at 20 μM Ca^{2+} in the presence of 1 mM Mg^{2+}. Data shown are from a single experiment performed in duplicate, which was repeated once.

TABLE 1

Effect of DXR on the equilibrium constants of [^{3}H]ryanodine binding at several calcium concentrations

Binding of 0.5 nM [^{3}H]ryanodine was measured as described before in the presence of 1 mM Mg^{2+}. Calcium concentrations were titrated with EGTA. Data shown are average of two experiments performed in triplicate ± range.

[Ca^{2+}]	[DXR]	K_d	B_{max}	Hill coefficient, n_H
μM	μM	nM	pmol/mg of protein	
12	0	7.4 ± 0.7	0.78 ± 0.06	0.94
12	1	6.3 ± 0.7	0.65 ± 0.04	0.97
12	10	3.4 ± 0.5	0.95 ± 0.05	0.86
12	30	2.2 ± 0.2	1.27 ± 0.08	0.91
30	0	4.2 ± 0.5	0.92 ± 0.05	0.93
30	1	3.8 ± 0.2	0.78 ± 0.05	0.94
30	10	2.3 ± 0.1	0.89 ± 0.07	0.93
30	30	1.7 ± 0.3	1.07 ± 0.06	0.85
50	0	3.1 ± 0.4	0.95 ± 0.04	0.84
50	1	2.2 ± 0.1	0.90 ± 0.03	1.01
50	10	1.9 ± 0.2	1.13 ± 0.04	0.93
50	30	1.5 ± 0.2	1.13 ± 0.07	0.87

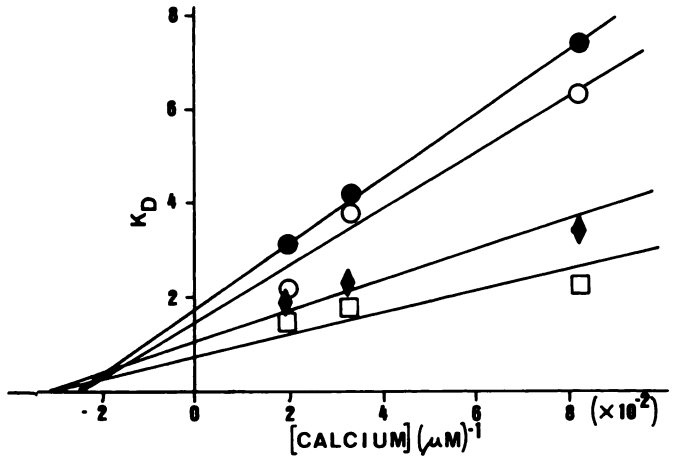


Fig. 5. Interaction of calcium and DXR; effect of DXR on the K_d of [^{3}H]-ryanodine binding at several Ca^{2+} concentrations. Experiments were performed as described in the legend to Table 2 at 0 (●), 1 (○), 10 (♦), and 30 (□) μM DXR.

allowing the Ca^{2+} to be fully accumulated by the vesicles between additions. Ca^{2+} release is induced by a 20 μM bolus of $CaCl_2$ to initiate Ca^{2+}-activated Ca^{2+} release (Fig. 6A) having an initial rate of 0.232 nmol/mg of protein/sec (±10%, 12 experiments). Ca^{2+} release studies were undertaken with daunorubicin, a potent congener of DXR, to reduce costs. Daunorubicin (30 μM) enhances the rate of Ca^{2+}-activated release of Ca^{2+} by 14-fold, to 3.3 nmol/mg of protein/sec (Fig. 6A). Daunorubicin-enhanced release is concentration dependent up to 100 μM and has an ED_{50} of 51 μM (Fig. 6C), which is in good agreement with its potency in the binding of [^{3}H]ryanodine (Table 2). Enhanced release of Ca^{2+} at 100 and 300 μM daunorubicin is completely inhibited by 1 μM ruthenium red, although the accumulated calcium can still be released by the ionophore A23187 (Fig. 6B). Daunorubicin-enhanced rates of Ca^{2+} release are extremely dependent on the free Ca^{2+} in the medium. Addition of daunorubicin (30 μM) at a Ca^{2+} concentration of approximately 30 μM (i.e., after Ca^{2+}-induced Ca^{2+} release is initiated) causes 5.8-fold higher release rates than those observed when the drug is added after the extravesicular Ca^{2+} concentration is allowed to drop to <5 μM (3.3 versus 0.58 nmol/mg of protein/sec, respectively) (see Fig. 9A).

DXR at 30 and 100 μM has no influence on the Ca^{2+}-dependent ATPase activity of SR membranes, whereas significant inhibition of this Ca^{2+} transporter by doxorubicinol is observed only at higher concentrations of 100 μM (Table 3), indicating that the effects of micromolar anthraquinone on the transport of Ca^{2+} across rat cardiac SR vesicles are primarily mediated by their channel activity, rather than by alteration of Ca^{2+}(+Mg^{2+})-ATPase function.

Interaction of anthraquinones and caffeine on the cardiac Ca^{2+} release channel. With skeletal SR, caffeine is known to allosterically sensitize the activation of [^{3}H]ryanodine binding sites by Ca^{2+}, which reflects an enhancement of channel activation by Ca^{2+} (13, 19). In cardiac SR, we find that the ED_{50} of caffeine for sensitization of the ryanodine binding site to activation by Ca^{2+} is 7.8 ± 0.8 mM (three experiments), which is approximately 240 times higher than that observed with DXR. This observation raises the question of whether caffeine and the anthraquinones are acting on the same site. To address this question, the activation of [^{3}H]ryanodine binding was measured with varying concentrations of one drug in the presence of fixed concentration of the other drug, and vice versa. Fig. 7A shows the activation of the [^{3}H]ryanodine binding site by caffeine in the absence and presence of three concentrations of DXR. Binding is elevated in the presence of DXR (the more potent activator) at low concentrations of caffeine; however, the ED_{50} concentration of caffeine is negligibly shifted from 6 mM in the absence of DXR to 10 mM in the presence of 60 μM DXR. Fig. 7B demonstrates that occupancy of [^{3}H]ryanodine sites at low concentrations of DXR is further enhanced by caffeine (up to 8 mM) but attains a maximum level of [^{3}H]ryanodine occupancy near 100 μM DXR. The ED_{50} concentrations of DXR in the absence and presence of 2.5 mM caffeine are 25 and 40 μM, respectively.

DXR (30 μM) or caffeine (12 mM), in the presence of 30 μM Ca^{2+} and 1 mM Mg^{2+}, decreases the K_d for [^{3}H]ryanodine binding from 3.98 to 1.11 and 1.37 nM, respectively. The simultaneous presence of 30 μM DXR and 12 mM caffeine results in a K_d of 1.19 nM, indicating that the effects of DXR and caffeine are not additive on the enhancement of the binding affinity of ryanodine for its site. Neither drug alone nor their combination has significant effect on the maximal binding capacity (Fig. 8). Consistent with [^{3}H]ryanodine binding data, caffeine (1.5 mM) inhibits daunorubicin-enhanced Ca^{2+} release rates from SR vesicles by 20% (from 3.3 to 2.7 nmol/mg of protein/sec) (Fig. 9B). This is not surprising, because 30 μM daunorubicin and 1.5 mM caffeine alone enhance the release rate by 14-fold (Fig. 6C) and 15% (data not shown), respectively. Applied together with daunorubicin, caffeine (1.5 mM) occupies a fraction of the daunorubicin sites. Hence, the fewer sites occupied by micromolar daunorubicin express less effect on the release rate of Ca^{2+} than in the absence of caffeine.

DXR exhibits cumulative lesions on the SR release channel. The activating effect of DXR on the binding of [^{3}H]-ryanodine to cardiac SR receptors is readily reversible, because preincubation of membranes with DXR (30 μM) for 2 hr followed by elimination of free DXR by repetitive washing and centrifugation does not change significantly the binding of [^{3}H]-ryanodine, compared with controls treated in the same manner but lacking DXR (0.88 and 0.78 pmol/mg of protein, respectively). SR membranes preincubated in the absence and presence of DXR (30 μM) for 5 and 24 hr on ice, in the presence of protease inhibitors, are assayed for their response to a second

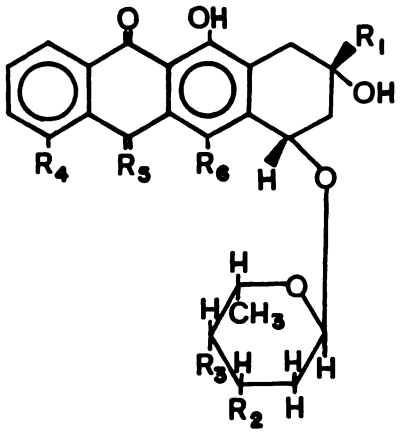
TABLE 2

Structure-activity relationships of anthraquinones in the activation of the binding of [^{3}H]ryanodine

Binding of 5 nM [^{3}H]ryanodine was measured at 1 μM Ca^{2+} concentration in the presence of 1 mM Mg^{2+}, as described in Materials and Methods. Values are average of three independent experiments assayed in duplicate ± standard error. Relative potency was calculated from the ED_{50} values by setting the value for doxorubicin to 1. DAU, daunorubicin.

Compound	R_1	R_2	R_3	R_4	R_5	R_6
1 DXR	—$COCH_2OH$	—NH_2	—OH	—OCH_3	=O	—OH
2 3′-Epiiso-DAU	—$COCH_3$	—OH	—NH_2	—OCH_3	=O	—OH
3 Doxorubicinol	—$COHCH_2OH$	—NH_2	—OH	—OCH_3	=O	—OH
4 Daunorubicin	—$COCH_3$	—NH_2	—OH	—OCH_3	=O	—OH
5 4-Demethyl-6-deoxy-DAU	—$COCH_3$	—NH_2	—OH	—OH	=O	—H
6 Esorubicin	—$COCH_2OH$	—NH_2	—H	—OCH_3	=O	—OH
7 5-Imino-DAU	—$COCH_3$	—NH_2	—OH	—OCH_3	—NH	—OH
8 4-Demethoxy-DXR	—$COCH_2OH$	—NH_2	—OH	—H	=O	—OH

Compound	ED_{50}	Hill coefficient, n_H	Relative potency
	μM		
1	32 ± 4	1.5 ± 0.1	1.0
2	37 ± 6	1.6 ± 0.2	0.86
3	39 ± 8	1.6 ± 0.2	0.82
4	48 ± 6	1.6 ± 0.1	0.67
5	52 ± 3	1.5 ± 0.1	0.62
6	57 ± 6	1.8 ± 0.1	0.56
7	>100		<0.3
8	>300		<0.1



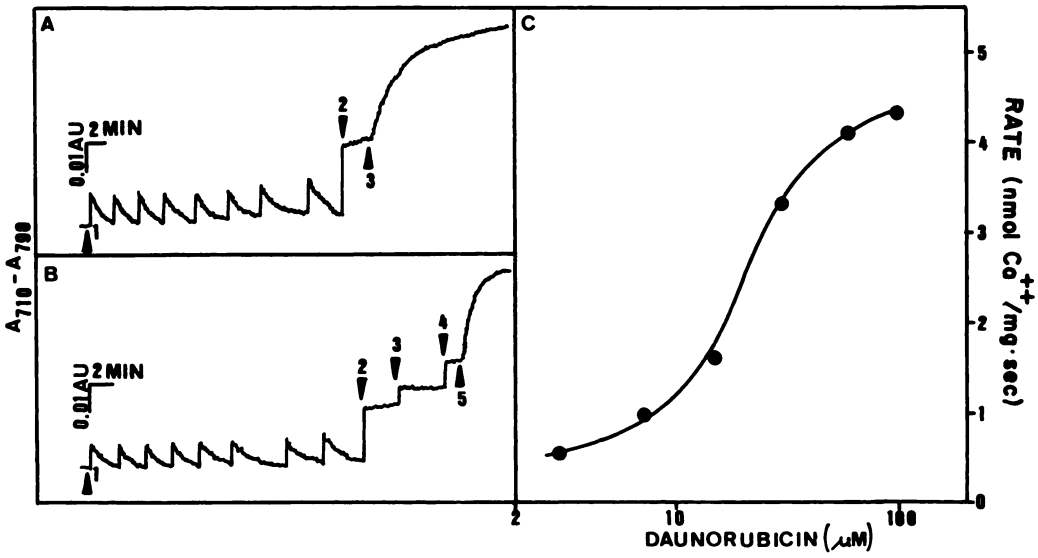


Fig. 6. Effect of daunorubicin on Ca^{2+}-activated Ca^{2+} release from rat cardiac SR microsomes. Ca^{2+} uptake and Ca^{2+}-activated Ca^{2+} release was performed as described in Materials and Methods. Additions in A, *1*, eight consecutive additions of 10 μM $CaCl_2$; 2, 20 μM $CaCl_2$; 3, 30 μM daunorubicin. Additions in B, *1*, eight consecutive additions of 10 μM $CaCl_2$; 2, 20 μM $CaCl_2$; 3, 100 μM daunorubicin plus 1 μM ruthenium red; *4*, 300 μM daunorubicin; *5*, 2 μg of A23187. C, Dose dependence of daunorubicin-enhanced rates of Ca^{2+}-activated Ca^{2+} release. Concentrations of daunorubicin were added after the release of calcium was triggered by 20 μM Ca^{2+} (at the level of 25 μM Ca^{2+}). Data shown are representative experiments, which were repeated once with similar results.

exposure of DXR following washout of the first DXR treatment, as described above. [^{3}H]Ryanodine binding subsequently assayed in the absence or presence of DXR (15 and 30 μM) with control and DXR-pretreated membrane vesicles shows that the DXR-pretreated membranes are significantly more sensitive to repeated exposure to DXR (i.e., are sensitized) and that the degree of sensitization depends on the initial length of exposure to the drug (Table 4). For example, SR membranes pretreated for 24 hr show a 7.7-fold enhancement of [^{3}H]-ryanodine binding upon repeated exposure to DXR (30 μM), when compared with pretreated membranes not re-exposed to the drug, whereas membranes not pretreated with the drug only exhibit a 2.7-fold enhancement upon exposure to the drug (Table 4). This represents a 2.9-fold sensitization. Membranes pretreated for shorter amounts of time (e.g., 5 hr) with DXR (30 μM) exhibit only a 1.7-fold sensitization (2.6- versus 4.5-fold enhancement with control and pretreated membranes, respectively (Table 4). In accordance with these findings, repeated exposure of SR vesicles pretreated for 5 hr with DXR (10 μM) sensitizes subsequent rates of Ca^{2+} release induced by

TABLE 3

Effect of DXR and doxorubicinol on the activity of Ca^{2+} ($+Mg^{2+}$)-ATPase

ATPase activity of 3.8 μg of cardiac SR microsomes was determined as described in Materials and Methods. DXR was added initially to the assay medium, which was blanked before measurements were made. Values are average ± standard error.

[Anthraquinone]	n	Activity
μM		μmol of P_i/mg of protein/min
DXR		
0	5	2.07 ± 0.25
30	3	2.78 ± 0.12
100	3	2.12 ± 0.13
Doxorubicinol		
0	4	1.79 ± 0.11
30	3	1.61 ± 0.31
100	3	1.28 ± 0.05*

* Significant difference ($p < 0.05$) from the control.

20 μM DXR by over 5-fold, compared with controls not pretreated with the drug (15.6- versus 3.1-fold enhancement with pretreated and control membranes, respectively). In contrast, only a slight (10%) enhancement is seen with a 0.5-hr pretreatment with DXR (20 μM) (Table 4). These findings demonstrate the time-dependent cumulative sensitization of the cardiac SR release channel by repeated exposures to DXR, in consonance with the etiology of cardiomyopathy.

Role of receptor thiols in anthraquinone-mediated SR lesions. The readily reversible nature of anthraquinone-induced changes at the ryanodine receptor cannot account for the long term, cumulative sensitization just described. Because direct covalent modification of the receptor protein by anthraquinone is unlikely, we have explored the possibility that DXR binding alters the redox state of receptor sulfhydryl groups, which appear to make a critical contribution to the normal gating of the Ca^{2+} release channel. To test this hypothesis, we examined the effect of DTT, and effective SH group-reducing agent, on the DXR-enhanced binding of [^{3}H]ryanodine. DTT (1 mM) has no significant effect on the binding of [^{3}H]ryanodine at 1 μM Ca^{2+}; however, it significantly potentiates the effect of 10, 20, 50, and 100 μM DXR (Fig. 10). DTT (1 mM) also increases the release rate of Ca^{2+} induced by 60 μM daunorubicin, from 1.65 to 2.44 nmol/mg of protein/sec (Fig. 11). Addition of 1 mM DTT after the DTT/daunorubicin-enhanced release reaches completion (i.e., near steady state) only slightly increases the ability of the vesicles to reaccumulate Ca^{2+} (Fig. 11A), whereas reaccumulation is significantly more apparent when DTT (2 mM) is added after the daunorubicin-induced (i.e., without DTT pretreatment) release reaches steady state. The influence of sulfhydryl oxidation by 4,4′-dithiodipyridine on the daunorubicin-enhanced binding of [^{3}H]ryanodine was assessed at 0.1, 0.6, and 2 μM concentrations (Fig. 12). Treatment reduces the maximal effect of daunorubicin on the activation of [^{3}H]ryanodine binding without changing the ED_{50} concentrations (ED_{50} values are 92.2, 103.8, 91.7, and 95.5 μM in the absence and in the presence of 0.1, 0.6, and 2 μM 4,4′-dithiodipyridine, respectively). In contrast to DTT, 4,4′-dithiodipyridine added once the daunomycin-induced Ca^{2+} release reaches steady state stimulates additional release of intravesicular Ca^{2+} (Fig. 11B).

Discussion

Rat cardiac SR, a model for studying anthraquinone-induced cardiomyopathies. Anthraquinones such as DXR are one of the most important groups of antineoplastic agents currently being investigated. Clinical use of DXR results in a dose- and time-related cardiotoxicity in patients if the cumulative dose exceeds 500 mg/m^2 (22, 42). The principle mechanism responsible for initiating the cumulative cardiac dysfunction associated with anthraquinone chemotherapy remains unknown. The present work establishes the first rodent cardiac model for investigating anthraquinone-mediated mechanisms altering the integrity of EC coupling at the level of the Ca^{2+} release channel of SR to ascertain their possible role in the etiology of cardiomyopathy. The rat cardiac preparations described here exhibit enrichment in [^{3}H]ryanodine receptor (7–8 pmol/mg of protein), which is comparable to SR preparations

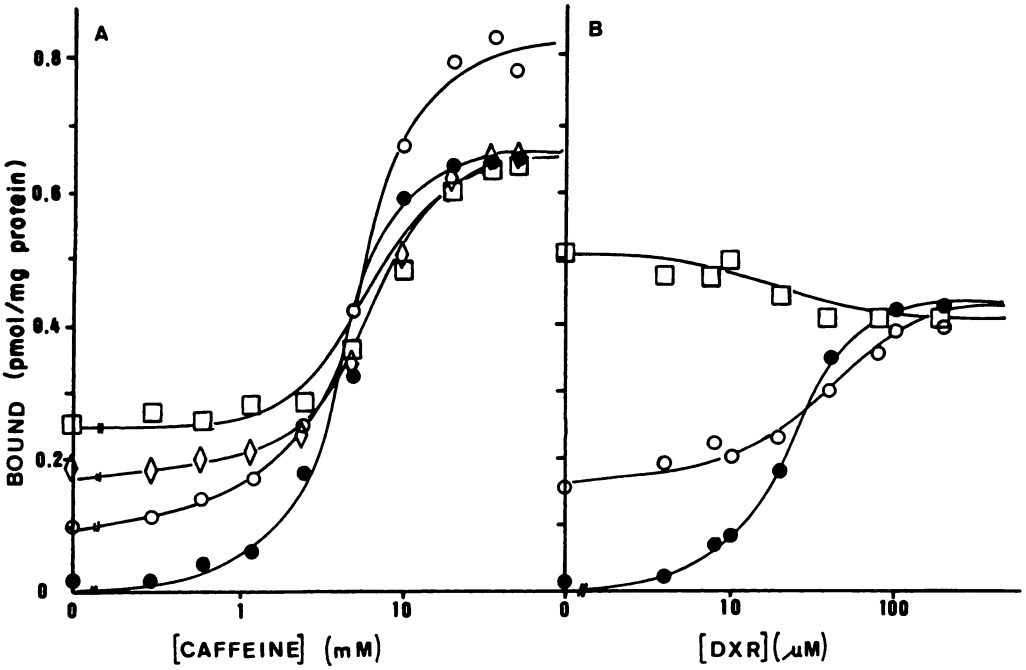


Fig. 7. A, Effect of DXR at 0 (●), 10 (○), 30 (◇), and 60 (□) μM concentrations on the caffeine-activated binding of [^{3}H]ryanodine. Binding of 1 nM [^{3}H]ryanodine was measured at 1 μM Ca^{2+} and 1 mM Mg^{2+}. B, Effect of caffeine at 0 (●), 2.5 (○), and 8 (□) mM concentrations on the DXR-activated binding of [^{3}H]ryanodine. Data shown are from single experiments performed in duplicate, which were replicated once with similar results.

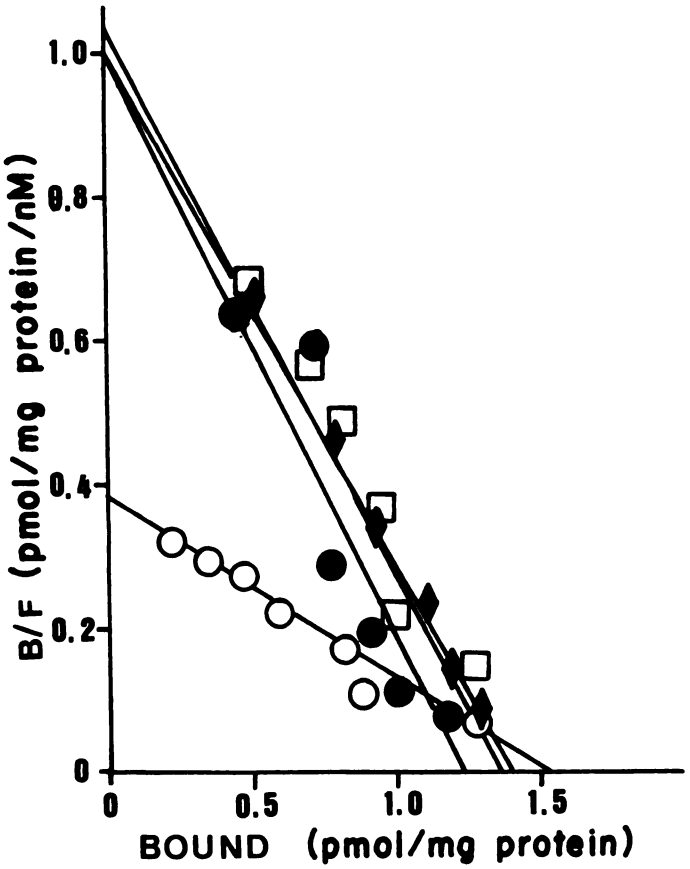


Fig. 8. Effect of DXR and caffeine on the K_d and B_{max} of [^{3}H]ryanodine binding alone and in combination. ○, Control; ●, 30 μM DXR; ♦, 12 mM caffeine; □, 30 μM DXR and 12 mM caffeine. The K_d values are in the text and the B_{max} values are 1.54, 1.16, 1.43, and 1.33 pmol/mg of protein, respectively. Data shown are from single experiments performed in triplicate. *B/F*, Bound/free.

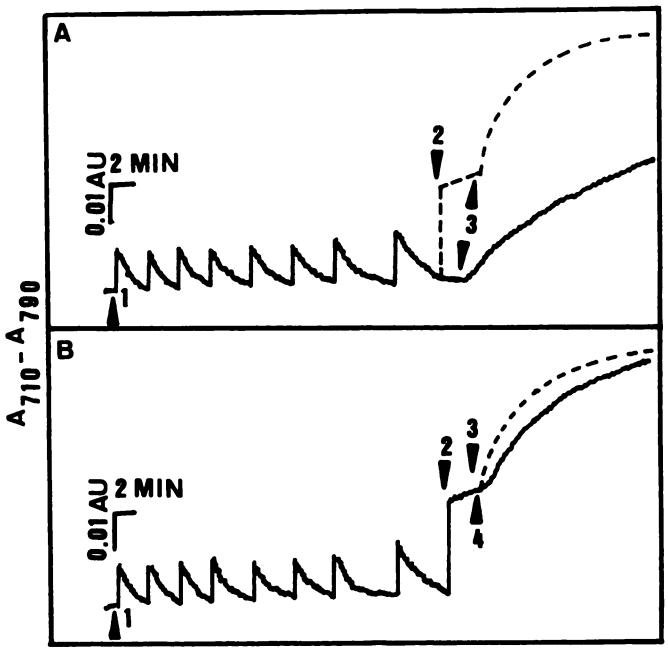


Fig. 9. Interaction of Ca^{2+}, daunorubicin, and caffeine on the Ca^{2+}-activated Ca^{2+} release of cardiac SR vesicles. Additions in A, *1*, eight consecutive additions of 10 μM $CaCl_2$; *2*, 20 μM $CaCl_2$; *3*, 30 μM daunorubicin. The addition of daunorubicin presented by *solid* and *dashed lines* occurred at 5 and 25 μM Ca^{2+}, respectively. Additions in B, *1*, eight consecutive additions of 10 μM $CaCl_2$; *2*, 20 μM $CaCl_2$; *3*, 30 μM daunorubicin (*dashed line*); *4*, 30 μM daunorubicin plus 1.5 mM caffeine (*solid line*). For rate values see text. Data shown are representative experiments, which were repeated once with similar results.

from canine ventricles (5, 43, 44), and rapid Ca^{2+} release rates on the order of 5 nmol/mg of protein/sec, which are similar to those previously described with SR vesicles from canine ventricles (45). SR preparations from canine ventricles currently used to study Ca^{2+} transport properties and the ryanodine receptor have obvious limitations, especially for performing *in vivo* toxicological studies to assess the relevance of receptor-mediated mechanisms of anthraquinone-induced cardiomyopathy, whereas the rodent model circumvents these limitations.

Anthraquinones alter cardiac SR function by direct interaction with the ryanodine receptor. The ability of DXR to induce release of Ca^{2+} from skeletal muscle SR has been reported by Zorzato and co-workers (29). Subsequent experiments with skeletal SR have demonstrated that these effects are the result of direct and reversible interaction of anthraquinones with the ryanodine receptor complex of junctional SR (19). Similar to the mechanism proposed for caffeine-induced release of Ca^{2+} (13), micromolar anthraquinone increases the apparent affinity by which Ca^{2+} activates the [^{3}H]-ryanodine binding site and enhances the rapid release of Ca^{2+} from SR vesicles, suggesting an allosteric site for anthraquinones on the ryanodine receptor complex that can modify channel gating (19).

In cardiac SR we find that DXR and daunorubicin enhance the release of intravesicular Ca^{2+} in a dose-dependent fashion (Fig. 6 and Table 4) by binding to an allosteric effector site on

TABLE 4

Sensitization of the effect of DXR on the binding of [^{3}H]ryanodine and on the release rate of Ca^{2+} with DXR-pretreated membranes

Membranes (SR microsomes for Ca^{2+} transport, purified for binding experiments) were preincubated with and without 30 μM (for binding) or 10 μM (for release) DXR on ice for 0.5, 5, and 24 hr, in the presence of 10 μg/ml aprotinin, 50 μg/ml pepstatin, and 50 μg/ml leupeptin, and then centrifuged at 30,000 × *g* for 30 min. The pellet was washed twice and the final pellet was resuspended in assay buffer containing 1 μM Ca^{2+} and 1 mM Mg^{2+} for binding and in 0.3 M sucrose, 10 mM imidazole buffer for transport studies and assayed for ryanodine binding and Ca^{2+} transport in the absence and in the presence of 15, 20, or 30 μM DXR. Vesicles were loaded actively with 4 × 10 μM $CaCl_2$ and DXR was added after calcium release was triggered by addition of 40 μM Ca^{2+}

Preincubation time	[DXR]	[^{3}H]ryanodine binding[a]			
		Control		30 μM DXR pretreatment	
hr	*μM*	*pmol/mg*	*enhancement*	*pmol/mg*	*enhancement*
5	0	0.32		0.35	
5	15	0.76	2.4	1.20	3.4
5	0	0.35		0.40	
5	30	0.90	2.6	1.81	4.5
24	0	0.68		0.48	
24	15	0.87	1.3	1.44	3.0
24	0	0.16		0.11	
24	30	0.43	2.7	0.81	7.7
Preincubation time	[DXR]	Rate of Ca^{2+} release[b]			
		Control		10 μM DXR pretreatment	
hr	*μM*	*nmol/mg/sec*	*enhancement*	*nmol/mg/sec*	*enhancement*
0.5	0	0.083		0.075	
0.5	20	0.323	3.89	0.318	4.24
5.0	0	0.105		0.127	
5.0	20	0.323	3.07	1.985	15.62

[a] Single experiments in triplicate.
[b] Average of two determinations.

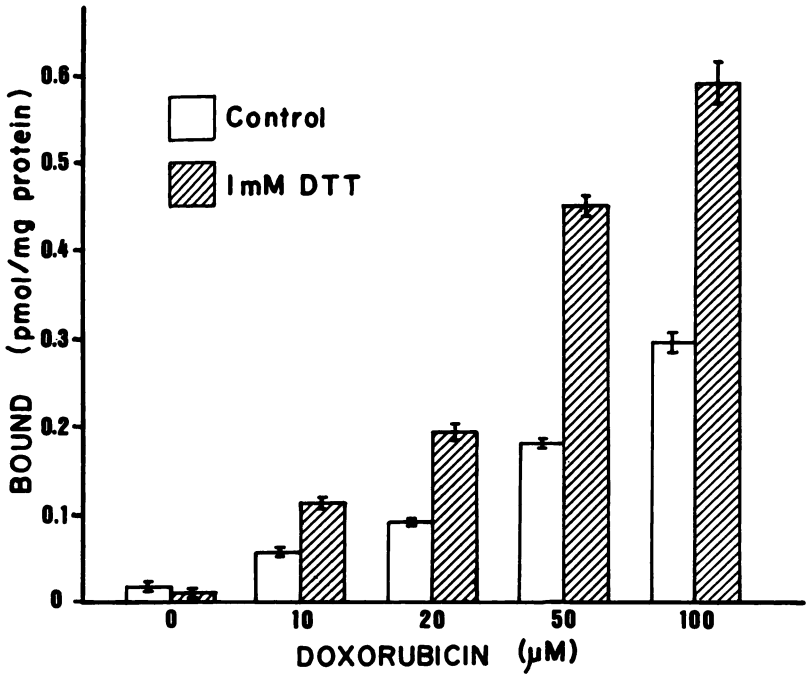


Fig. 10. Effect of DTT on the DXR-enhanced binding of [^{3}H]ryanodine. Binding of 1 nM [^{3}H]ryanodine was measured at 1 μM Ca^{2+} and 1 mM Mg^{2+} in the absence and presence of 10, 20, 50, and 100 μM DXR alone (□) and in combination with 1 mM DTT (▨). The experiments were performed in triplicate and repeated once.

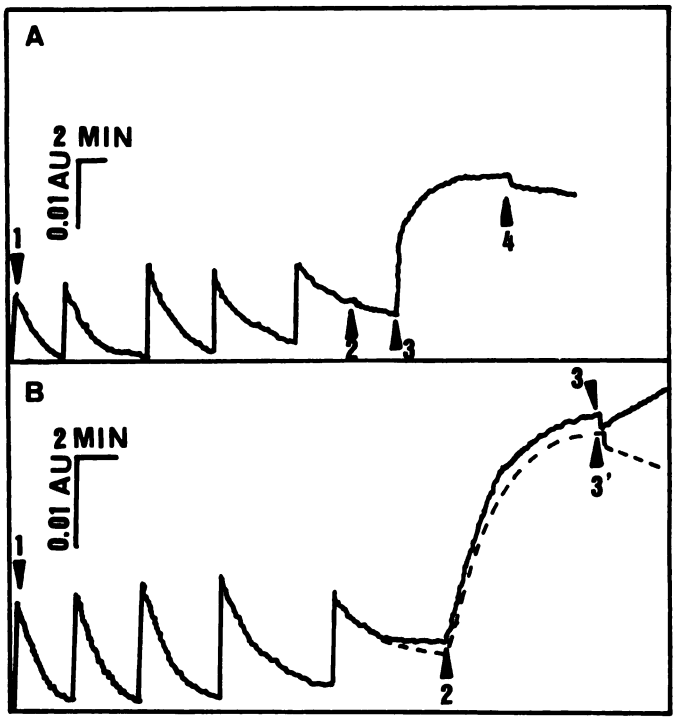


Fig. 11. Effect of DTT or 4,4′-dithiodipyridine on the daunorubicin-enhanced release rate of Ca^{2+} from SR microsomes. Seventy-five μg of SR microsomes were actively loaded by five consecutive additions of 10 μM $CaCl_2$ (addition *1*). Additions in A, *2*, 1 mM DTT; *3*, 60 μM daunorubicin (Ca^{2+} release rate is 2.44 nmol/mg of protein/sec); *4*, 1 mM DTT. Additions in B, *2*, 60 μM daunorubicin [Ca^{2+} release rate is 1.65 (± 10%) nmol/mg of protein/sec]; *3*, 3 μM 4,4′-dithiodipyridine. In a separate experiment (*dashed line*), 2 mM DTT was added (addition *3′*) after daunorubicin (60 μM)-induced Ca^{2+} release reached steady state. Data shown were representative experiments, which were repeated once with similar results.

the [^{3}H]ryanodine receptor that influences the sensitivity of the [^{3}H]ryanodine binding site to activation by Ca^{2+} (Fig. 2). Because the rate of binding of [^{3}H]ryanodine to its receptor is regulated by the free Ca^{2+} and Mg^{2+} concentrations in the assay medium, anthraquinones would be expected to dramatically enhance the rates of association of [^{3}H]ryanodine with its binding site, especially at suboptimal Ca^{2+} and in the presence of a physiological level of Mg^{2+}. As is shown in Fig. 3, the effect of DXR is to increase the association rate of ryanodine, which fully accounts for the increase in affinity of ryanodine for its site. Anthraquinone-stimulated rates of Ca^{2+} release are markedly influenced by the free Ca^{2+} concentration (Fig. 9A), coincident with their effects on the equilibrium binding of [^{3}H]-ryanodine, which are pronounced at suboptimal Ca^{2+} (Fig. 5). The possibility that anthraquinones induce Ca^{2+} release from SR by interacting with a specific site on the ryanodine receptor complex of cardiac SR is further supported by 1) the enhancement of [^{3}H]ryanodine binding to the solubilized receptor (Fig. 4, *inset*), 2) the rapidly reversible nature of the interaction on short term exposure (Table 4), and 3) a strong structural requirement of anthraquinones for the activation of ryanodine binding (Table 2). For example, substitutions at C_4 are especially important for activity, because 4-demethoxy-doxorubicin has less than one tenth the activity of doxorubicin. 5-Imino-daunorubicin has an imino group instead of the reactive keto group at C_5, which dramatically reduces its potency at the receptor, demonstrating the importance of the quinone moiety. Interestingly, this analog is 4 times less cardiotoxic than daunorubicin (46), which is consonant with its potency at the receptor. These results support the hypothesis that anthraquinones directly sensitize the SR release channel to activation by Ca^{2+}, a physiologically relevant transmitter involved with EC coupling in cardiac muscle.

Olson and co-workers (31) have suggested that doxorubicinol, a primary metabolite of DXR, is responsible for cardiotoxicity, based on its potency in inhibiting a number of ion transporters (including the Ca^{2+}-dependent ATPase) in canine SR and

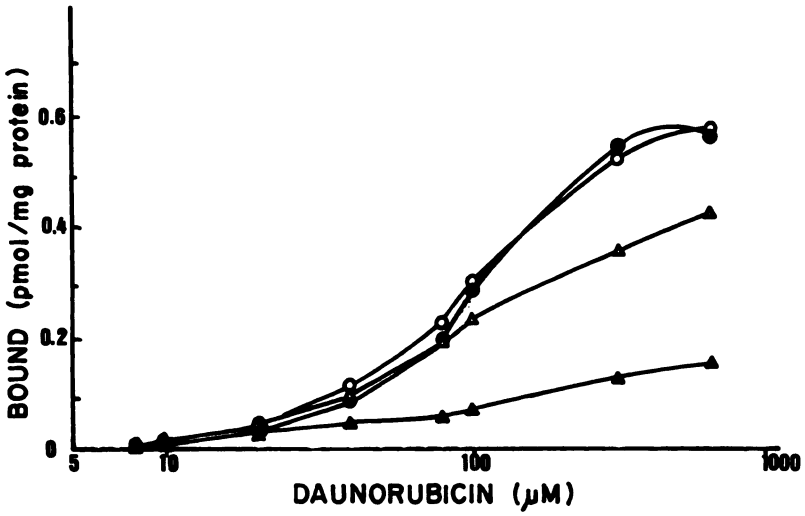


Fig. 12. Effect of 4,4′-dithiodipyridine on the daunorubicin-enhanced binding of [^{3}H]ryanodine. Binding of 1 nM [^{3}H]ryanodine was measured at 1 μM Ca^{2+} and 1 mM Mg^{2+}, in the absence (○) and presence of 0.1 (●), 0.6 (△), and 2 (▲) μM 4,4′-dithiodipyridine. Data shown are from single experiment performed in duplicate, which was repeated once with similar results.

plasma membrane fractions and on its ability to alter cardiac contractility. Our results with rat SR show that DXR has no significant influence on the Ca^{2+}-dependent ATPase of SR at concentrations that saturate effects mediated by the ryanodine receptor (100 μM) (Table 3). Furthermore, high concentrations of doxorubicinol are required to cause marginal inhibition of Ca^{2+}-dependent ATPase (30% inhibition at 100 μM). Because doxorubicinol is one of the most potent structures at the ryanodine receptor (ED_{50} = 39 μM) and attains tissue levels of 2 to 11% of DXR in the heart (31), the Ca^{2+} release channel may be the more plausible target for the observed changes in cardiac function.

Caffeine and DXR act at competitive sites. In contrast to the conclusion reached with skeletal SR concerning the noncompetitive mechanism by which caffeine inhibits anthraquinone-stimulated release of Ca^{2+} (19), more detailed studies of this interaction in cardiac SR strongly suggest that caffeine and DXR indeed compete for the same binding site on the ryanodine receptor complex, because these drugs in combination influence the K_d of [^{3}H]ryanodine to its binding site to the same extent seen with each drug alone (i.e., a nonadditive effect) (Fig. 8). DXR-activated binding of [^{3}H]ryanodine is inhibited by caffeine, and the ED_{50} concentration of the caffeine-activated binding of ryanodine is shifted to the right (Fig. 7), which can account for the inhibition of anthraquinone-stimulated Ca^{2+} release by caffeine (Fig. 9B). A common site for DXR and caffeine is also supported by Zorzato *et al.* (47), who have demonstrated that photolabeling of a M_r 170,000 protein from skeletal junctional SR with DXR is inhibited by caffeine.

DXR causes time- and dose-dependent cumulative lesions at the ryanodine receptor. Anthraquinones exhibit both acute and chronic cardiotoxicity. Chronic exposure to anthraquinones results in cumulative dose-dependent lesions, which are irreversible and progressive despite withdrawal of the drug (21–23, 32) and may be the result of accumulation of toxic metabolites such as doxorubicinol in cardiac tissues (31, 48).

We have demonstrated that a single acute (0.5- to 2-hr) exposure of cardiac SR membranes to DXR enhances the binding of [^{3}H]ryanodine and the release of Ca^{2+} from actively loaded vesicles in a fully reversible manner. However long term (5- to 24-hr) treatment of SR with DXR and subsequent elimination of the drug by repeated washes dramatically sensitizes the Ca^{2+} release channel and the binding of [^{3}H]ryanodine to repeated exposure to the drug (Table 4). Repeated anthraquinone exposure appears to increase the potency of subsequent challenges with DXR. This mechanism may be significant in the etiology of cardiomyopathy observed with current clinical strategies to control neoplasms with anthraquinones, because 1) cyclic fluctuations in DXR levels in the blood (and heart) with repeated doses may progressively sensitize the Ca^{2+} release channel, with subsequent (low) doses having greater effects on Ca^{2+} leakage from SR, and 2) the accumulation of the primary metabolite doxorubicinol in cardiac tissue (48) is itself very potent at the ryanodine receptor.

Finally, we address one possible molecular mechanism that may be involved in long term sensitization of the ryanodine receptor to repeated anthraquinone exposure. Aryldisulfides (e.g., 4,4′-dithiodipyridine) specifically oxidize protein thiols to form mixed disulfides and exhibit a potent biphasic effect on the binding of [^{3}H]ryanodine, stimulating occupancy at ≤1 μM while fully inhibiting it at 10 μM (49). Oxidation of critical thiols on the ryanodine receptor has been correlated with the functional state of the channel (49, 50). Our results clearly demonstrate that expression of anthraquinone-induced sensitization of the SR Ca^{2+} release channel is markedly influenced by the oxidation state of thiols that are functionally important to the ryanodine receptor complex (Figs. 10–12). Treatment of SR with DTT to promote the reduction of receptor thiols does not itself induce Ca^{2+} release but enhances both the initial rates of Ca^{2+} release and [^{3}H]ryanodine binding induced by daunorubicin (Figs. 10 and 11), supporting the idea that anthraquinones promote the oxidation of thiols critical to the gating of this channel and that these thiols may be allosteric with respect to the anthraquinone (caffeine) effector site.

Detailed study of this aspect of the effect of anthraquinones on a receptor-mediated shift in the redox equilibrium of critical thiols at the ryanodine receptor complex, which in turn may lead to long term sensitization of the Ca^{2+} release channel to activation by endogenous signal molecules (Ca^{2+}, ATP), is in progress in our laboratory. Further investigations of the molecular mechanism of anthraquinones *in vivo* is required for complete understanding of the cardiotoxicity and could make pos-

sible the bio-rational treatment of the serious side effect of these valuable anticancer agents.

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