Patterns of Interaction Between Anthraquinone Drugs and the Calcium-Release Channel From Cardiac Sarcoplasmic Reticulum

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We have studied the effects of clinically useful anthraquinones on the cardiac sarcoplasmic reticulum calcium-release channel. Micromolar concentrations of doxorubicin and other anthracyclines at the cytosolic face of the channel significantly and reversibly increase the open probability of single channels in artificial phospholipid bilayers. Lifetime analysis shows that anthracyclines and calcium are synergistic activators of the calcium-release channel. Radiolabeled ryanodine binding suggests that all the anthracyclines studied are equally potent as channel activators in vitro. Mitoxantrone, an anthracycinedione derivative, variably increases channel open probability at low (1–10 μM) concentrations. Higher concentrations are associated with the appearance of channel currents with lower amplitudes than the fully open state, and normal openings are reduced in frequency. At these concentrations, the interaction of mitoxantrone with the channel reduces the level of ryanodine binding. Abnormal function of the cardiac calcium-release channel will alter calcium handling within the myocyte and may be the basis for anthraquinone-related cardiotoxicity. (Circulation Research 1990;67:272–283)

Anthraquinones are highly effective chemotherapeutic agents with a wide spectrum of antitumor activity. Their clinical use is limited principally by a dose-dependent cardiotoxicity, which is produced both by anthracyclines (e.g., doxorubicin, epirubicin, and idarubicin) and by anthracyclenes (e.g., mitoxantrone), although the histological features produced by each group are reported to show significant differences. Among the anthracyclines, epirubicin and idarubicin are less cardiotoxic than doxorubicin. The subcellular effects of doxorubicin in the heart have been extensively investigated, but the mechanism of cardiotoxicity remains unclear.

Doxorubicin can undergo a reduction reaction to a semiquinone species that is a potential generator of oxygen-derived free radicals. It has been proposed that this might initiate generalized membrane system disruption through lipid peroxidation, leading to intracellular calcium overload. Abnormalities of the sarcoplasmic reticulum (SR) are characteristic early features of doxorubicin cardiotoxicity, and Zorzato et al. provided evidence of a specific interaction between the drug and the terminal cisternal regions of skeletal SR. They also demonstrated that this effect was not associated with the production of the semiquinone and proposed that doxorubicin selectively activated a calcium efflux pathway in the SR.

Working on skeletal muscle preparations, Abrams et al. extended these findings by showing that doxorubicin interacted specifically with the ryanodine receptor complex, which is part of the SR calcium-release channel (CRC). Ryanodine binds to the channel only in its open state, and doxorubicin was shown to increase [3H]ryanodine binding to heavy SR vesicles. In addition, doxorubicin and mitoxantrone were both found to be potent stimuli to the efflux of Ca2+ from preloaded vesicles. By incorporating skeletal heavy SR vesicles into artificial planar phospholipid bilayers and observing current fluctuations under voltage-clamped conditions, Nagasaki and Fleischer found that doxorubicin increased transmembrane current, providing additional evidence for direct activation of the CRC by the drug. They proposed that a similar effect in cardiac muscle would interfere with intracellular calcium homeostasis and could contribute to cardiac dysfunction.

In this report, we investigate the interactions between clinically useful anthraquinones and the
CRC from cardiac SR by studying the activity of single ion channels in phospholipid bilayers and radiolabeled ryanodine binding. The results show that the effects of drugs within the group are not homogeneous, which may relate to the different patterns of cardiotoxicity that are encountered in clinical practice.

Materials and Methods

Materials

[^3]H]Ryanodine (60 Ci/mmol) was purchased from New England Nuclear, Dupont, Stevenage, Hertfordshire, UK. Unlabeled ryanodine was purchased from Progressive Agri-Systems, Wind Gap, Pa. Doxorubicin, epirubicin, andidarubicin were generous gifts from Farmitalia Carlo Erba, St. Albans, Hertfordshire, UK, and mitoxantrone was the generous gift from Lederle Ltd., Gosport, Hampshire, UK. Phosphatidylethanolamine (bovine heart) was purchased from Avanti Polar Lipids, Pelham, Alabama. Aqueous counting scintillant was purchased from Amersham International, Amersham, Buckinghamshire, UK. All other chemicals were of AnalaR or best available grade from BDH Ltd. (Poole, Dorset, UK) or Sigma Chemical Co Ltd. (Poole, Dorset, UK).

Membrane Preparation

Sheep hearts were obtained from a local abattoir and transported directly to the laboratory in an ice-cold cardioplegic solution composed of (mM) NaCl 102, sodium lactate 29, KCl 20, MgCl2 16, and CaCl2 2. Heavy SR vesicles were prepared from left ventricular muscle using the method of Meissner and Henderson14 with minor modifications. Muscle was minced and then homogenized in 4 vol solution containing (mM) sucrose 300, phenylmethylsulfonyl fluoride (PMSF) 1, and PIPES/KOH 20, pH 7.4. The homogenate was centrifuged at 10,500g for 20 minutes in a Sorvall GSA rotor (Dupont Biotechnology, Stevenage, Hertfordshire, UK), and then the supernatant was resuspended at 90,000g for 60 minutes in a Sorvall A641 rotor. The pellet was resuspended in a solution containing (mM) KCl 400, MgCl2 0.5, CaCl2 0.5, EGTA 0.5, and PIPES/KOH 25, pH 7.0, with 10% (wt/vol) sucrose. This preparation was subfractionated on discontinuous sucrose gradients; the suspension was layered over similar salt solutions containing 20%, 30%, and 40% (wt/vol) sucrose and centrifuged at 140,000g for 120 minutes in a Sorvall AH629 rotor. The fraction sedimenting at the 30%/40% interface was collected and pelletted by centrifugation at 90,000g for 60 minutes in a Sorvall A641 rotor in 400 mM KCl and then resuspended in a solution containing 400 mM sucrose and 5 mM HEPES/Tris, pH 7.2. Vesicles were snap frozen in liquid N2 and stored at −70°C.


Heavy SR vesicles (60–100 μg protein) were incubated at 37°C for 90 minutes with 5 mM[^3]H]ryanodine in 1 ml buffered medium containing 1 M KCl, 5 μM PMSF, 1 mM EGTA, and 25 mM PIPES/KOH, pH 7.4; calcium was added to produce desired free calcium concentrations as determined by the method of Bers.13 Other additions to the medium are described for individual experiments. At the completion of the incubation, the medium was diluted with 5 ml ice-cold buffer and filtered through Whatman GF-B filters (Maidstone, Kent, UK) that had been presoaked in buffer. Filters were washed with three 5-mL aliquots of buffer and counted in 10 mL aqueous counting scintillant the following day. All incubations were performed in triplicate. Nonspecific binding was determined using matching control media to which 2.5 μM unlabeled ryanodine had been added; these counts (~1–5% of total) were subtracted from total binding to produce specific binding (see “Results”). Protein assays were performed using the modification of the Lowry method described by Markwell et al.16

Single-Channel Recordings

Experiments were performed at 22°C. Planar phospholipid bilayers, composed of phosphatidylethanolamine dispersed in n-decane at a concentration of 30 mg/mL, were painted across a 200-μm diameter hole in the styrene copolymer septum between two experimental chambers containing 50 mM choline chloride and 10 mM HEPES/Tris, pH 7.2. Vesicles were added to the designated cis chamber, and the solution was fortified with choline chloride to produce a 7:1 gradient across the membrane. Vesicle fusion was detected by the appearance of a chloride-selective conductance.17 Calcium channels were observed after perfusing the cis chamber (corresponding to the cytosolic side of the channel)18 with 250 mM HEPES/Tris, pH 7.4, and the trans chamber (corresponding to the SR luminal face of the channel) with 250 mM glutamic acid and 10 mM HEPES titrated to pH 7.4 with Ca(OH)2 ([Ca2+]76 mM). A single fusion event was typically associated with the incorporation of 0–3 calcium channels, and bilayers usually remained sufficiently stable to permit recording data for up to 30 minutes. The trans chamber was held at ground, whereas the cis chamber was voltage-clamped at different holding potentials. For experiments with cis-calcium greater than 10 μM, unbuffered solutions were used, and the Ca2+ was measured directly with a calcium electrode; for lower concentrations, solutions were buffered with 1 mM EGTA, and the free Ca2+ was calculated as described by Bers.15

Channel opening results in a flow of ions across the bilayer, which was amplified19 and recorded on FM tape. Single-channel data were displayed on a Hewlett-Packard 7475A plotter after digitization at 2 kHz using a PDP 11/73–based computer system (Indec, Sunnyvale, Calif.). Single-channel open probabilities and open and closed lifetimes were determined using 50% amplitude threshold analysis (Intracel, Cambridge, UK) of 3 minutes continuous
data digitized at 2 kHz after low-pass filtering at a front panel setting of 1 kHz with a four-pole RC-mode filter. Lifetimes are displayed in noncumulative histograms together with probability density functions obtained using the method of maximum likelihood.20 Lifetimes of less than 1 msec were not fully resolved and were therefore excluded from the fitting procedure, and a missed events correction was used before analysis.20,21 Fits to double and triple exponentials were compared using a likelihood ratio test.22,23

Results

Single-Channel Recordings: Anthracyclines

Figure 1 shows representative recordings of current fluctuations from a bilayer with a single calcium channel. The cis chamber contains 0.1 μM Ca2+ plus 1 mM ATP (Figure 1a); increased channel opening is seen after the addition of 10 μM doxorubicin to the cis chamber (Figure 1b). From another experiment at 10 μM cis-Ca2+ (Figure 2a), the addition of 100 μM trans-doxorubicin has no effect on channel openings (Figure 2b). The same channel is subsequently activated by 10 μM cis-doxorubicin (Figure 2c), and this effect is reversed after drug washout (Figure 2d). Epirubicin and idarubicin both produced qualitatively similar reversible channel activation.

The mechanism of the interaction between doxorubicin and the CRC was investigated using solutions in the cis chamber containing calcium as the only other activating ligand. Figure 3 shows representative recordings of current fluctuations from a bilayer with a single calcium channel in control medium containing 10 μM Ca2+ (Figure 3a) and after the addition of 10 μM and 25 μM doxorubicin (Figures 3b and 3c, respectively). Channel open probability (Po) rose with increasing doxorubicin concentration (Po 0.03, 0.09, and 0.75, Figures 3a–c, respectively); the Po of nearly all the channels studied under these conditions could be increased to near unity by further increasing the doxorubicin concentration (data not shown). Single-channel conductance was 98 pS in control media and was not affected by the addition of doxorubicin.

Figure 4 displays the lifetime analysis of recordings of channel activity from which the traces in Figure 3 were taken. This shows that the initial increase in Po is predominantly due to more frequent opening events, with a similar duration distribution to that of the control period; the most obvious change, therefore, is a shortening of closed times. As the doxorubicin concentration was increased, open events increased both in frequency and in duration. For the open times in Figures 4a and 4b, the most likely fits were obtained with double exponentials, whereas in Figure 4c, a triple exponential was required. All closed times were best fitted to triple exponentials.
Figure 3. Current fluctuations in a bilayer containing a single calcium channel with 10 \( \mu M \) \( Ca^{2+} \) cis before (panel a) and after the addition of 10 \( \mu M \) (panel b) and 25 \( \mu M \) (panel c) doxorubicin to the cis chamber. Channel openings are upward deflections, and current levels of the open and closed channel states are indicated by the dashed lines. All recordings were made with the cis chamber clamped at 0 mV relative to ground.

Figure 4. Left panel: Open and closed lifetime histograms and maximum likelihood fits for a single calcium channel with 10 \( \mu M \) \( Ca^{2+} \) cis before (panel a) and after the addition of 10 \( \mu M \) (panel b) and 25 \( \mu M \) (panel c) doxorubicin to the cis chamber. Maximum likelihood fits are obtained with probability density functions (shown by the solid curves) with the equation \( f(t) = a_1(1/s_1)\exp(-t/s_1) + \ldots + a_n(1/s_n)\exp(-t/s_n) \), where \( a \) and \( s \) refer to relative areas and time constants, respectively. Right panel: Values for \( a \) and \( s \) with open and closed lifetimes.
Figure 5 shows the effect of *cis*-doxorubicin at 100 
\( \mu M \) *cis*-Ca\(^{2+} \). Representative recordings of current fluctuations from a bilayer with a single calcium channel are displayed in control medium (Figure 5a) and after the addition of 5 \( \mu M \) and 10 \( \mu M \) doxorubicin (Figures 5b and 5c, respectively). \( P_o \) rose with increasing doxorubicin concentration (P, 0.12, 0.58, and 0.82, Figures 5a–c, respectively). Lifetime analysis from this experiment is shown in Figure 6. At this *cis*-calcium concentration, an increase in open event duration contributes to the rise in \( P_o \) even with low doxorubicin concentrations; the most likely fits to Figures 6b and 6c required triple exponentials.

**Single-Channel Recordings: Mitoxantrone**

The effect of *cis*-mitoxantrone on channel activity is shown in Figure 7. Representative recordings of current fluctuations from a bilayer with at least two calcium channels are displayed in control medium containing 0.1 \( \mu M \) Ca\(^{2+} \) plus 1 mM ATP (Figure 7a) and after the addition of 1 \( \mu M \) and 5 \( \mu M \) mitoxantrone (Figures 7b and 7c, respectively). Channel opening is stimulated by 1 \( \mu M \) mitoxantrone and then inhibited when the concentration is increased. An initial increase in channel opening, shown in this experiment, was not always observed; 35% of channels (\( n = 20 \)) showed an immediate reduction in opening events.

A typical effect of higher mitoxantrone concentrations is shown in Figure 7d. The bilayer contains a single channel with 0.1 \( \mu M \) Ca\(^{2+} \) plus 1 mM ATP and 30 \( \mu M \) mitoxantrone added to the *cis* chamber. Current fluctuations are seen, some of low amplitude and others to the fully open channel level. These represent open events with a modified conductance that is due either to a conformational change in the channel producing a subconductance state or to a partial block of the channel pore to ionic flow. Low amplitude events were not seen in the absence of mitoxantrone and were specifically associated with the incorporation of CRCs into the bilayer. The addition of up to 100 \( \mu M \) *cis*-mitoxantrone to bilayers before or after vesicle fusion events that did not incorporate a CRC had no effect on membrane conductance.

Low amplitude openings are further illustrated in Figure 8. The current fluctuations of a single channel in a bilayer are shown on an expanded time-scale in the presence of control medium containing 10 \( \mu M \) Ca\(^{2+} \) (Figure 8a), and after the addition of 10 \( \mu M \) (Figure 8b) and 50 \( \mu M \) (Figure 8c) mitoxantrone; 2 \( \mu M \) ruthenium red was added to the *cis* chamber immediately before the recording in Figure 8d. In the control trace, deflections of an amplitude less than the full channel open state represent unresolved opening events. However, after the addition of 10 \( \mu M \) mitoxantrone, there are low amplitude events that are sufficiently long to be fully resolved. In this experiment, 50 \( \mu M \) mitoxantrone effectively abolished normal openings, but the increase in small amplitude fluctuations appears to make the baseline closed current level “noisy.” The addition of ruthenium red,\(^{24} \) as a specific ligand to reduce channel opening, confirms that this action of mitoxantrone is channel mediated rather than being a nonspecific membrane effect. These recordings were made at −20 mV, as low amplitude events were more easily observed at increasingly negative holding potentials.

The mechanism of the interaction between *cis*-mitoxantrone and the CRC was investigated using *cis* solutions containing calcium as the only other activating ligand. Figure 9 shows representative recordings of current fluctuations from a bilayer with a single calcium channel in control medium containing 10 \( \mu M \) cis-Ca\(^{2+} \) (Figure 9a) and after the addition of 5 \( \mu M \) (Figure 9b) and 10 \( \mu M \) (Figure 9c) mitoxantrone. \( P_o \) values are 0.03, 0.22, and 0.07, respectively. Lifetime analysis, shown in Figure 10, demonstrates that at the lower mitoxantrone concentration, the increase in \( P_o \) is mainly due to an increased frequency of channel opening, whereas at the higher drug concentration, although open event duration is increased, the \( P_o \) falls as opening events are less frequent. For the open times in Figures 10a and 10b, the most likely fits were obtained with double exponentials, whereas in Figure 10c, a triple exponential was required. All closed times were best fitted to triple exponentials.

Washout of mitoxantrone results in a loss of the small current fluctuations and a reappearance of normal channel openings. In some cases, however, usually after exposure to high (>20 \( \mu M \)) concentrations, the effect of the drug was not fully reversible; there was often a persistence of longer opening events, and \( P_o \) might be either increased or decreased. Mitoxantrone added to the *trans* chamber had no effect (data not shown).

**Radiolabeled Ryanodine Binding**

Single-channel data provide important information about the mechanisms of interaction between drugs and the channel complex. Radiolabeled ryanodine binding can be used to add quantitative information about drug action on channel populations. Previous reports using skeletal muscle SR have shown that ryanodine only binds to the open channel,\(^{12} \) and calcium is, therefore, an important activator of binding.\(^{25} \) Figure 11 (left panel) demonstrates the effect of doxorubicin on [*H]ryanodine binding at different calcium concentrations. In the presence of 100 \( \mu M \) Ca\(^{2+} \), binding is not significantly increased by the addition of doxorubicin. At 10 \( \mu M \) Ca\(^{2+} \), however, 25 \( \mu M \) doxorubicin increases [*H]ryanodine binding nearly to levels seen with 100 \( \mu M \) Ca\(^{2+} \), which appear to be maximal. Scatchard analysis of the data was performed to estimate \( B_{\text{max}} \) and \( K_D \) values for binding under the different conditions, and Hill coefficients were calculated to investigate the possibility of cooperativity with [*H]ryanodine binding (Figure 11, right panel). Under conditions that produce nearly maximal binding, Hill coefficients of less than 1.0 were obtained, suggesting that there was no cooperativity.
Figure 5. Current fluctuations in a bilayer containing a single calcium channel with 100 μM Ca\textsuperscript{2+} cis before (panel a) and after the addition of 5 μM (panel b) and 10 μM (panel c) doxorubicin to the cis chamber. Channel openings are upward deflections, and current levels of the open and closed channel states are indicated by the dashed lines. All recordings were made with the cis chamber clamped at 0 mV relative to ground.

Table: Open and closed lifetime histograms and maximum likelihood fits for a single calcium channel with 100 μM Ca\textsuperscript{2+} cis before (panel a) and after the addition of 5 μM (panel b) and 10 μM (panel c) doxorubicin to the cis chamber. Maximum likelihood fits are obtained with probability density functions (shown by the solid curves) with the equation \( f(t) = a_1(1/s_1)\exp(-t/s_1) + \ldots + a_n(1/s_n)\exp(-t/s_n), \) where \( a \) and \( s \) refer to relative areas and time constants, respectively. Right panel: Values for \( a \) and \( s \) with open and closed lifetimes.
between binding sites. However, at 10 μM Ca²⁺ without doxorubicin, Scatchard analysis of the data was nonlinear, and a Hill coefficient of 1.42 was obtained.

Figure 12 shows the effect of different anthracyclines on [³H]ryanodine binding in 5 μM free Ca²⁺. The addition of 1–100 μM anthracycline results in an incremental rise that is quantitatively similar for all three drugs. Figure 13 compares the effects of doxorubicin and mitoxantrone at 5 and 10 μM Ca²⁺. In these experiments, 10 μM Ca²⁺ plus 25 μM doxorubicin stimulated maximal binding, and so a further increase in drug concentration is without additional effect. Mitoxantrone, in 5 μM Ca²⁺, produces very little change in binding, although there is a small initial increase at low concentrations followed by a slight decrease. This pattern is illustrated more clearly in the presence of 10 μM Ca²⁺; under these conditions, the binding at 100 μM mitoxantrone is reduced considerably below the baseline value. None of the anthraquinones investigated stimulated specific ryanodine binding in the presence of subactivating (10 nM) calcium concentrations.

Discussion

The contractile state of the heart is determined by the cytosolic calcium concentration. In the mammalian cardiac myocyte, the calcium that initiates contraction is released from the SR in response to the action potential. Therefore, the CRC has a central role in the process of excitation-contraction coupling. Previous work has shown that a rise in the calcium concentration at the cytosolic face of the channel is the principal ligand to stimulate channel opening.²⁴

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**FIGURE 7**. Panel a: Current fluctuations in a bilayer containing at least two calcium channels with 0.1 μM Ca²⁺ plus 1 mM ATP cis. Panels b and c: 1 μM and 5 μM mitoxantrone have been added to the cis chamber, respectively. Recordings were made with the cis chamber clamped at 0 mV relative to ground. Panel d: From a different experiment, current fluctuations in a bilayer containing a single calcium channel with 0.1 μM Ca²⁺ plus 1 mM ATP plus 30 μM mitoxantrone cis. The recording was made with the cis chamber clamped at −20 mV relative to ground. All channel openings are shown as upward deflections, and current levels of the fully open and closed states are indicated by the dashed lines.

**FIGURE 8**. Current fluctuations in a bilayer containing a single calcium channel with 10 μM Ca²⁺ cis before (panel a) and after the addition of 10 μM (panel b) and 25 μM (panel c) mitoxantrone to the cis chamber; 2 μM ruthenium red was then added to the cis chamber (panel d). Channel openings are upward deflections, and current levels of the fully open and closed states are indicated by the dashed lines. All recordings were made with the cis chamber clamped at −20 mV relative to ground.
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FIGURE 9. Current fluctuations in a bilayer containing a single calcium channel with 10 μM Ca\(^{2+}\) cis before (panel a) and after the addition of 5 μM (panel b) and 10 μM (panel c) mitoxantrone to the cis chamber. Channel openings are upward deflections, and current levels of the open and closed states are indicated by the dashed lines. All recordings were made with the cis chamber clamped at 0 mV relative to ground.

FIGURE 10. Left panel: Open and closed lifetime histograms and maximum likelihood fits for a single calcium channel with 10 μM Ca\(^{2+}\) cis before (panel a) and after the addition of 5 μM (panel b) and 10 μM (panel c) mitoxantrone to the cis chamber. Maximum likelihood fits are obtained with probability density functions (shown by the solid curves) with the equation \(f(t) = a_1(1/s_1)\exp(-t/s_1) + \ldots + a_n(1/s_n)\exp(-t/s_n)\), where \(a\) and \(s\) refer to relative areas and time constants, respectively. Right panel: Values for \(a\) and \(s\) with open and closed lifetimes.

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FIGURE 11. Effect of calcium and doxorubicin on \[^{3}H\]ryanodine binding at different \[^{3}H\]ryanodine concentrations (left panel). Results shown are the means and standard errors from three separate experiments. Scatchard analysis was used to obtain \(K_d\) and \(B_{max}\) from which the Hill coefficients (\(n_h\)) were calculated (right panel) (see text for details). Dox, doxorubicin.

This observation is consistent with the hypothesis that the small influx of calcium during the slow inward current is the trigger for the release of SR calcium stores: calcium-induced calcium release.\(^{26,27}\) Physiological concentrations of ATP and magnesium\(^{24}\) have also been shown to be significant modulators of channel activity, respectively increasing and decreasing channel \(P_o\). In this study, the effect of clinically useful anthraquinones has been investigated on single CRCs, both under conditions approximating the physiological environment at the cytosolic face of the channel (submicromolar \(Ca^{2+}\) plus millimolar ATP), and using calcium as the sole other activating ligand, which allows for more detailed investigation of the mechanism of drug interaction.

Similar patterns of drug effect are seen under both sets of conditions.

Doxorubicin and the other anthracyclines produce significant, reversible activation of the cardiac SR CRC. Inappropriate channel opening would result in increased cytosolic calcium concentrations, which is consistent with the suggestion that anthracycline cardiotoxicity is mediated through intracellular calcium overload. Stimulation of SR calcium release is also consistent with observations that low concentrations of doxorubicin increase the force of contraction in isolated papillary muscles,\(^{28}\) whereas higher doses are associated with decreased force production,\(^{29}\) possibly because the amount of calcium released exceeds the capacity of pumps in the SR membrane.

FIGURE 12. Effect of anthracyclines on equilibrium \[^{3}H\]ryanodine binding in incubation media containing 5 \(\mu M\) calcium. Results shown are the mean of three separate experiments for doxorubicin and epirubicin and two separate experiments for idarubicin. The dashed line indicates the level of binding with 5 \(nM\) \[^{3}H\]ryanodine in 100 \(\mu M\) \(Ca^{2+}\).
to reaccumulate it. The depletion of SR calcium stores, in itself, would reduce contractile force, and in addition, the impairment of calcium clearance from the cytosol during diastole would interfere with relaxation.

The data extend previous reports of the effects of anthracyclines on skeletal SR to the cardiac CRC. The previously reported stimulation of Ca\(^{2+}\) efflux is shown to be due to a rise in single-channel open probability. Abramson et al.\(^{10}\) have proposed that this effect of doxorubicin is mediated through a sensitization of the calcium activator site of the CRC for calcium, but the results of the present study suggest that anthracyclines and calcium act synergistically to increase channel opening. Activation of the CRC, with calcium as the sole ligand, has been investigated with channels from both skeletal\(^{30}\) and cardiac\(^{31,32}\) SR. The increase in \(P_o\), seen with elevation of the cis-calcium concentration, is almost exclusively due to an increased frequency of opening events, whereas their duration is unchanged. A comparison of the control lifetimes in 10 and 100 \(\mu M\) cis-calcium (shown in Figures 4a and 6a) is consistent with this mechanism. In the present study, doxorubicin is shown to raise \(P_o\), through an increase in both open event frequency and duration. Furthermore, doxorubicin is able to increase \(P_o\) to values approaching unity, whereas with calcium alone, the sheep cardiac CRC has been shown to exhibit a maximum \(P_o\) of approximately 0.4.\(^{32}\) The results obtained at 10 \(\mu M\) cis-Ca\(^{2+}\) with low (<10 \(\mu M\)) concentrations of doxorubicin are consistent with the possibility that, under these conditions, the drug is sensitizing the CRC to calcium. However, the finding that 5 \(\mu M\) doxorubicin at 100 \(\mu M\) cis-Ca\(^{2+}\) affects open event duration suggests that, even at low concentrations, the drug and calcium interact synergistically with the CRC.

The relation between ryanodine binding and channel open probability has not been investigated fully, but previous work has shown that ligands that stimulate or inhibit single-channel opening have similar effects on ryanodine binding.\(^{33,34}\) In the presence of 100 \(\mu M\) Ca\(^{2+}\), \(^{3}H\)ryanodine binding is not increased by the addition of doxorubicin, although at this calcium concentration, the drug does increase single-channel \(P_o\). At 10 \(\mu M\) Ca\(^{2+}\), however, doxorubicin stimulates both single-channel \(P_o\) and \(^{3}H\)ryanodine binding. These results suggest that, although ryanodine binds to a channel open state, a single-channel \(P_o\) approaching unity is not required to recruit all the binding sites. Thus, \(^{3}H\)ryanodine binding is only related to channel activity under conditions when \(P_o\) is relatively low.

The data in Figure 12, which show similar quantitative stimulation of \(^{3}H\)ryanodine binding by the different anthracyclines, suggest that these drugs are equipotent in their effect on CRC activation. In clinical use, however, epirubicin is considerably less cardiotoxic than doxorubicin, and the same has been claimed for idarubicin.\(^{4}\) These observations suggest that, although activation of the cardiac CRC may be related to drug cardiotoxicity, other factors are also involved. First, Olson et al.\(^{35}\) have shown that higher concentrations of doxorubicin can also inhibit the SR Ca\(^{2+}\)-ATPase and other ion pumps. Second, the production of certain metabolites may be important in the generation of cardiotoxicity; doxorubicinol is the major metabolite of doxorubicin\(^{33}\) and has similar effects to doxorubicin both on the skeletal muscle CRC\(^{13}\) and on other ion pumps.\(^{35}\) Third, anthracyclines may also vary with regard to the concentrations that are accumulated by cardiac cells. By attaching doxorubicin to an insoluble agarose support,\(^{36}\) it has been shown that the drug can exert a cytotoxic action at the cell surface, so that intracellular drug concentration and anticancer activity are factors that are not necessarily closely associated.

The interaction of mitoxantrone with the CRC is complex. In 65% of channels, low concentrations (<10 \(\mu M\)) produced an increase in \(P_o\). All channels,
however, show a decreased $P_o$ associated with longer opening events as the drug concentration is increased. Low-amplitude current fluctuations were only observed with higher concentrations of mitoxantrone and were not seen in the presence of physiological ligands alone or with anthracyclines. The amplitude of these events was too small to permit further analysis to determine whether they represent a partial block to ion flow through the open channel or the appearance of a subconductance state. As our analysis used a threshold of 50% of the full channel open current level to register an opening event, channel opening in the presence of mitoxantrone will be underestimated. It is not possible to assess accurately the contribution that low-amplitude openings would make to total calcium release from the SR, but, as mitoxantrone has been found to be more potent than doxorubicin at low concentrations in stimulating Ca$^{2+}$ efflux from skeletal SR, it is likely that they are significant.

The inhibition of specific $[^3H]$ryanodine binding in the presence of high concentrations of mitoxantrone suggests that the low-amplitude current fluctuations do not represent channel opening events of a nature that allows ryanodine to bind to its receptor. The effect on Ca$^{2+}$ efflux of mitoxantrone at concentrations above 10 $\mu$M has not been reported, but this would be of interest in the light of our observations of single-channel activity and ryanodine binding.

In conclusion, anthracyclines produce a dose-dependent increase in both CRC open probability and specific $[^3H]$ryanodine binding. This effect is consistent with the development of intracellular calcium overload, which in turn may lead to the reported histological changes associated with cardiotoxicity. Mitoxantrone has been reported to produce a cardiomyopathy with different histological characteristics; although the complexity of the drug interaction with the CRC does not allow us to propose a specific mechanism, our findings provide a basis for the heterogeneity of the effects of the anthracyclines and the anthracingodines. The ability of novel anthraquinones to interact with the CRC may well be important in the future development of drugs in this class with reduced cardiotoxicity. Finally, it is important to emphasize the observation that drugs that can stimulate the release of intracellular calcium stores are associated with cardiotoxicity. Some inotropic drugs in clinical use, for example, phosphodiesterase inhibitors, elevate the calcium concentration within the myocyte. As the capacity of the SR to accumulate calcium has been shown to be depressed in patients with congestive cardiomyopathy, there is likely to be an increased vulnerability to intracellular calcium overload. There is, therefore, at least a theoretical basis to question the rationale of using such agents for the long-term treatment of contractile failure.

References

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