Biphasic Effects of Doxorubicin on the Calcium Release Channel From Sarcoplasmic Reticulum of Cardiac Muscle

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To define the mechanism of doxorubicin cardiotoxicity, the effects of doxorubicin and caffeine were examined on calcium release channels from cardiac sarcoplasmic reticulum. We found that calcium release from cardiac sarcoplasmic reticulum vesicles was induced by both compounds. When sarcoplasmic reticulum vesicles were incorporated into planar lipid bilayers, calcium-permeable channels were observed. Addition of caffeine (2.5–10 mM) increased channel open probability from less than 0.1% to 40%, and this effect persisted for a mean of 44 minutes. In contrast, doxorubicin (2.5–10 μM) had a biphasic effect; initially, doxorubicin activated the channel, whereas after a mean of 8 minutes, the channel became irreversibly inhibited. Although the degree of channel activation by doxorubicin was concentration dependent, the time needed to inactivate the channel was concentration independent. Pretreatment with dithiothreitol (0.2 mM) prevented doxorubicin-induced channel inactivation, and channel activity persisted for an average of 58 minutes. Dithiothreitol alone did not alter channel open probability. Our results support the hypotheses that 1) the integrity of sulfhydryl groups is important for some aspects of calcium release channel function and 2) activation and inactivation of the channel are separable processes. The biphasic effect of doxorubicin on channel function may also correspond to the clinically observed adverse effects of doxorubicin, a widely used chemotherapeutic agent that, after prolonged usage, causes a dilated cardiomyopathy. (Circulation Research 1990;67:1167–1174)

Calcium stored in the sarcoplasmic reticulum (SR) of cardiac or skeletal muscle is mobilized to initiate a contraction by activating ion channels called calcium release channels. These channels, which are concentrated in the T tubule/SR junction of skeletal muscle,1–3 can be purified1–3 and reconstituted.3 In addition, the channel protein has recently been cloned.4 Comparisons of biochemical and molecular properties of the channels from cardiac and skeletal muscle have shown that these channels are similar.5–7 However, the physiological trigger for opening the channel appears to be different in the two types of striated muscle,8,9 and differences in some of the functional properties of the channels from the two types of muscle have been described (compare Smith et al5 and Rousseau et al6).

Calcium release from SR vesicles made from both muscles can be initiated by a number of compounds, although different sensitivities to the compounds have been reported.10 Calcium and ATP appear to be important for the activity of the calcium release channel under physiological conditions, particularly in cardiac muscle, where the trigger for excitation–contraction coupling is thought to be calcium from the extracellular solution.9 Other compounds such as caffeine, halothane, and doxorubicin have effects on calcium release from vesicles, although this activation may be related to the adverse effects of the compound.

Doxorubicin (Adriamycin, Adria Laboratories, Dublin, Ohio), a widely used chemotherapeutic agent, activates calcium release from SR vesicles and has been used as a photoaffinity label for the calcium release channel complex.11 Clinically, this compound is cardiotoxic.12–14 It has been hypothesized that the doxorubicin-induced cardiotoxicity is related to lipid peroxidation, oxidation of critical sulfhydryl groups, and/or triggering of calcium efflux from SR.10,15–18 These effects may be interrelated. For example,
oxidation of sulfhydryl groups on the calcium release channel of SR may alter the activity of the channel.

The experiments in this report show that doxorubicin exerts a biphasic effect on the calcium release channel of cardiac SR. The initial effect of doxorubicin is to activate the channel, whereas prolonged exposure irreversibly inhibits the channel. This contrasts with the action of caffeine, which produces a prolonged activation of the calcium release channel. In addition, we found that it was possible to "protect" the channel from doxorubicin-induced inhibition by pretreating the channels with dithiothreitol (DTT), an agent that maintains sulfhydryl groups in the reduced form.

Materials and Methods

SR vesicles prepared from canine heart as described previously were suspended in a buffer containing 150 mM KCl, 20 mM 3(N-morpholino)propanesulfonic acid (MOPS) (pH 6.8), 0.1 mM phenylmethylsulfonyl fluoride, 10 mg/l aprotinin, 0.8 mg/l antipain, 2 mg/l trypsin inhibitor, and 0.3 M sucrose and quickly frozen in liquid nitrogen and kept at −80°C. Vesicles were used within 4 weeks after preparation. Protein concentrations were determined by the Lowry method, using bovine serum albumin as a standard.

Calcium release was monitored using 45Ca2+ as described previously. Briefly, the SR vesicles (0.05 mg/ml) were incubated at 37°C for 5 minutes in a reaction mixture containing 0.15 M KCl, 20 mM MOPS (pH 6.8), 5 mM MgCl2, 10 mM NaN3, 0.26 mM EGTA, and 0.065 mM 86CaCl2 (104 cpm/mmol). Active calcium uptake was started by addition of 2 mM ATP to the reaction mixture. At a steady state of calcium loading, calcium release was initiated by addition of doxorubicin or caffeine and stopped at various times by filtration of aliquots of the reaction mixture with 0.45-μm filters (Millipore Corp., Bedford, Mass.). After filtration, each filter was immediately washed twice with 2.5 ml of 0.15 M KCl, 20 mM MOPS (pH 6.8), 30 mM EGTA, and 15 μM ruthenium red. The filters were then dried, and their radioactivity was counted.

SR vesicles also were incorporated into preformed planar lipid bilayers that were formed by painting a lipid/decane solution across a hole in a Teflon partition that separated two lucite compartments. Channel incorporation was accomplished using the method outlined by Smith et al. Briefly, vesicles were added to one compartment after the membrane was formed, usually the cis compartment. Incorporation was monitored in the presence of a chloride and an osmotic gradient across the membrane [cis solution: 600 mM N-methyl-D-glucamine chloride, 20 mM HEPES, 10 mM CaCl2, 0.2 mM EGTA, pH 7.3; trans solution: 250 mM HEPES, 53 mM Ca(OH)2, pH 7.3]. The insertion of a chloride-permeable channel was the signal that a vesicle had fused with the bilayer. These channels are convenient markers because they are present in SR membranes, they are large conductance channels, and they are open most of the time. To monitor calcium release channels, the bilayer chamber was then perfused with a chloride-free solution: 250 mM HEPES-Tris, 1 mM EGTA, and 0.5 mM CaCl2 (0.1 μM free calcium), pH 7.3. In addition, the chloride-free solution was isosmotic to the trans solution to prevent incorporation of additional vesicles into the bilayer.

Channel insertion and subsequent experiments were monitored under voltage-clamp conditions with a pair of Ag/AgCl electrodes contacting the solutions via CsCl junctions. The channel currents were amplified using a patch-clamp amplifier (Yale model MK-5, Warner Instruments, Hamden, Conn., or Axopatch Ib, Axon Instruments, Burlingame, Calif.) and recorded on chart (General Scanning, Watertown, Mass.) and tape (Dagan Corp, Minneapolis, Minn.) recorders. Data were analyzed after filtering with an eight-pole Bessel filter (Frequency Devices, Haverton, Mass.) to 300 Hz and digitizing at 1 kHz to transfer to a PDP 11/73 (Indec Systems, Sunnyvale, Calif).

Doxorubicin and caffeine were purchased from Sigma, St. Louis. Lipids were purchased from Avanti Polar Lipids, Birmingham, Ala. All other reagents used were of analytical grade.

Results

Doxorubicin or Caffeine Induces Rapid Calcium Release From Cardiac SR Vesicles

Caffeine and doxorubicin initiated release of calcium from cardiac SR vesicles that had been loaded with 45Ca2+ (~15 nmol/mg SR) in the presence of 2 mM ATP. At 7 minutes after addition of ATP, when the calcium loading had reached a steady state, addition of 2 mM caffeine caused approximately 30% of the loaded calcium to be released from the SR within 10 seconds (Figure 1, left panel), while 40% of the loaded calcium was released by addition of 25 μM doxorubicin (Figure 1, right panel). Further increase in the drug concentrations did not change the amount of calcium released. Similar results have been obtained with caffeine and doxorubicin when calcium efflux from cardiac SR vesicles was monitored with the calcium indicator antipyrilazo III (Reference 24 and data not shown).

Doxorubicin or Caffeine Opens Calcium Release Channels From Cardiac SR Incorporated Into Planar Lipid Bilayers

To show that caffeine- and doxorubicin-induced calcium release from SR vesicles was channel-mediated, SR vesicles from cardiac muscle were incorporated into planar lipid bilayers and channel currents were recorded. Channel activity in the presence of 0.1 μM free calcium is shown in Figure 2 (top two tracings of each panel). In the absence of drugs, the probability of finding the channel open under these conditions was generally less than 1%. Addition of either caffeine (Figure 2, bottom two tracings of left
Caffeine-Induced Opening of Calcium Release Channels Is Maintained

Addition of caffeine to the cytoplasmic side of the channel increased channel activity, and in eight of 15 experiments, caffeine opened more than one channel. In the control period of the experiment (Figure 4, top plot of upper left panel) the channel was open less than 0.1%. Only a 2-minute control segment is shown, but this behavior was maintained for 10 minutes. Addition of 2.5 mM caffeine at the beginning of the segment shown led to increased channel activity (Figure 4, second plot of upper left panel). The third and fourth plots show channel activity 10 and 20 minutes after caffeine addition. Although the mean open time varied between 52% and 29% in this experiment, it does not indicate that the activity is decreasing with time. Variation in the mean activity level as seen in this figure was found in virtually all experiments performed, regardless of the concentration of caffeine used. This variability appears to be a property of the calcium release channel from SR. For example, two additional experiments are shown in Figure 5, in which the degree of activation varied between 18% and 82%.

The duration of activation was similar after addition of either 2.5 or 5 mM caffeine (Figure 5). In 10 experiments in which caffeine was the activator, the channels were active for a period greater than 15 minutes (16–90 minutes; mean, 44 minutes). A second addition of caffeine reactivated the channel.
During the active time, channel openings maintained a square, “boxcar” appearance. In addition, there were no shifts in the baseline current, indicating the absence of nonspecific current leaks. In five experiments in which caffeine was the activator, the experiment was terminated prematurely, due to rupture of the bilayer while the channel was still active (at 2–27 minutes). These experiments have not been included in the determination of the mean time of activation because inclusion of these experiments would lead to an underestimation of the longevity of caffeine activation.

**Doxorubicin-Induced Openings of Calcium Release Channels Is Biphasic**

Addition of doxorubicin to the cytoplasmic side of the channel increased activity, and over the concentration range studied (1–10 μM), usually only one channel was activated. In the control period, the channel open probability was less than 0.1% (Figure 4, upper right panel, top plot). Only a 2-minute control segment is shown, but this behavior was maintained for 10 minutes, after which doxorubicin was added. In the second plot, 2.5 μM doxorubicin was added at the beginning of the segment shown. The third and fourth plots show that channel activity was virtually absent 10 and 20 minutes after doxorubicin addition. Addition of caffeine or calcium to the chamber after channel inactivation did not reopen the channel.

Although there was some variation in the absolute magnitude of channel activation, we found that activation, but not the rate of inactivation, appeared to depend on the concentration of doxorubicin (Figure 6). In all 23 experiments in which doxorubicin was added to the chamber, there was an initial activation of the channel similar to that shown in Figure 4, upper right panel. After prolonged exposure of the channels to the drug, 22 of the experiments showed one or a combination of the following: 1) The channel became irreversibly inhibited, and drugs known to activate the channel could not reactivate the channel. The duration of activation before irrevers-

### Discussion

In this paper we have evaluated the effects of caffeine and doxorubicin on the properties of the calcium release channel from cardiac SR. We found that, while both compounds activate the channel, the response of the channel to prolonged SR. We found that, while both compounds activate the channel, the response of the channel to prolonged exposure to the compounds differed. After caffeine opened the channel, the channel remained active as long as caffeine was present. In contrast, after doxorubicin opened the channel, the channel became irreversibly inhibited after prolonged exposure to this compound.
Doxorubicin-induced inactivation could be prevented by pretreating the channel with DTT, an agent that maintains sulfhydryl groups in the reduced form. DTT, when added alone, had no effect on channel activation. A similar lack of effect of DTT on anthraquinone activation of calcium release from SR vesicles from skeletal muscle has been reported previously. These results suggest that the activation of the calcium release channel by doxorubicin is regulated by a different biochemical process than the inhibition by doxorubicin.

Caffeine has been shown to activate calcium release channels from SR vesicles prepared from skeletal and cardiac muscle in vesicle release assays. Caffeine also has been shown to open channels from SR vesicle made from skeletal and cardiac muscle that had been incorporated into planar lipid bilayers. In this series of experiments, we used channels from cardiac muscle SR and found that caffeine induced a dramatic increase in channel activity, often opening several channels in the bilayer, and that this activity would persist in the continued...
doxorubicin is activating the calcium release channel from experiments showing that doxorubicin binds to the same protein as ryanodine in both skeletal and cardiac muscle. However, the inhibitory effect of doxorubicin could not have been easily observed using an assay that measures calcium release from vesicles. Only in a system such as the bilayer, in which the activity of the channel can be followed over time, could this effect be detected.

In several cases, prolonged exposure of the channels to doxorubicin increased the leakiness of the membrane to the degree that it was not possible to distinguish individual channel openings. Neither ruthenium red nor calcium addition was effective in reversing the leak. Although the mechanism of this change in membrane permeability is unknown, our experiments suggest that the target is some component of the SR membrane, because the doxorubicin-induced increased leakiness was seen only after SR vesicles were incorporated into the membrane. The target may not be the calcium release channel itself, because the increased leakiness was seen in only approximately 40% of the experiments.

Although the calcium release channels of cardiac and skeletal muscle are similar, the main clinical side effects are associated with cardiac muscle. This may be because the heart has low levels of superoxide dismutase, catalase, and nonselenium-dependent glutathione peroxidase. The decreased levels of these enzymes compared with other tissues would make cardiac muscle more susceptible than skeletal muscle to doxorubicin.

The biphasic response of the channel to the addition of doxorubicin is reminiscent of the biphasic cardiototoxic response seen after doxorubicin is administered to patients for the treatment of many solid tumors and hematologic malignancies. Doxorubicin is given in small daily doses of 20–30 mg/m²/day (approximately equal to a plasma concentration of 0.4–0.1 μM/dose) until a cumulative dose of 500 mg/m² is achieved. Different cardiovascular responses to doxorubicin have been observed acutely and after repeated administration. The abnormalities associated with intravenous administration of doxorubicin, or acute administration, include tachycardia, hypotension, and arrhythmias. After prolonged use of doxorubicin, irreversible congestive heart failure develops. These irreversible changes occur in a high percentage of patients who receive high doses (>500 mg/m²) of doxorubicin over the course of treatment, suggesting that the toxicity is cumulative and dose dependent.

The ability of doxorubicin to open calcium release channels may account for the acutely observed clinical effects of the drug, which at low concentrations increases left ventricular end-diastolic pressure and increases the force of contraction in cardiac muscle. Both of these changes could arise from increased intracellular free calcium and might be caused by activation of calcium release channels in the SR.
orubicin-induced inactivation of the channel was prevented by pretreating the system with DTT. These experiments show that activation and inactivation of the calcium release channel can be separated, suggesting that different biochemical mechanisms are involved in the two responses to doxorubicin addition. These experiments also show that the biphasic effects of doxorubicin on the calcium release channel can be related to the observed cardiotoxicity.

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Figure 7. Graph showing that pretreatment with dithiothreitol (DTT) prevents doxorubicin (Dox) inactivation. After the addition of 200 μM DTT to the cis chamber at –5 minutes, 2.5 μM Dox was added at time 0. The initial activity is similar to the activity seen after the addition of Dox alone, but the activity after pretreatment persists for a mean of 58 minutes. Note that DTT alone did not alter channel activity. This graph shows data from a single experiment without pretreatment (○) and a single experiment with pretreatment (●).

The delayed effects of doxorubicin to inhibit channel opening observed in this report may be related to the cardiomyopathy observed after long-term administration of doxorubicin. Damage to cardiac muscle by doxorubicin is irreversible, and once clinically evident heart failure occurs, changes in the cardiac muscle and clinical deterioration are irreversible.14,16,17 This observation is reminiscent of our inability to reverse the doxorubicin-induced inhibition of the calcium release channel (Figure 4, upper right panel).

The long-term, cumulative cardiotoxic effects of doxorubicin have been proposed to be related to the formation of free radicals, which can lead to peroxidation of membrane phospholipids and subsequently alter membrane permeability or channel function.30,31 Another proposal is that doxorubicin interacts with critical thiol groups associated with the channel complex16,30–32 that alter channel function. These hypotheses are supported by the present findings that DTT, a reducing agent, when added before the administration of doxorubicin, preserved channel activity after doxorubicin addition (Figure 7). Although doxorubicin is a natural photoaffinity label, it is unlikely that our results could be explained by light-dependent cross-linking of doxorubicin to the membrane proteins, despite the fact that our experiments were done under room lights. To obtain cross-linking of doxorubicin to SR proteins, 5-minute exposure to a high-intensity ultraviolet light (7,000 μW/cm²) is used,11 whereas we observed complete inhibition of channel activity in 8 minutes under room lights (>200 times less intense in the ultraviolet range).

In summary, we have shown that both caffeine and doxorubicin can activate the calcium release channel from cardiac SR but that doxorubicin induced inhibition of the channel after prolonged exposure. Dox-
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