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Multiple actions of the anthracycline daunorubicin on cardiac ryanodine receptors.

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Running title: Daunorubicin and cardiac ryanodine receptors.

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NON-STANDARD ABBREVIATIONS.
CSQ1 – skeletal isoform of calsequestrin; calsequestrin type 1
CSQ2 – cardiac isoform of calsequestrin; calsequestrin type 2.
DAD – delayed after depolarization
EAD – early after depolarization
I'_F – fractional mean current
NHMRC – National Health and Medical Research Council
P_o – open probability
RyR1 – skeletal isoform of the ryanodine receptor; ryanodine receptor type 1
RyR2 – cardiac isoform of the ryanodine receptor; ryanodine receptor type 2
SR – sarcoplasmic reticulum

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ABSTRACT

Our aim was to examine the molecular basis for acute effects of the anthracycline daunorubicin on cardiac ryanodine receptor (RyR2) channels and cardiac calsequestrin (CSQ2). Cardiotoxic effects of anthracyclines preclude their chemotherapeutic use in patients with pre-existing heart conditions. To address this significant problem the mechanisms of anthracycline toxicity must be defined, but at present are poorly understood. RyR2 channel activity was assessed by measuring Ca^{2+} release from cardiac sarcoplasmic reticulum vesicles and by examining single RyR2 channels inserted into artificial lipid bilayers. We show that 0.5 to 10 μM daunorubicin increases the activity of RyR2 channels after 5 to 10 min and that activity then declines to very low levels when channels are exposed daunorubicin concentrations of ≥2.5 μM for a further 10 to 20 min. Extensive dissection of these effects shows for the first time that the activation results from a redox-independent binding of daunorubicin to the RyR2 complex. Novel data includes the demonstration of daunorubicin binding to RyR2. We provide compelling evidence that RyR2 channel inhibition is due to the oxidation of free SH groups. The oxidation reaction is prevented by the presence of 1 mM dithiothreitol (DTT). We also present novel data showing that CSQ2 modifies the response of RyR2 to daunorubicin, but that the response of RyR2 is not dependent on daunorubicin binding to CSQ2. We suggest that binding of daunorubicin to RyR2 and CSQ2, and oxidation of RyR2, are all likely to contribute to anthracycline-induced cardiotoxicity during chemotherapy.

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INTRODUCTION

Cardiac and skeletal muscle contraction is triggered by excitation-contraction (EC) coupling, the process which links depolarization of the surface membrane with Ca^{2+} release from the internal sarcoplasmic reticulum (SR) Ca^{2+} store. Ca^{2+} is released from the SR through the ryanodine receptor (RyR) Ca^{2+} channel which forms the hub of a macromolecular complex that spans the SR membrane and includes cytoplasmic proteins and proteins in the lumen of the SR. The efficacy of Ca^{2+} release is set by the integrated effects on RyR channel activity of associated proteins/ligands, covalent modification by redox reactions and phosphorylation and by ionic conditions. In the heart, single point mutations in the cardiac isoform of the RyR (RyR2) or the calcium binding protein calsequestrin (CSQ2, located within the lumen of the junctional SR) lead to ventricular tachycardia and sudden death (Brini, 2004; Lahat et al., 2001), while therapeutic drugs such as the anthracyclines can seriously disrupt RyR2 function.

Anthracyclines, including daunorubicin and doxorubicin are highly effective in the treatment of a variety of cancers. The drugs have a long half-life, accumulate in the heart at concentrations in the nM and low μM range and induce an ~8-fold increase in the risk of death due to cardiac complications (Mertens et al., 2001). The cardiac complications manifest as acute arrhythmias and hypotension and chronic conditions such as dilated cardiomyopathy and congestive heart failure (Menna et al., 2007). The cardiotoxicity is thought to result from the synergistic actions of the acute and chronic effects of the drugs on SR proteins. The acute effects are attributed to the accumulated anthracycline binding to target protein(s), while the chronic effects include changes in expression of Ca^{2+} handling proteins such as RyR2, CSQ2 and SERCA (the sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase) (Arai et al., 1998; Gambliel et al., 2002).

Anthracycline binding to SR proteins results in reduced SR Ca^{2+} storage capacity and modified Ca^{2+} release. The anthracyclines cross the surface and SR membranes, bind to CSQ2 (Charlier et al., 2005; Kim et al., 2007; Park et al., 2005) and alter RyR1 and RyR2 activity

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(Abramson et al., 1988; Feng et al., 1999; Ondrias et al., 1990; Pessah et al., 1990). Daunorubicin at 2.5 – 10 μM initially activates native RyR2 and then irreversibly inhibits the channels (Ondrias et al., 1990). Ca^{2+} release from SR vesicles is inhibited by 1 – 30 μM daunorubicin (Olson et al., 2000).

The mechanism of daunorubicin actions on RyR2 are not well understood and are likely to include redox modification of the protein. Daunorubicin contains a quinone moiety which undergoes redox cycling and induces oxidative tissue damage (Menna et al., 2007). This could alter RyR2 activity by decreasing the number of reactive thiol groups, either by oxidizing free -SH groups to disulphides (Abramson et al., 1988), or by transiently withdrawing electrons and shifting the redox potential of reactive thiols to more negative values (Marinov et al., 2007; Pessah et al., 2002). The RyR2 monomer contains 89 cysteine residues, 21 of which are available for oxidation with functional consequences. Doxorubicin reduces the number of free thiols on RyR1 (Feng et al., 1999). The contribution of redox modifications to anthracycline action on RyR2 remains controversial. The thiol-reducing agent, dithiothreitol (DTT) prevents doxorubicin-induced inhibition of RyR2 channels (Ondrias et al., 1990), but not inhibition of Ca^{2+} release from the SR (Olson et al., 2000). The ability of daunorubicin to bind to RyR2 and directly modify channel gating has not previously been reported and is examined here.

CSQ2 is a second SR anthracycline target. Anthracyclines bind to CSQ2 with micromolar affinity (Kim et al., 2005), altering its Ca^{2+} binding capacity. Given that CSQ2 is a regulator of RyR2 (Wei et al., 2009), anthracyclines might impact SR Ca^{2+} homeostasis through modifying CSQ2 and its interaction with RyR2. There have been few studies of the effects of anthracyclines on single RyR2 channel activity. Most studies have used cardiac SR Ca^{2+} release or [^{3}H]ryanodine binding as an index of RyR2 channel function, but not the molecular mechanism of drug action on RyR2 channel activity. The possibility that CSQ2 may influence the action of anthracyclines on RyR2 channel function has not previously been investigated.

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In this manuscript we examine the actions of daunorubicin on Ca^{2+} release from cardiac SR vesicles and on the activity of single RyR2 channels in lipid bilayers. We explore the possibility that the action of the drug is due to redox modification of reactive thiols on the RyR2 complex, or to redox-independent ligand binding. We examine the effect of daunorubicin on the Ca^{2+} binding capacity of CSQ2 and whether this contributes to the anthracycline effects on RyR2 channel activity.

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MATERIALS AND METHODS

Materials. The monoclonal anti-RyR antibody was obtained from Abcam (Cambridge, United Kingdom). Phospholipids were obtained from Avanti Polar Lipids (Alabama, United States). All other chemicals were obtained from Sigma-Aldrich (Castle Hill, Australia).

SR vesicle isolation, RyR2 and CSQ2 purification. Cardiac SR vesicles were prepared from sheep heart (Laver et al., 1995). RyRs were solublized and purified from SR as described by (Dulhunty et al., 2005). CSQ2 isolated from cardiac SR was purified by gel elution (Beard et al., 2008). CSQ2 was eluted from large vertical slab SDS polyacrylamide gels in a denaturing buffer using an 8.5% resolving gel (containing 8.5% acrylamide/bis (29:1), 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, 0.05% APS, and 0.1% TEMED) and a 4% stacking gel consisting of 4% acrylamide/bis (29:1), 0.125 M Tris-HCl, pH 6.8, 0.1% SDS 0.05% APS and 0.1% TEMED. SDS-PAGE and Western Blot are described in (Beard et al., 2002). The Ca^{2+} binding capacity of CSQ2 was determined using a $^{45}Ca^{2+}$ spin dialysis binding assay (Maruyama et al., 1984).

Single channel recording and analysis. Planar bilayers separating two baths (*cis* and *trans*) were formed as previously described (Beard et al., 2005; Beard et al., 2002). SR vesicles (50 μg) were added to the *cis* solution so that the cytoplasmic surface of the SR and RyRs faced the *cis* solution after incorporation into the lipid bilayer. For vesicle incorporation, the solution compositions were: *cis*: 230 mM CsMS, 20 mM CsCl, 1 mM $CaCl_2$, and 10 mM TES (pH 7.4); and *trans*: 30 mM CsMS, 20 mM CsCl, 1 mM $CaCl_2$, and 10 mM TES (pH 7.4). After incorporation of a channel, *trans* $[Cs^+]$ was raised to 250 mM with addition of 200 mM CsMS and the *cis* solution was altered by the addition 1.32mM BAPTA (free $[Ca^{2+}]$ = 1 μM). In some experiments, endogenous CSQ2 was dissociated from the RyR/triadin/junctin complex by exposure to a high ionic strength solution (Beard et al., 2002). Triadin and junctin are transmembrane proteins that bind to the luminal domain of RyR2. CSQ2 associates with the RyR2/triadin/junctin complex by binding to junctin and triadin (Gyorke and Terentyev, 2008; Wei et al., 2009). Electrical

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potentials are expressed here as cytoplasmic relative to luminal. The luminal solution was maintained at virtual ground. Single channel currents were recorded at +40 mV and -40 mV. The data was filtered at 1 kHz and sampled at 5 kHz. The mean open times of channels varies from 1 ms to 10 ms and the measured dead times for the filter/s (using a 20% discriminator), was <200 μs and the dead time calculated for an ideal 4^{th} order low pass bessel filter (as in the Axopatch 200A) is no more than 200 μs. Thus missed events would have been few and would not have altered the average parameters (Beard et al., 2008). Single channel parameters were obtained using the Channel 2 program (developed by P.W. Gage and M. Smith, John Curtin School of Medical Research, Canberra, Australia) from 60 to 180 s of channel activity. Open probability (P_o) was measured as fractional mean current (I'_F) or as the fraction of time that the channel was open using threshold discriminators. I'_F, is the average of all data points in a recording period, divided by the maximum single channel current. I'_F is approximately equal to the P_o measured by threshold discrimination. Experiments were performed at 23 ± 2 ^oC.

Calcium release assay. A Cary 3 spectrophotometer was used to monitor extravesicular Ca^{2+} at 710 nm, using the Ca^{2+} indicator antipyrylazo III (Dulhunty et al., 1999; Jalilian et al., 2008). Cardiac SR vesicles (200 μg/ml) were added to a solution containing 100 mM KH_2PO_4, 0.4 mM antipyrylazo III, 1 mM Na_2ATP and 4 mM $MgCl_2$ with temperature controlled at 25 ^oC. The cuvette solution was magnetically stirred throughout the experiment. Cardiac SR vesicles were added to the cuvette and the Ca^{2+} ATPase was activated by MgATP in the solution. The SR was loaded with four additions of 7.5 μM Ca^{2+}. Thapsigargin (300 nM) was added to block the Ca^{2+}-ATPase (Sagara and Inesi, 1991), so that Ca^{2+} release could be specifically measured. Ruthenium red was then added to confirm that Ca^{2+} release was through the RyR and finally Ca^{2+} ionophore (A23187) was added to release Ca^{2+} remaining in the SR. In one series of experiments daunorubicin was added 30 s after thapsigargin (Fig. 1A), to examine the ability of high concentrations of the drug to immediately stimulate Ca^{2+} release. In a second series, SR vesicles

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were incubated for 20 min with daunorubicin (or with vehicle buffer alone for control experiments) before they were added to the cuvette. The concentration of daunorubicin or vehicle buffer in the cuvette was adjusted to the incubation concentration and the vesicles loaded with Ca^{2+} in the usual way. Ca^{2+} release in this experiment was stimulated by 5 mM caffeine, following addition of thapsigargin (Fig. 1B). Calibration curves of optical density changes with addition of 12.5 μM to 50 μM $CaCl_2$ to the cuvette solution showed that neither daunorubicin, caffeine nor ruthenium red affected the calibration. The initial rate of daunorubicin or caffeine induced Ca^{2+} release was measured. The total amount of Ca^{2+} loaded into the SR was not altered by incubation of SR vesicles with daunorubicin.

Statistics. Average data are given as mean ±SEM. Statistical significance was evaluated using paired or unpaired Student's t-test as appropriate or ANOVA. Numbers of observations (n) are given in Tables and Figure legends. RyR channel activity is notoriously variable, possibly because of differences between channels in the degree phosphorylation, nitrosylation or oxidation (Copello et al., 1997; Marengo et al., 1998). To reduce effects of variability in control open probability (P_oc), and to evaluate parameters after daunorubicin addition (P_{oD}) or NEM addition (P_{oN}), data are expressed as the difference between the $\log_{10} P_{oC}$ and $\log_{10} P_{oD}$ or Po_N for each channel (eg $\log_{10} P_{oC}$ - $\log_{10} P_{oD}$). The difference from control was assessed with a paired t-test applied to $\log_{10} P_{oC}$, $\log_{10} P_{oD}$ or $\log_{10} P_{oN}$. The difference between relative data obtained at different times or concentrations was assessed using ANOVA with the multidimensional Mahalanobis test. A P value of <0.05 was considered significant for all tests.

Daunorubicin coupled CNBr-activated Sepharose. Daunorubicin was coupled to CNBr-activated Sepharose 4B following manufacturer's instructions. Purified RyR2 (0.5 mg/ml) in 50 mM Tris-HCl, pH 8 was incubated with the mixture with rotation overnight at 4° C. Unbound RyR2 was removed by washing with 50mM Tris-HCl, pH 8. Proteins bound to the daunorubicin coupled CNBr-activated Sepharose 4B were eluted by boiling for 1 min in sample buffer (0.05 M Tris-

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HCl (pH 6.8), 2% SDS, 0.01 M EDTA, 10% glycerol, 0.6 M mercaptoethanol , 0.02% bromophenolblue) and 0.15% Triton-X 100. The resultant supernatant was loaded on 4-12% SDS Page gels, transferred and probed with an anti RyR2 antibody (34C). In one experiment, non-specific binding sites on RyR2 were saturated by preincubating purified RyR2 with activated Sepharose for 2 h at 4° C with rotation prior to their addition to daunorubicin-coupled sepharose.

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RESULTS

Ca^{2+} release from cardiac SR vesicles. The records of full experiments are shown in Fig. 1. There were no consistent differences between the amounts of Ca^{2+} taken up into the SR when was daunorubicin was added after (Fig. 1A) or before (Fig. 1B) store loading. This is indicated by the vertical arrows at the end of each record in Fig. 1. The height of the arrow indicates the total Ca^{2+} released after thapsigargin addition, by daunorubicin or caffeine and then by the ionophore. The amount released is equal to the amount taken up and stored and is similar in the two types of experiment. To demonstrate that daunorubicin interacted with the Ca^{2+} release mechanism in our hands, we examined the immediate effect of the drug on Ca^{2+} release using concentrations ≥10 μM (Fig. 2A). The drug concentrations used to stimulate immediate Ca^{2+} release were higher than those required to activate release after longer incubations in subsequent studies with SR vesicles and single RyR2 channels, and higher than clinical concentrations. This was expected as the activation by lower concentrations takes several minutes to develop (see Fig. 3 below). The initial rate of Ca^{2+} release increased in a concentration-dependent manner to rates of ~120 nmoles/mg/min with 500 μM daunorubicin (Fig. 2B, upper trace). The release was through RyR2 as it was blocked by ruthenium red. When the rate was measured 10 min after addition of 10 μM daunorubicin, there was a significant increase in Ca^{2+} release rate, in contrast to the initial rate (measured within 10 s of daunorubicin addition) which was not changed (insert Fig. 2B).

The effect of longer exposure to daunorubicin was examined by incubating vesicles with daunorubicin for 20 min before Ca^{2+} uptake was initiated and ~30 min before Ca^{2+} release was examined. Although the rate of Ca^{2+} release after thapsigargin addition was low, there was a trend towards an increase in release after addition of 5 – 10 nM daunorubicin and a significant decrease seen with 10μM daunorubicin. To more accurately measure Ca^{2+} release, a stronger release was initiated with caffeine (Fig 1B and 2C-E). The rate of caffeine-induced Ca^{2+} release, measured ~30 min after initial exposure to daunorubicin, increased significantly with only 5 nM

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and 10 nM of the drug, then declined towards control levels as the concentration increased to 50 nM and was significantly depressed with 10 μM (Fig. 2C-E). The drug concentrations required to alter Ca^{2+} release during prolonged exposures were similar to those required to alter channel activity during exposure lasting several minutes (following section). The Ca^{2+} release experiments show that daunorubicin either stimulated or inhibited Ca^{2+} release depending on the concentration and the duration of exposure to the drug.

RyR2 channels in lipid bilayers. Daunorubicin was added to the luminal (*trans)* solution bathing RyR2 channels in lipid bilayers. Daunorubicin concentrations of 500 nM and 1 μM increased RyR2 activity after several minutes (Fig. 3A&C) and the increased activity was maintained for the duration of exposure to duanorubicin (up to 40min). Activity increased more rapidly with higher drug concentrations (compare the rates of change in P_o during exposure to 1 μM and 2.5 μM daunorubicin (Fig. 3C&D) and average data in Table 1). With higher drug concentrations (2.5 μM and 10 μM), activity first increased and then declined (Fig. 3B&D), to levels which were significantly less than the maximum activity and less than that before daunorubicin addition. The very low activity was maintained for lifetime of the experiment (up to 60 min). The activity of the channel in Fig. 3C was enhanced between 7.5 and 20 min exposure to 1 μM daunorubicin and then declined after 20 min to levels that were less than the maximum, but higher than before drug addition. This fall in activity may reflect a slow onset inhibition with 1 μM daunorubicin. We apply the term "activation phase" to the period within ~3-8 min after daunorubicin application when P_o was first enhanced compared to preceding "control" levels before drug application and the term "inhibitory phase" as periods between ~11 and 13 min with ≥2.5 μM when P_o was depressed compared to initial levels.

Channel open probability (P_o) with daunorubicin (P_{oD}) relative to the control (P_{oC}, before drug addition) for each channel was calculated and average relative P_o increased significantly

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after incubations with ≥500 nM daunorubicin (Fig. 3E). Concentrations >1 μM produced a significant inhibition at longer times. The relative P_o during the inhibition phase was significantly lower than the previously activated P_o, and significantly lower than control activity (Fig. 3F). The channels remained inhibited for the lifetime of the bilayer (Fig. 3D).

Reversibility of daunorubicin-induced activation and inhibition. To determine if the effects of daunorubicin on RyR2 were reversible, we examined the effect of perfusing the *trans* chamber with *trans* solution lacking daunorubicin. Disulphide formation cannot be reversed by removing the oxidizing reagent, although it can be reversed by addition of reducing reagents. Thus recovery after perfusion of the *trans* chamber (removal of daunorubicin) would indicate that the effect had not been due to oxidation of free –SH groups to –S-S- moieties. We found that perfusion after activation by 1 μM daunorubicin was followed (after 4.0±0.8 min) by a reduction in channel activity to control levels that were maintained for the duration of the experiment (Fig. 4A&B). This washout reversibility suggested that the increase in activity was likely to be due either to the drug binding to RyR2 or to transient redistribution of electrons in the presence of the drug without chemical modification (Marinov et al., 2007), as opposed to the oxidation of free –SH groups to form a -S-S-. Many non-thiol reagents have a redox potential and can also alter RyR2 function by electron redistribution within free -SH groups without making or breaking disulphide bonds (Marinov et al., 2007; Pessah et al., 2002). The effects of electron redistribution are reversed by removing the agents or by adding oxidizing or reducing reagents to normalize the redox potential of the environment surrounding the free –SH group. The washout experiments do not distinguish between cytoplasmic, transmembrane or luminal binding/modification sites because daunorubicin is lipid soluble and equilibrates across the bilayer and could therefore bind to sites located on the luminal, intramembarne or cytoplasmic face of the RyR2 complex. Daunorubicin would re-equilibrate across the bilayer after perfusion of the *trans* chamber and the concentration in the lipid and cytoplasmic compartments would be similarly reduced.

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To determine whether daunorubicin increased RyR2 activity by binding to the protein or by causing a transient redistribution of electrons, we examined drug binding to RyR2. Daunorubicin was coupled to CnBr-activated sepharose and incubated with purified RyR2. The eluate was electrophoresed and following western blot the RyR2 was detected with an anti-RyR antibody. The strong immunostaining (Fig. 4E) indicated that daunorubicin bound specifically to the RyR2 protein. After preincubating RyR2 with CNBr-activated sepharose to eliminate non-specific binding, the RyR2 bound strongly to the daunorubicin coupled CNBr-activated sepharose (data not shown). RyR2 did not bind to activated sepharose in the absence of daunorubicin (Fig. 4E).

In contrast to daunorubicin-induced activation, inhibition by 2.5 μM daunorubicin was not relieved by perfusion of the *trans* chamber (Fig. 4C&D). Thus daunorubicin-induced inhibition is likely to be due to either a chemical modification such as oxidation of free –SH groups to –S-S- bonds, which cannot be reversed (reduced) simply by drug removal, or to ligand binding to a high affinity site or an inaccessible site that becomes buried after binding.

DTT protection of free –SH groups and reduction of -S-S-. The potential role of –SH modification was examined using DTT, a strong reducing agent used to protect free thiols from oxidation to –S-S- groups and from electron withdrawal, or to reduce –S-S- bonds (Cleland, 1964; Eager et al., 1997). Prevention or reversal of either activation or inhibition by DTT would indicate effects due to redox modification of free thiol groups. DTT at 1 mM prevents RyR2 oxidation (Eager and Dulhunty, 1999; Eager et al., 1997) and its addition to the *cis* or *trans* chamber can indicate the location of the reactive –SH groups as DTT is not highly lipid soluble at pH 7.4 and does not cross the bilayer in significant amounts. Since the thiol groups of DTT have pKa values of 9.1 and 10.2 (Zhang et al., 1991) and the pH of the bilayer solutions was buffered

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to 7.4, DTT would be predominantly in its water soluble, ionized form (White, 1999) and would be unable to easily cross the lipid bilayer (Rang, 2007; White, 1999).

DTT in the trans solution. DTT (1 mM) added to the *trans* solution before 10 μM daunorubicin (Fig 5A&B) did not alter RyR2 activity or prevent the usual increase and then decrease in channel activity. Similarly, 1 mM DTT added to the *trans* chamber after daunorubicin-induced inhibition did not reverse the inhibition (Fig. 5C&D). Therefore, if -SH groups are modified to alter RyR2 activity, they are not located on the SR luminal side of the RyR2 complex.

DTT in the cis solution. DTT in the *cis* solution did not alter activation by 10 μM daunorubicin, but did prevent the inhibitory action of the drug (Fig. 6A). Channel activity increased and remained greater than control for up to 25 min after 10 μM daunorubicin addition (Fig. 6B). This result suggests that the inhibitory effect of daunorubicin is caused by oxidation of free thiol groups on the cytoplasmic domain of the RyR2 complex. The persistence of activation with DTT in the *cis* or the *trans* solution indicated that activation was not due either to oxidation of free –SH groups to –S-S- or to electron withdrawal effects. Surprisingly, channel activity remained at very low values when DTT was added to the *cis* chamber after inhibition by 10 μM daunorubicin (Fig. 6C&D), indicating that DTT did not reverse inhibition once it had been established. This apparent contradiction might be explained if the –S-S- became buried in the protein after its formation and were not accessible to DTT (see Discussion).

N-Ethylmaleimide (NEM) probe for modification of free sulphydryl groups. The results thus far show two actions of daunorubicin on the RyR2 channel complex. Firstly, an activation that is not dependent on redox modification and is most likely the consequence of a direct interaction between daunorubicin and the RyR2 complex. The second delayed inhibitory effect appears to be redox sensitive in that it can be prevented if DTT is present in the cytoplasmic solution before

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daunorubicin is added. To further explore the involvement of free –SH groups, we modified (alkylated) free –SH groups on the RyR2 complex with NEM before adding daunorubicin.

NEM alone influenced RyR2 channel activity. P_o fell progressively for 30 min following addition of 10 mM NEM to the *cis* solution (Fig. 7A). NEM is highly lipid soluble (Abbott and Schachter, 1976) and presumably equilibrated across the bilayer to alkylate –SH groups on luminal, cytoplasmic and transmembrane parts of the RyR2 complex. When 10 μM daunorubicin was added ~9 min after NEM (Fig. 7B), the decline in activity seen with NEM alone appeared to be halted. Channel activity remained less than control, but did not decline to levels seen with exposure to NEM alone (Fig. 7A). To evaluate the effect of daunorubicin in the presence of NEM, P_o with daunorubicin was normalized to P_o in NEM prior to daunorubicin addition (NEM control) in each channel and average data shown in Fig. 7C. This normalization procedure facilitated interpretation of the effects of daunorubicin alone on the NEM background, by removing the specific effect of NEM. Relative P_o increased above the NEM control after 10 min and remained greater than the NEM control for the next 20 min. The increase in P_o was reminiscent of the activation phase seen in the absence of NEM (Figs. 3-6 above).

Relative P_o during the activation and inhibitory phases following 10 μM daunorubicin application in normal and NEM-alkylated channels are compared in Fig. 7D. There is a significant increase in relative P_o during the activation phase in both cases with no significant difference between the relative increases in activity. The significant fall in P_o to less than control levels in the inhibition phase in normal channels is not seen in the NEM-modified channels, where relative P_o in the inhibitory phase was not significantly different from that in the activation phase. There was a trend towards a decline in P_o (Fig. 7C&7D) which may have been due to a residual inhibitory effect of daunorubicin, but was more likely due to continued NEM action shown in Fig. 7A. Notably, relative P_o during the inhibitory phase in NEM modified channels did

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not fall below the control level recorded before daunorubicin application. The difference between relative P_o in the normal and NEM-modified channels in the inhibitory phase was significant, thus the normal daunorubicin-induced inhibition appears to have been largely prevented by NEM-alkylation. This is consistent with the possibility that free –SH groups that are normally oxidized by daunorubicin and produce channel inhibition were modified by NEM and no longer available to be oxidized by daunorubicin.

Influence of CSQ2 on daunorubicin–induced changes in RyR2 activity. Since anthracyclines bind to CSQ2 (Kim et al., 2005) and CSQ2 modifies RyR2 activity (Wei et al., 2009), the effects of daunorubicin on RyR2 may have been mediated by daunorubicin binding to CSQ2 which remains associated with RyR2 channels when they are incorporated into the bilayer with SR vesicles (Beard et al., 2002; Wei et al., 2009). To determine whether CSQ2 influenced the response of RyR2 to daunorubicin, endogenous CSQ2 was selectively dissociated from the RyR/triadin/junctin complex by exposing the luminal side of native RyR2 channels to a high ionic strength solution (500 mM Cs^+) following channel incorporation into lipid bilayers (Beard et al., 2002). A decrease in activity associated with CSQ2 dissociation from RyR2 was observed (Wei et al., 2009) and then the *trans* chamber was perfused to remove dissociated CSQ2 and return the ionic strength to the usual 250 mM. The RyR2 channels lacking CSQ2 responded to 2.5 μM daunorubicin in a similar manner to native RyR2s containing CSQ2 (Fig. 8). An initial increase in channel activity (from a P_o of 0.06 ± 0.01 to 0.15 ± 0.03) was followed by a decline in P_o to 0.05 ± 0.01. Relative P_o in the activation phase was similar in normal channels and channels lacking CSQ2 (Fig. 8). Relative P_o fell significantly in the inhibitory phase in the absence of CSQ2, but was not significantly less than control and was significantly greater than the relative P_o during the inhibitory phase in normal channels. Thus the absence of CSQ2 reduced the magnitude of daunorubicin-induced inhibition.

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Although the absence of CSQ2 had no significant effect on the degree of activation induced by daunorubicin, the time taken to reach maximum activity was significantly longer than in RyR2 channels from which CSQ2 had not been dissociated (Table 1). The time to reach a minimum during the inhibitory phase of daunorubicin action also tended to be longer in the absence of CSQ2, but the change was not statistically significant. These results suggest that daunorubicin's interactions with RyR2 are modulated by CSQ2, but that the interactions do not primarily depend on daunorubicin's association with CSQ2. Daunorubicin's interaction with RyR2 may be modified by changes in the channel induced by CSQ2 binding to triadin and/or junctin.

Daunorubicin could influence Ca^{2+} release from cardiac SR by modifying the Ca^{2+} binding capacity of CSQ2 and hence the amount of Ca^{2+} stored in the SR. We found that 2.5 μM daunorubicin reduced the capacity of CSQ2 for Ca^{2+} binding to >70% of that in the absence of the drug at all concentrations of Ca^{2+} tested (Table 2). It is not clear from the present experiments how the effects on Ca^{2+} binding capacity may translate into alterations in the way CSQ2 regulates Ca^{2+} release through RyRs, but is likely to reduce the amount of Ca^{2+} available for release during systole.

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DISCUSSION

We show two separate actions of the chemotherapeutic agent daunorubicin on RyR2 channels, with novel observations on the nature, time and concentration dependence of these effects. We show for the first time that daunorubicin-induced activation of RyR2 is not due to redox modification, but likely follows daunorubicin binding to RyR2, which we also directly demonstrated for the first time. The results indicate that the slower inhibitory phase of daunorubicin's action that is mediated by -S-S- formation at a site or sites accessible from the cytoplasmic side of RyR2. Unexpectedly, CSQ2 binding to RyR2 modulates the effects of daunorubicin on the channel, but the effects are not due to daunorubicin binding to CSQ2. However daunorubicin may reduce Ca^{2+} release through RyR2 by reducing the Ca^{2+} binding capacity of CSQ2 and hence the Ca^{2+} load in the SR.

The results do not exclude the possibility that the effects of daunorubicin on RyR2 are mediated by influences of the drug on associated proteins which modulate RyR2 activity, although preliminary data indicates that purified RyR2 channels respond to daunorubicin in the same way as the native channels.

The activation phase. The daunorubicin-induced increase in activity was reversed when the drug was removed, which suggested that the increased activity was due either to drug binding or to transient electron redistribution within reactive thiol groups (Marinov et al., 2007; Pessah et al., 2002) as distinct from oxidation of thiols to disulphides (Eager and Dulhunty, 1999). The increase in P_o was observed more quickly after application at higher daunorubicin concentrations and apparently saturated with 1 μM daunorubicin. The saturation may have been due the onset of inhibition masking further increases in activity. However activity did not increase beyond this maximum when inhibition was prevented either by DTT in the *cis* chamber or by NEM treatment, suggesting that the maximum response was achieved with 1 μM daunorubicin. The activation site could not be identified as being on the cytoplasmic, transmembrane or luminal domain of the

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RyR2 complex as the hydrophobic drug would have equilibrated rapidly across the bilayer and could bind to a hydrophobic pocket within the RyR2 protein complex, either within membrane spanning regions, luminal residues or microdomains in the cytoplasmic assembly.

It should be noted oxidation of "exposed" -SH groups by low concentrations of oxidants leads to RyR2 activation. When the oxidant concentration increases, oxidation of less accessible -SH groups leads to inhibition and both activation and inhibition are prevented by DTT (Eager and Dulhunty, 1999; Eager et al., 1997). That we did not see oxidation-induced activation in the present experiments is curious. One possibility is that the anthracyclines bind to RyR2 near the "exposed" SH groups and prevent their oxidation to disulphides, perhaps by sterically hindering the required close interaction between two –SH groups.

The inhibitory phase. Inhibition was not reversed by drug washout, indicating that it either depended on oxidation of free –SH goups to -S-S-, or that the inhibition depended on binding which was very high affinity or that the site was inaccessible to the bulk solution. The fact that inhibition was prevented by the presence of DTT and by NEM-modification indicated that it depended on -SH oxidation. Although DTT is weakly lipid soluble at pH ~7.4 (see Results and (Rang, 2007; White, 1999; Zhang et al., 1991)), it does not cross the bilayer within in the duration of a bilayer experiment and is thus useful for studying the sidedness of the inhibitory interaction (Eager and Dulhunty, 1999). Since DTT in the *cis*, but not *trans*, solution prevented inhibition, the –S-S- was likely formed on the cytoplasmic side of the channel complex, or in a region accessible to the cytoplasmic solution. Other redox modifications such as S-glutathionylation or S-nitrosylation (Hidalgo and Donoso, 2008), or reactions involving reactive oxygen species produced by redox cycling of the daunorubicin quinone moiety (Menna et al., 2007; Minotti et al., 2004), would not be significant in our system because the substrates and/or necessary mitochondrial enzymes are not present in the SR vesicle preparation.

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Curiously, although DTT in the *cis* solution prevented the inhibition of RyR2 with daunorubicin, addition of DTT to the *cis* solution after inhibition had been established failed to restore channel activity. There are numerous examples of DTT preventing disulphide formation but not reducing the disulphide (Eager et al., 1997)(Terentyev et al., 2008). This apparent contradiction is explained by the –S-S- groups becoming buried in the protein and inaccessible to the solution.

CSQ2 as an anthracycline target. The interaction between daunorubicin and RyR2 did not depend on CSQ2 because both the activation and inhibitory phases were seen when CSQ2 was either bound to RyR2 and after CSQ2 dissociation from RyR2. However CSQ2 did modify rate of activation and the extent of inhibition. We speculate that CSQ2 binding may cause a conformational change in the RyR2, exposing the daunorubicin activation site and allowing faster activation by daunoruicin. CSQ2 binding to junctin and/or triadin for example may induce secondary conformational changes in RyR2 which expose the daunorubicin binding site. The lesser inhibition of RyR2 in channels lacking CSQ2 could be explained if CSQ2 removal altered RyR2 structure in a manner that reduced the impact of disulphide formation on channel gating.

Daunorubicin binding to CSQ2 reduces its Ca^{2+} binding capacity. As CSQ2 polymers have a higher Ca^{2+} binding capacity than monomers or dimers, daunorubicin might prevent CSQ2 polymerization and thus lower its Ca^{2+} binding capacity. However CSQ2 (in contrast to CSQ1) is monomeric at physiological ionic strength (~150 mM) and Ca^{2+} concentrations (~1 mM) (Park et al., 2005; Wei et al., 2009). Thus the reduced Ca^{2+} binding capacity is unlikely to be linked to polymerisation, but it could be associated with some unfolding of the monomer induced by daunorubicin binding.

Effects of altered RyR2 activity on SR function and heart failure. Anthracyclines cause severe disruption of SR Ca^{2+} handling, depleting the SR of its Ca^{2+} load and causing cytoplasmic Ca^{2+}

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overload (Holmberg and Williams, 1990; Shadle et al., 2000; Shan et al., 1996). The elevated cytoplasmic Ca^{2+} triggers delayed after depolarisations (DADs), tachycardia and arrhythmias which are symptomatic of acute anthracycline-induced cardiotoxicity and heart failure (Gyorke and Terentyev, 2008; Shan et al., 1996). Prolonged Ca^{2+} overload also leads to some of the chronic effects of anthracycline treatment and heart failure including cardiomyocyte apoptosis and necrosis (Jessup and Brozena, 2003; Kumar et al., 2001; Minotti et al., 2004; Nakayama et al., 2007; Zucchi and Ronca-Testoni, 1997).

Our results indicate that the RyR2-dependent contribution to disruption of Ca^{2+} handling by daunorubicin would depend on the accumulated concentration of the drug in the myocytes. Increased RyR2 activity with low concentrations would contribute to DADs and arrhythmia in the early acute phase of daunorubicin toxicity. The inhibitory phase would become apparent after longer exposures and with higher accumulated drug concentrations. Inhibition of RyR2 channels can also lead to ventricular fibrillation through the generation of early after-depolarizations (EADs) when the action potential is prolonged (Bers, 2002; Gomez and Richard, 2004). EADs occur when L-type Ca^{2+} channels inactivation is slowed as a result of reduced Ca^{2+} release through RyR2 and a consequently lower than normal cytoplasmic Ca^{2+} concentration (Bers, 2002). This is seen in heart failure when Ca^{2+} stores are depleted or with loss-of-function mutations in RyR2 (Gomez and Richard, 2004) and could occur during the inhibitory phase of daunorubicin action. The reduced Ca^{2+} binding capacity of CSQ2 in the presence of daunorubicin would further contribute to EADs, by reducing SR Ca^{2+} load and Ca^{2+} available for release. The extent that daunorubicin metabolites alter RyR2 function and contribute to the effects of the parent compound remains to be determined.

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To conclude, we describe a biphasic modulation of single RyR2 channels by clinically relevant concentrations of daunorubicin. Channels are initially activated and then inhibited by the drug. We provide evidence that the activation is caused by a redox-independent interaction between daunorubicin and RyR2, while inhibition is due to the oxidation of thiol groups. Daunorubicin reduces the Ca^{2+} binding capacity of CSQ2 but this does not mediate the effects of daunorubicin on RyR2. Our study provides important new information on the functional consequences of anthracycline binding to two of its targets in the SR of cardiomyocytes which may help in the eventual design of anthracyclines that do not detrimentally alter Ca^{2+} signalling in the heart.

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AUTHORSHIP CONTRIBUTION

Participated in research design: Hanna A.D., Beard N.A., Dulhunty A.F.

Conducted experiments: Hanna A.D., Beard N.A., Janzcura M., Cho E.

Performed data analysis: Hanna A.D., Beard N.A., Dulhunty A.F.

Wrote or contributed to the writing of the manuscript: Hanna A.D., Beard N.A., Dulhunty A.F.

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Footnotes

* these authors made equal senior author contributions to the work

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FIGURE LEGENDS

Figure 1. Measurement of Ca^{2+} release from cardiac SR vesicles. The recordings show optical density as a function of time after vesicle addition during a Ca^{2+} release experiment. An increase in optical density indicates an increase in extravesicular [Ca^{2+}] detected by antipyrylazo III absorbance at 710nm. Cardiac SR vesicles were first added to the cuvette. [Ca^{2+}] increased with vesicle addition and then fell as Ca^{2+} was taken up into the vesicles upon activation of SERCA. The SR was then loaded with four additions of 7.5 μM Ca^{2+}. Thapsigargin (300 nM) was added to block SERCA, and then 100 μM daunorubicin in **A** or 5 mM caffeine in B to stimulate Ca^{2+} release. Ruthenium red was then added followed by the Ca^{2+} ionophore which allowed Ca^{2+} remaining in the vesicles to equilibrate with the extravesicular solution. The final large increase in [Ca^{2+}] with ionophore indicates (a) that Ca^{2+} had not been maximally released from the vesicles and (b) the antipyralazo response was not saturated. The arrows at the end of each trace indicate the total amount of Ca^{2+} released from the level at the time of thapsigargin addition (broken line) to the maximum release after ionophore addition. The total release is also indicative of the total amount accumulated by the SR. In **A,** the immediate effect of daunorubicin on Ca^{2+} release was measured. In **B,** the effect of 30 min incubation in daunorubicin on caffeine-induced Ca^{2+} release was evaluated. The shaded areas in **A** and **B** encircle the regions of the experiment shown in Fig 2A and Figs 2C & 2D respectively.

Figure 2. Effects of daunorubicin on Ca^{2+} release from cardiac SR. **A**) Daunorubicin added at concentrations of ≥50 μM (using the protocol in Fig. 1A), had an immediate action in increasing the rate of Ca^{2+} release. Daunorubicin concentration is shown to the left of each trace and the initial rate of Ca^{2+} release is shown to the right. **B**) Average rates of Ca^{2+} release with indicated concentration of daunorubicin. Note the comments in the text regarding the intentionally high daunorubicin concentrations used to evoke an immediate effect of daunorubicin addition. The inset in **B** shows the average initial rates of Ca^{2+} release (open circles)

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and the rate of Ca^{2+} release measured 10 min (closed circles) after addition of vehicle (0 μM daunorubicin) or 10 μM daunorubicin in one set of experiments in which daunorubicin-induced release was monitered for ~11 min. (**C & D**) Caffeine-induced Ca^{2+} release from vesicles equilibrated for ~30 min in daunorubicin (20 min before the start of the experiment plus an additional 10 min during the Ca^{2+} loading steps). Daunorubicin concentration is shown to the left of each trace. **C**) Records showing the effect of equilibrating SR vesicles in 10 to 50 nM daunorubicin on caffeine-induced Ca^{2+} release. **D**) Records showing the effect of equilibrating SR vesicles 0.1 to 10 μM daunorubicin on caffeine-induced Ca^{2+} release. **E**) Average relative rates of Ca^{2+} release after addition of thapsigargin (grey symbols) and caffeine-induced Ca^{2+} release (black symbols) after incubation with indicated concentrations of daunorubicin. The dotted lines under the records in **A, C and D** indicate the slopes used in determining the rates of release shown in **B** and **E.** The asterisks (*) indicate a significant difference from the control rates of release measured in the absence of daunorubicin (n = 5 for all concentrations). In E, the asterisks (*) over data obtained after incubation with of 5 and 10 nM daunorubicin indicate a significant difference in caffeine-induced Ca^{2+} release only.

Figure 3. The activity of RyR2 is modified by daunorubicin. A) & B) 3s traces of native RyR2 channel activity at +40mV. Channels open upward from zero current (c, continuous line) to maximum open conductance (o, broken line). **(A)** *Top panel* – Control recording; *middle and bottom panel* – after the addition of 1 μM daunorubicin to the *trans* chamber, maximal RyR2 activity was measured at 11 min in this channel, with no further change in activity recorded for 20 min after daunorubicin addition. **B)** *Top panel* – Control recording from different native RyR2 channel; *middle and bottom panel* – after the addition of 10 μM daunorubicin to the *trans* chamber, maximal RyR2 activation was measured at 8 min, and maximal RyR2 inhibition was recorded 16 min after daunorubicin addition. **C)** & **D)** P_o was measured every 10s throughout

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experiments with 1 μM daunorubicin (**C**) or 2.5 μM daunorubicin in (**D**) at +40 mV (dark grey bins) and -40 mV (light grey bins). **E**) & **F**) Combined data from measurements of P_o at +40 mV and -40 mV (n = 8 – 12 at each concentration). Data is presented as average relative P_o (log rel P_o, i.e. $\log_{10}P_{oD}$ -$\log_{10}P_{oC}$ (see Methods). Average relative P_o during the activation phase is shown in **E**) and average relative P_o during the inhibitory phase shown in **F**). The asterisk (*) indicates a significance difference from the control P_o recorded before adding daunorubicin. The ($^{\#}$) indicates a significant difference between P_o during the activation and inhibitory phase.

Figure 4. Daunorubicin-induced activation is reversible upon washout. A) & **C)** 3s traces of native RyR2 channel activity at +40mV. Channels open upward from zero current (c, continuous line) to maximum open conductance (o, broken line). **A)** *Top panel* – Control recording; *middle panel* – after the addition of 1 μM daunorubicin to the *trans* chamber, maximal activity was measured (8 min after daunorubicin addition); *bottom panel* –after the *trans* chamber was perfused with control recording solution. **C)** *Top panel* – Control recording; *middle panel* – after the addition of 2.5 μM daunorubicin to the *trans* chamber, maximal RyR2 inhibition was measured (14 min after daunorubicin addition) ; *bottom panel* – washout, after the *trans* chamber was perfused with control solution. **B)** & **D)** Combined data from measurements of P_o at +40 mV and -40 mV (n = 9 in **B;** n = 12 in **D).** Average P_o before daunorubicin addition, during the activation phase with 1 μM daunorubicin and then after washout of 1 μM daunorubicin are shown in **B**). Average relative P_o before daunorubicin addition, during the inhibitory phase with 2.5 μM daunorubicin and then after washout of 2.5 μM daunorubicin are shown in **D**). The asterisk (*) indicates a significance difference from the control P_o recorded before adding daunorubicin. The ($^{\#}$) indicates a significant difference between P_o in the presence of daunorubicin and after daunorubicin washout. **E)** Western blot showing RyR2 bound to CNBr-activated sepharose (lane 1) and to daunorubicin coupled CNBr-activated sepharose (lane 2). **F)**

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Average density of RyR2 of control (bin 1) and daunorubicin coupled CNBr-activated sepharose (bin 2). Results are from three experiments, including one where RyR2 was pre-incubated with CNBr-activated sepharose. The asterisk (*) indicates a significance difference in densitometry units between the control and daunorubicin coupled CNBr-activated sepharose.

Figure 5. Effect of DTT in the *trans* solution on the action of daunorubicin –A) & **C)** 3s traces of native RyR2 channel activity at +40mV. Channels open upward from zero current (c, continuous line) to maximum open conductance (o, broken line). **A)** *1st panel* – control activity; *2nd panel* - addition of 1 mM DTT to the *trans* solution; *3rd and 4th panels* –10 min (3rd panel) and 25 min (4th panel) after the addition of 10 μM daunorubicin to the *trans* solution. **C)** *1st panel* – Control activity; *2nd and 3rd panel* - 6 min (2nd panel) and 12 min (3rd panel) after the addition of 10 μM daunorubicin to the *trans* solution; *4th panel* – after the addition of 1 mM DTT to the *trans* solution. **B)** & **D)** show average combined data for P_o at +40 mV and -40 mV (n = 14 in **B**) and n = 10 in **D)).** The asterisk (*) indicates a significant difference from the control P_o recorded before adding daunorubicin. The ($^{\#}$) indicates a significant difference between P_o during the activation and inhibitory phase of daunorubicin action.

Figure 6. Effect of DTT in the *cis* solution on the action of daunorubicin – A) & **C)** 3s traces of native RyR2 channel activity at +40mV. Channels open upward from zero current (c, continuous line) to maximum open conductance (o, broken line). **(A)** *1st panel* – control activity; *2nd panel* - after adding 1 mM DTT to the *cis solution*, 3rd *and 4th panels* – 10 min (3rd panel) and 25 min (4th panel) after adding 10 μM daunorubicin to the *cis* solution. **C)** *1st panel* – Control activity; *2nd and 3rd panel* - 5 min (2nd panel) and 13 min (3rd panel) after adding 10 μM daunorubicin to the *cis* solution; *4th panel* – after adding 1 mM DTT to the *cis* solution. **B)** & **D)** show average combined data for P_o at +40 mV and -40 mV (n = 8 in **B** and n = 10 in **D).** The ($^{\#}$)

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indicates a significant difference between P_o during the activation and inhibitory phase of daunorubicin action.

Figure 7. Effect of NEM alkylation on the actions of daunorubicin. **A**) to **C**) Combined data from measurements of P_o at +40 mV and -40 mV. The effect of NEM alone is shown in **A**) (n = 6). Data in **A**) is presented as average relative P_o (log rel P_o, i.e. $\log_{10}P_{oN}$ -$\log_{10}P_{oC}$): P_o in NEM relative to P_o before adding NEM (see Methods). The effect of adding 10 μM daunorubicin after 9 min exposure to NEM is shown in **B**) and **C**) (n = 14). Data in **B**) is presented as P_o after adding daunorubicin relative to P_o at the start of the experiment before NEM addition (log rel P_o, i.e. $\log_{10}P_{oD}$ -$\log_{10}P_{oC}$). Data in **C**) is presented as P_o after adding daunorubicin relative to P_o in NEM before daunorubicin addition (log rel P_o, i.e. $\log_{10}P_{oD}$ -$\log_{10}P_{oN}$). Data in **D**) is presented as relative P_o after adding daunorubicin alone (relative to P_o prior to adding daunorubicin: log rel P_o, i.e. $\log_{10}P_{oD}$ -$\log_{10}P_{oC}$), or P_o after adding daunorubicin in the presence of NEM (relative to P_o in NEM prior to adding daunorubicin: log rel P_o, i.e. $\log_{10}P_{oD}$ -$\log_{10}P_{oN}$).

Figure 8. Daunorubicin effects on RyR2 do not depend on CSQ2. Combined data for P_o at -40mV and +40mV for native RyR2 channels (RyR2+CSQ2) (n = 13) and for RyR2 after dissociation of CSQ2 (RyR2-CSQ2) (n = 10). Data is presented as average relative P_o (log rel P_o, i.e. $\log_{10}P_{oD}$ -$\log_{10}P_{oC}$). The asterisk (*) indicates a significant difference from control activity before adding daunorubicin. The (#) indicates a significant difference between P_o during the activation and inhibitory phases of daunorubicin action. The ($^{@}$) indicates a significant difference between average P_o in native RyR2 and CSQ(-) RyR2 in the inhibitory phase.

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TABLES

Table 1. Time-dependent activation and inhibition of native RyR2 (containing CSQ2) and RyR2 after CSQ2 dissociation (CSQ(-) RyR2) by daunorubicin.

[Daunorubicin]	Time to activation phase (min)		Time to inhibition phase (min)	
	Native RyR2	CSQ(-) RyR2	Native RyR2	CSQ(-) RyR2
10 nM	15.0 ± 4.0	-	-	-
500 nM	7.9 ± 2.5	-	-	-
1 μM	8.1 ± 2.0	-	-	-
2.5 μM	3.0 ± 3.3	11.1 ± 1.3*	12.6 ± 4.3	17.6 ± 0.7
10 μM	4.0 ± 1.1	-	11.5 ± 1.9	-

Asterisk (*) indicates a significant difference between time to activation in native RyR2 (containing CSQ2) and RyR2 channels lacking CSQ2 (CSQ(-)RyR2). Dash (-) indicates daunorubicin concentrations not tested.

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Table 2. Ca^{2+} binding capacity of CSQ2 in the presence and absence of 2.5 μM daunorubicin.

$[Ca^{2+}]$ mM	Ca^{2+} binding capacity (nmol Ca^{2+}/mg purified CSQ2)	
	No daunorubicin	2.5 μM daunorubicin
0.1	131 ± 15	97 ± 9 *
1.0	168 ± 16	120 ± 11 *
1.5	194 ± 19	134 ± 14 *

Asterisk (*) indicates a significant difference between Ca^{2+} binding capacity of CSQ2 in the presence and absence of 2.5 μM daunorubicin (n=8-16).

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Figure 1

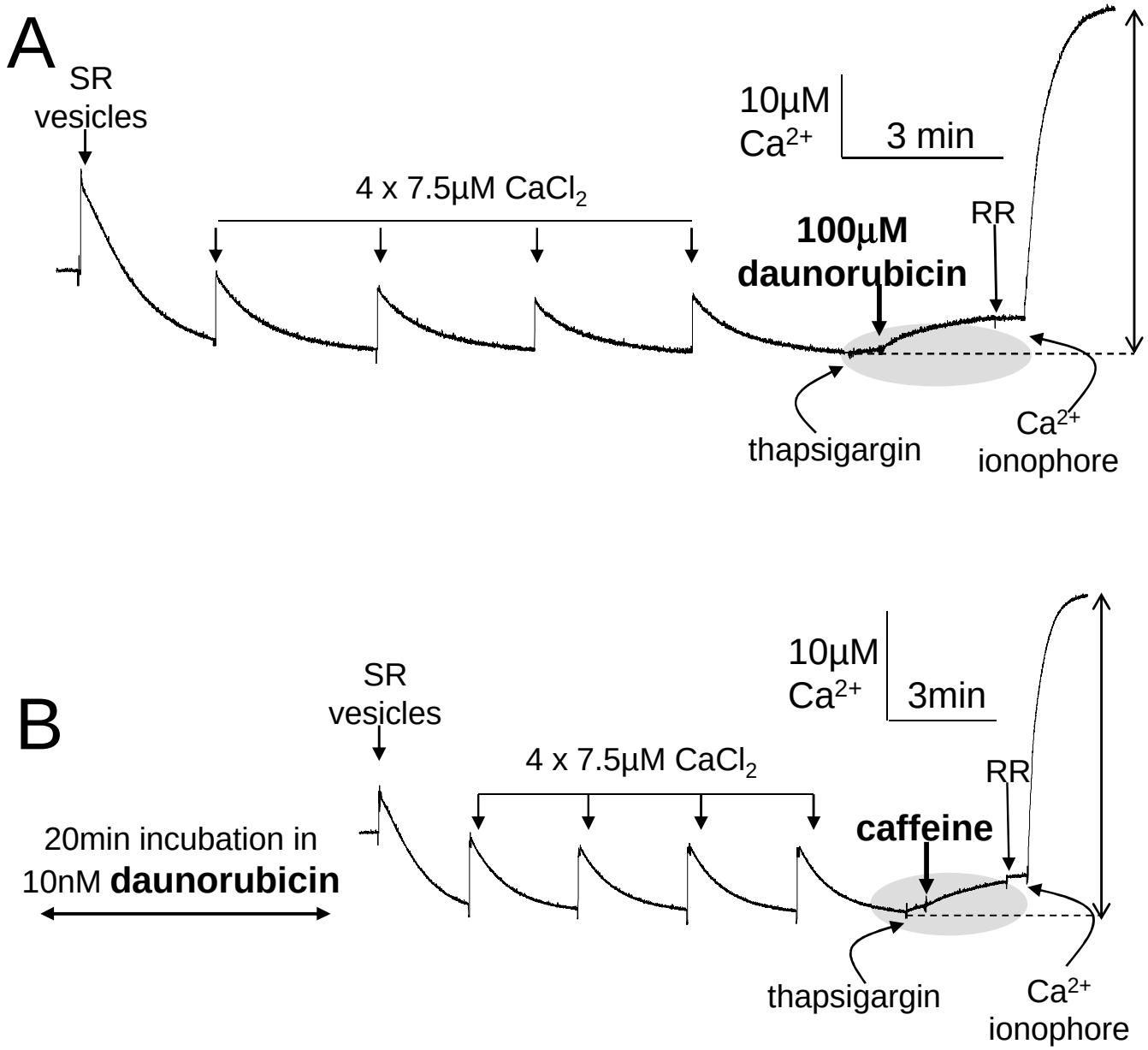

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Figure 2

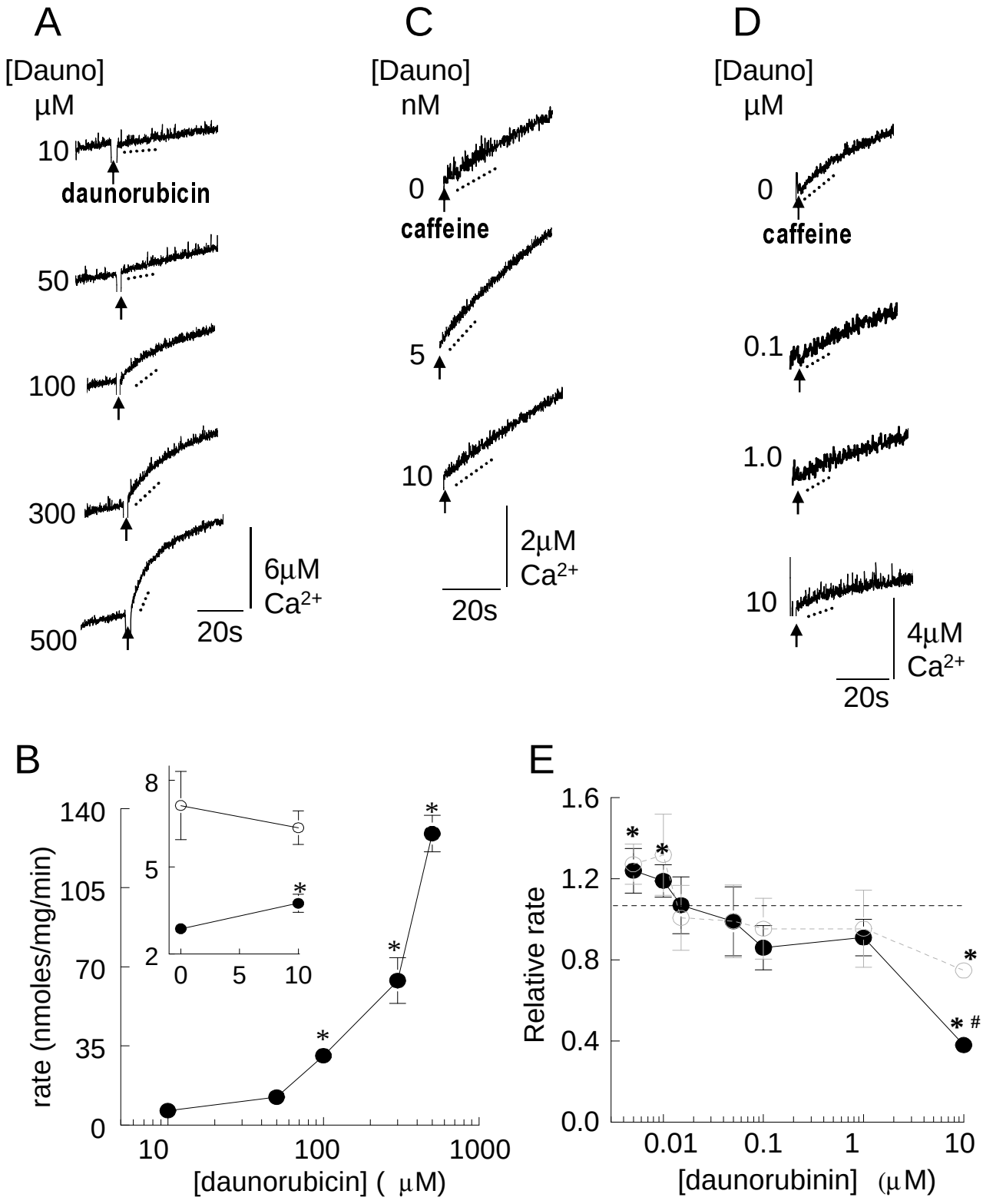

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Figure 3

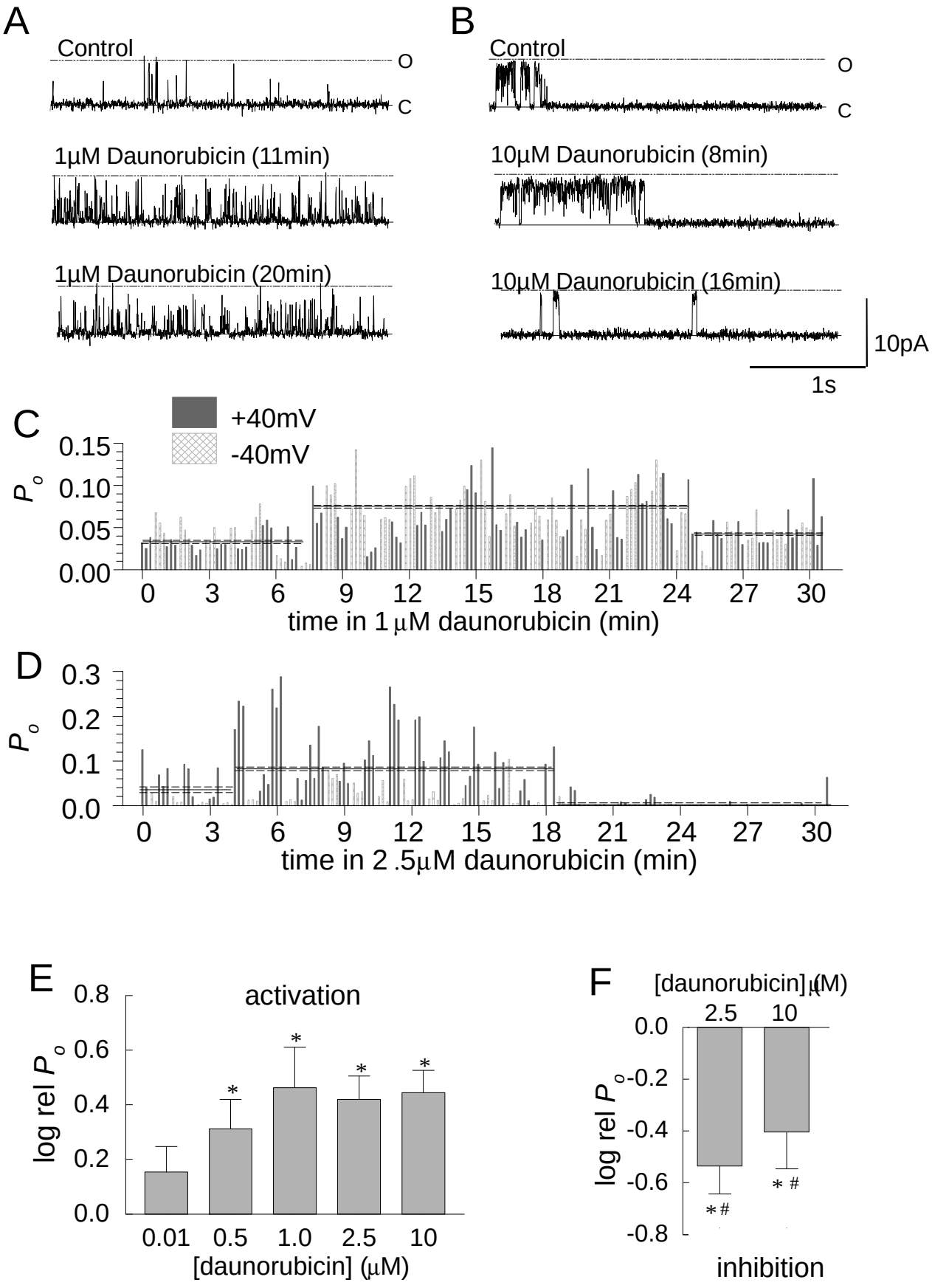

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Figure 4

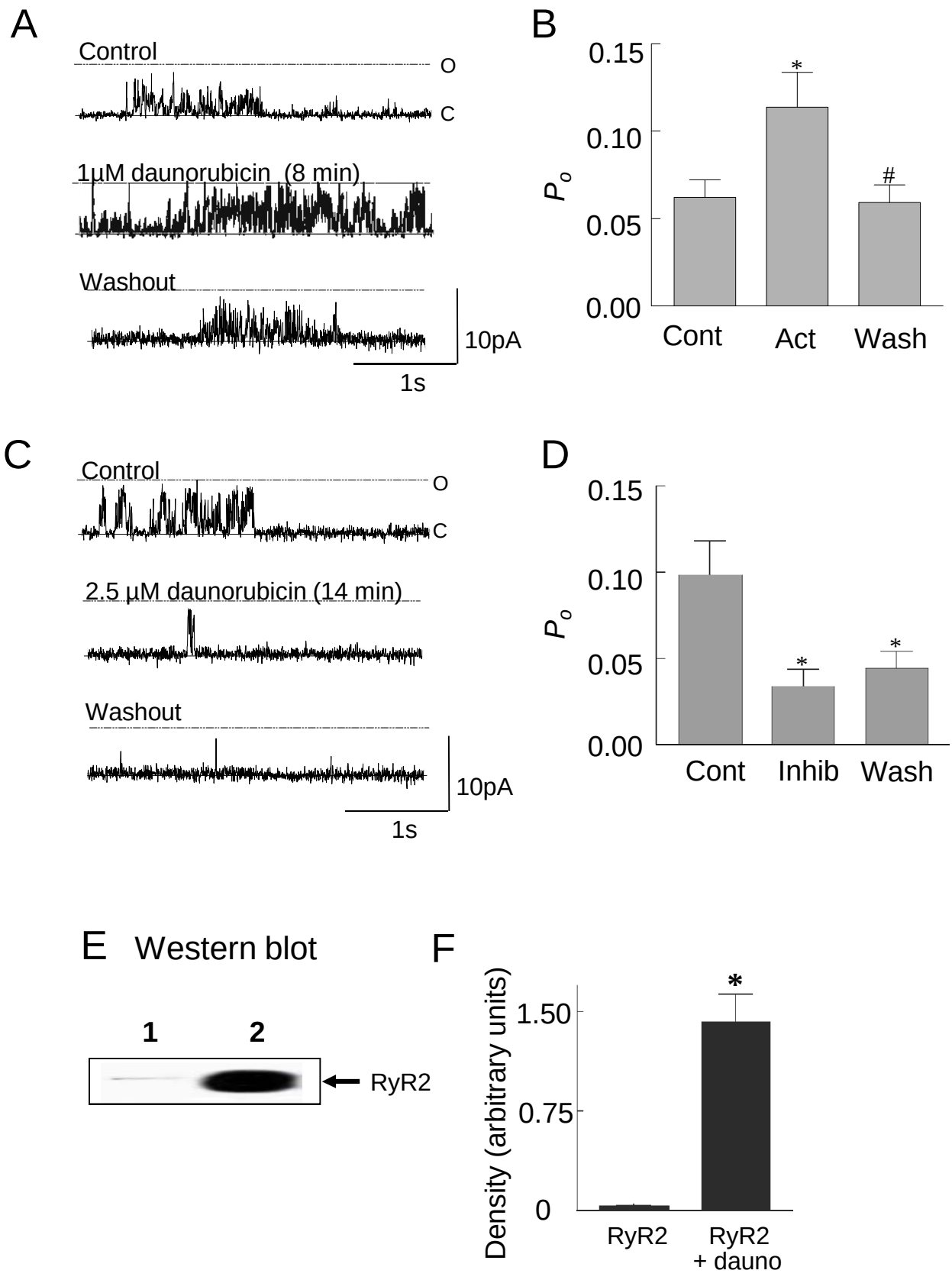

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Figure 5

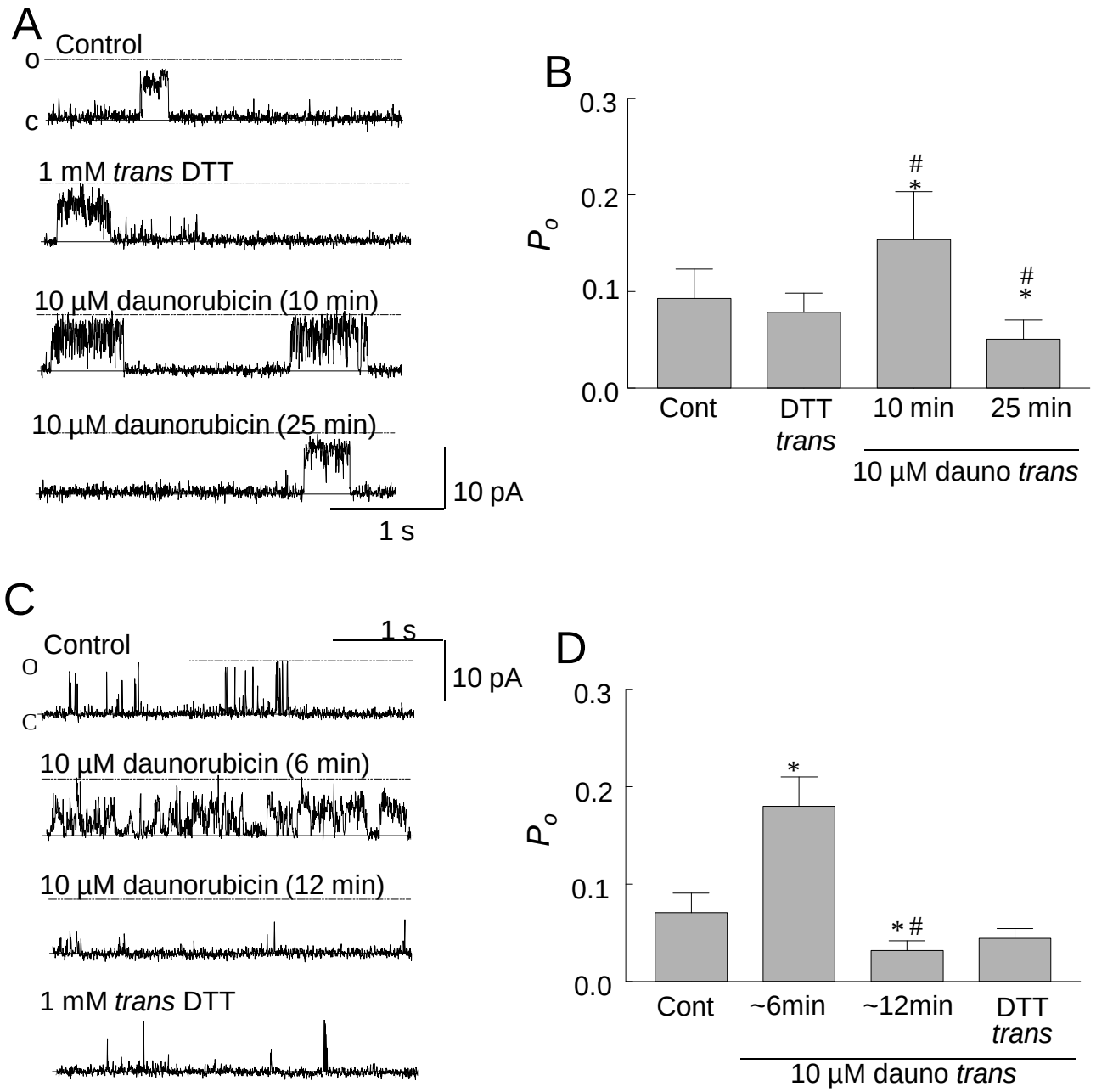

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Figure 6

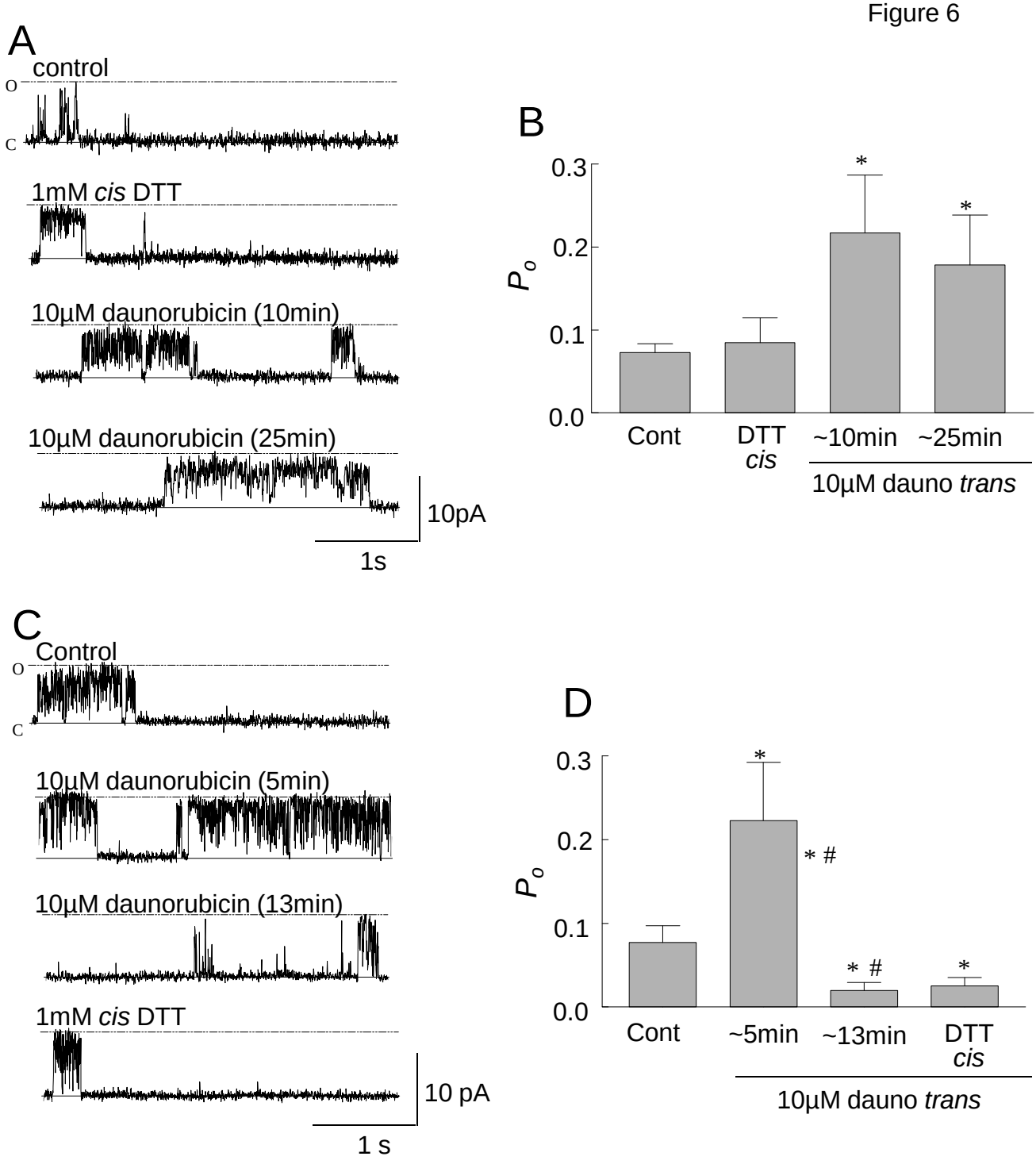

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Figure 7

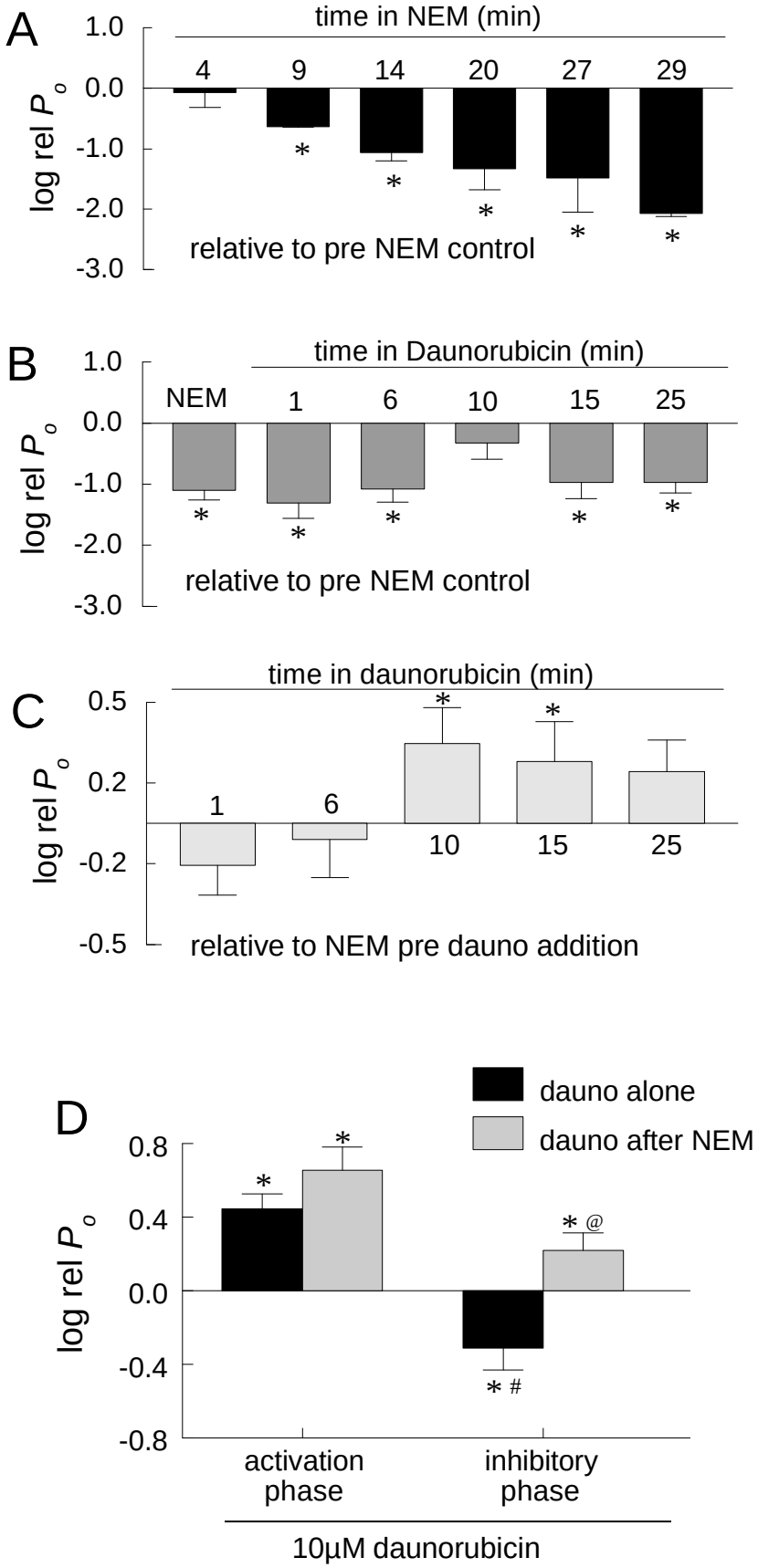

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Figure 8