A digitized-fluorescence-imaging study of mitochondrial Ca^{2+} increase by doxorubicin in cardiac myocytes

Enrique CHACON,* Roger ULRICH† and Daniel ACOSTA*‡

*Division of Pharmacology and Toxicology, College of Pharmacy, University of Texas, Austin, TX 78712, and †Upjohn Laboratories, Kalamazoo, MI 49001 U.S.A.

The objective of the present study was to investigate the role of mitochondrial Ca^{2+} in doxorubicin-induced cell injury. The effect of doxorubicin on cultured cells was investigated by digitized fluorescence imaging. The Ca^{2+} sensitive fluorescent dye fura-2 was used to estimate cytosolic, mitochondrial and total cellular Ca^{2+} . Rhodamine 123 was used to estimate the mitochondrial membrane potential, and cellular ATP was determined by h.p.l.c. The data showed that doxorubicin induced greater-than-2-fold increases in mitochondrial Ca^{2+} before changes in cytosolic Ca^{2+} could be detected. An increase in mitochondrial Ca^{2+} paralleled the observed dissipation in mitochondrial membrane potential. Cellular ATP levels appeared to decrease as a result of mitochondrial dysfunction, which in turn produced greater-than-2-fold increases in cytosolic Ca^{2+} . The data suggest that doxorubicin-induced alterations in mitochondrial Ca^{2+} homeostasis are associated with a dissipation in energy conservation, which may result in cell injury.

INTRODUCTION

Doxorubicin is a highly effective cancer chemotherapeutic agent, but its clinical use is severely restricted by its serious cardiotoxicity [see Singal *et al.* (1987) and Olson & Mushlin (1990) for reviews]. Several mechanisms have been suggested to explain the cardiotoxicity of doxorubicin, yet no single hypothesis adequately describes the observed cardiotoxic actions of the drug. One theory is an intercalation of the drug between base-pairs of DNA helices, with subsequent inhibition of DNA replication and RNA synthesis (DiMarco, 1975). Generally, no correlation is observed between antitumour potency and intercalative affinity, because various potent intercalators lack antitumour properties (Johnson *et al.*, 1979; Kapuscinski & Darynkiewicz, 1986). In addition, an analogue of doxorubicin, *N*-trifluoroacetyl Adriamycin 14-valerate, is an active antitumour agent, but does not enter the nucleus or bind DNA (Krishan *et al.*, 1976). However, Ito *et al.* (1990) have shown an early onset of selective inhibition of cardiac myofibril gene expression by doxorubicin. Another hypothesis proposed to explain doxorubicin cardiotoxicity is the intracellular- Ca^{2+} -overload hypothesis (Olson & Mushlin, 1990). Treatment of rabbits with doxorubicin showed the formation of Ca^{2+} inclusions in mitochondria of ventricular myocardium (Olson *et al.*, 1974). However, papillary muscles of rabbits treated with doxorubicin showed a decreased contractility, suggestive of low cytoplasmic Ca^{2+} (Jensen, 1986). Over the years the free-radical hypothesis for doxorubicin-induced cell damage has received considerable attention (Powis, 1989). Doxorubicin is believed to generate toxic oxygen radicals by way of oxidation–reduction cycling. Nonetheless, many observations appear inconsistent with this free-radical hypothesis [see Olson & Mushlin (1990) for a review]. In addition, we have recently observed that mitochondrial release of superoxide radical anions by doxorubicin was dependent on mitochondrial Ca^{2+} transport (Chacon & Acosta, 1991). These data suggested that the release of superoxide radical anions from mitochondria may be the result of altered mitochondrial Ca^{2+} cycling.

The main objective of the present study was to investigate further the role of mitochondrial Ca^{2+} in doxorubicin-induced cardiotoxicity. Employing the use of digitized fluorescence imaging (DFI) and the Ca^{2+} -sensitive dye fura-2, we estimated cytosolic and mitochondrial Ca^{2+} in cultured myocardial cells treated with doxorubicin. We show here that mitochondrial Ca^{2+} overload is an early event in the onset of doxorubicin-induced cell damage and relate these changes to a dissipation in mitochondrial membrane potential and a decline in cellular ATP, both of which precede a rise in cytosolic Ca^{2+} .

EXPERIMENTAL

Materials

Fura-2 acetoxymethyl ester (fura-2 AM), was purchased from Molecular Probes (Eugene, OR, U.S.A.). Rhodamine 123 (rh-123) was purchased from Eastman Kodak. Ruthenium Red, diltiazem, digitonin, 2,4-dinitrophenol (DNP) and imidazole (99% purity) were purchased from Sigma Chemical Co. Doxorubicin was purchased from Adria as a sterilized Adriamycin PFS (1 mg/ml) solution. All other reagents were of the highest available purity from commercially obtainable sources.

Myocardial-cell cultures

Primary myocardial cell cultures were prepared as previously described (Mbuga *et al.*, 1988) from Sprague–Dawley rats aged 3–5 days. The animals were obtained from a breeding colony maintained at the University of Texas in accordance with the regulations and guidelines established by the National Institutes of Health and the United States Department of Agriculture. Cells (10^6 /ml) were plated in 50 ml (25 cm²) culture flasks. Muscle cells were separated from non-muscle cells by a pour-off technique based on the differential rate of attachment of the cells to the culture flasks (Wenzel *et al.*, 1970). After 3 h, the resultant myocyte supernatant was poured off, and either 0.5 ml aliquots were plated on 25 mm-diameter round glass coverslips for imaging studies or 3 ml were used to plate 35 mm culture dishes for ATP experiments. The myocyte cultures were maintained in

Abbreviations used: DFI, digitized fluorescence imaging; fura-2 AM, fura-2 acetoxymethyl ester; rh-123, rhodamine 123; DNP, 2,4-dinitrophenol; DMSO, dimethyl sulphoxide; R-KRHI, respiratory Krebs–Ringer–Henseleit/imidazole; PTI, Photon Technology International; EMEM, Eagle's minimum essential medium.

‡ To whom correspondence should be addressed.

Eagle's minimum essential medium (EMEM) supplemented with 5% newborn-calf serum and antibiotics [penicillin G potassium salt (0.5 units/ml), streptomycin sulphate (0.05 mg/ml) and amphotericin B (0.5 μ g/ml)]. The cells were grown in a humidified atmosphere (37 °C) of CO₂/air (1:19) to maintain a pH of 7.2–7.4. The medium was replaced 24 h before the day of the experiments.

Drug treatments

Experiments were conducted 4 days after the initial plating of the myocytes. Myocardial-cell cultures were treated with 50 μ M-doxorubicin in order to compare the effects of doxorubicin on intracellular Ca²⁺ with our previous studies (Chacon & Acosta, 1991). The doxorubicin drug treatments were made by diluting aliquots of the 1 mg/ml Adriamycin PFS solution.

DFI

We have tried to include a detailed description of the instrumentation procedures throughout the experimental section, since the present paper is one of the first to report the use of a Photon Technology International (PTI-IMAGESCAN) system. DFI was performed using a PTI-IMAGESCAN system attached to a Zeiss IM-35 epifluorescence microscope. The illumination unit consisted of a 75 W xenon arc lamp whose illumination path first encounters a rotating chopper disc that can alternately reflect or transmit the beam through one of two monochromators. The monochromator outputs (excitation source) are combined into a bifurcated optical fibre and conducted to an epifluorescence microscope. A Hamamatsu silicon-intensified-target camera detects the low-level fluorescence, amplifies the signal and directs the signal to the video workstation. The video workstation (80386-based microcomputer) accepts and digitizes the analog signal for subsequent analysis and resulting digital images. The actual video processing is done by two specialized boards within the computer. Video images from the camera are transported to the computer through a video-input-system board and relayed to the monitor through the video-output-system board. Image processing immediately occurs in 16-bit arithmetic logic units and/or in an array of Look Up Tables. The video-output-system board contains its own Look Up Tables, frame memory and red/green/blue display interface. Therefore ratioed images can be viewed in almost real time.

For fluorescent-intensity values of single-excitation/single-emission probes such as rhodamine-123, thresholding was applied to remove all information at less than a specified cut-off level. However, with ratioed images of dual-excitation/single-emission compounds such as fura-2, background suppression utilized a more sophisticated technique known as intensity-weighted ratio presentation. This technique was developed by Lipscombe and co-workers (Lipscombe *et al.*, 1988; Tsien & Harootunian, 1990). In most digital-imaging systems a pseudocolour is assigned to a ratioed value obtained by dividing the intensity signals received from the alternating wavelengths. However, a problem arises where the numerator and denominator of the intensity ratio are both nearly zero, as is the case between cells. The most common technique is to set a threshold value where low intensities are cut off. Unfortunately, such thresholding may suppress important features if set too high. PTI-IMAGESCAN software incorporates an intensity-weighted ratio presentation. In essence, this background-suppression technique forms a ratioed image in which each pixel value encodes not only its ratio value but also a weighting parameter specifying the mean intensity of the signal. Hence the higher the mean intensity, the more significant the data are likely to be and the more brightly the image is displayed

with complete background suppression. An intensity-weighted ratio presentation allows one to judge the spatial thickness of a cell as well as dye accumulation in localized regions within a cell.

Selective compartmental localization of fura-2

Acetoxymethyl ester fluorescent probes loaded into cells may localize into cellular compartments (Davis *et al.*, 1987; Steinberg *et al.*, 1987; Gores *et al.*, 1989). Compartmental localization can be viewed after selective permeabilization of the plasma membrane to release the cytosolic contents. We determined the loading conditions in our cardiac cells that were required to load fura-2 into both the cytosolic and mitochondrial compartments. Cytosolic fura-2 loading was accomplished by incubating the myocytes with 1 μ M fura-2 acetoxymethyl ester (AM) for 30 min at 25 °C. Mitochondrial localization of fura-2 was evaluated by a modified procedure of Gores *et al.* (1989). Briefly, myocytes grown on glass coverslips were co-loaded with 2.5 μ M-fura-2 AM and 1 μ M-rh-123 for 1 h at 37 °C in culture medium (EMEM) supplemented with 2.5% (v/v) newborn-calf serum. After loading, the cells were washed three times with a respiratory Krebs–Ringer–Henseleit medium buffered to pH 7.4 with imidazole (R-KRHI; 110 mM-NaCl/5 mM-KCl/1 mM-MgSO₄/1.25 mM-CaCl₂/8 mM-NaHCO₃/0.5 mM-Na₂HPO₄/0.5 mM-KH₂PO₄/10 mM-glucose/15 mM-imidazole/1.3 mM-malate/5 mM-glutamate/5 mM-succinate). After washing, the cells were mounted in a microscope-stage incubation chamber for subsequent analysis by DFI. Single-excitation intensity-emission images of either fura-2 or rh-123 were captured using the appropriate excitation wavelength and emission filters. The Ca²⁺-sensitive wavelength of fura-2 was chosen (monochromator 1 was set at 350 nm) for imaging the spatial distribution of Ca²⁺ probe. Rh-123 was excited by using monochromator 2 set at 470 nm. The fura-2 excitation wavelength was reflected to the sample by a 400 nm dichroic long-pass filter. A 490–530 nm band-pass filter was used to select the fura-2 emission signal. The rh-123 excitation source was reflected by a 510 dichroic long-pass filter, whereas the emission signal was selected using a 520 nm long-pass filter. After acquiring intensity images representing each probe, the plasma membrane was selectively permeabilized with a 25 μ M-digtonin solution containing 5 mM-EGTA, 1 μ M-Ruthenium Red and 350 μ M-diltiazem (added as a 1:1 dilution from a twice-concentrated stock solution). After cell lysis (4 min) intensity images were again recorded for each probe. The mitochondrial membrane potential was dissipated using 200 μ M-DNP, and intensity images were recorded after 4 min.

DFI of cytosolic Ca²⁺ content using fura-2

Cytosolic Ca²⁺ was estimated by using fura-2 AM. Intracellular esterases hydrolyse the acetoxymethyl ester bonds and release the cell-impermeant free fura-2 into the cytosol. Fura-2 exhibits a Ca²⁺-dependent fluorescence (350 nm excitation) and a Ca²⁺-independent fluorescence (380 nm excitation) (Grynkiewicz *et al.*, 1985). Cardiac myocytes grown on glass coverslips in 2 ml of MEM were loaded with 1 μ M fura-2 AM dissolved in dimethyl sulphoxide (DMSO) (5 μ l of DMSO/2 ml of MEM). The cells were incubated with fura-2 AM for 30 min at 25 °C. Drug-treated myocytes were incubated with 50 μ M-doxorubicin at 37 °C in MEM containing 2.5% newborn-calf serum. After 3, 6, and 10 h of doxorubicin exposure, the myocytes were loaded with fura-2 AM. The cells were washed three times with R-KRHI buffer, placed in the chamber and analysed by DFI at room temperature to inhibit beating. Using the average of four frames for each wavelength, we used ratioed images (350 nm/380 nm) to determine the cytosolic free Ca²⁺. Control myocytes (non-drug-treated cells) loaded with fura-2 were used to adjust the slit widths of the excitation monochromators such

Fig. 1. Intensity images of cardiac cells co-loaded with fura-2 (FURA-2) and rh-123 (Rh123)

DFI images selective for fura-2 (A, B and C) were recorded using 350 nm excitation, whereas selective rh-123 images D, E and F were recorded using 470 nm excitation. The respective emission filters are described in the Experimental section. Images A and D represent intact cells, whereas images B and E were recorded after selective permeabilization of the plasma membrane with 25 μ M-digitonin. Images C and F were recorded after the addition of the uncoupler 2,4-DNP.

that the ratio was set to 1 in order to minimize the dynamic range in which the camera must work (Tsien & Harootunian, 1990). The adjustment was made under an intensity-display format of both the 350 nm and 380 nm signals and using the video-adjust colour-map display to adjust the sensitivity of the camera. The slit widths were adjusted such that the intensity from both the 350 and 380 signals were equal. The sensitivity of the camera gain was adjusted such that little to no flecks of red appear, which represented the high end of the camera. The low end of the camera (offset) was set before adjusting the sensitivity by closing off any light to the camera and adjusting the offset such that a 50/50 mixture of blue and black was being displayed on the colour monitor. If the histogram mode was activated, for the low-end adjustment, we tried to ensure that half of the intensity histogram peak was below the detection limits of the camera (i.e. a 50:50 mixture of the signal should be on and off scale). Final slit widths were 2 and 1.5 nm for monochromators 1 and 2, corresponding to the excitation wavelengths 350 and 380 nm respectively. Alternating excitation wavelengths were reflected to the sample by a 400 nm dichroic long-pass filter. A 490–530 nm band-pass filter was used to select the fura-2 emission signal. Autofluorescence in the presence or absence of doxorubicin at either 350 or 380 nm excitation did not interfere with the total emission signal.

DFI of total cellular Ca^{2+}

Total cellular Ca^{2+} was estimated using fura-2 AM and cardiac

myocytes grown on glass coverslips. After 0, 3, 6 and 10 h of doxorubicin exposure, myocytes were loaded with 2.5 μ M-fura-2 AM for 1 h at 37 °C. After loading, cells were washed three times with R-KRHI buffer and mounted in a chamber for subsequent analysis by DFI as described above.

DFI of mitochondrial membrane potential

Mitochondrial membrane potential in intact myocytes was estimated using rh-123 (Chen, 1988) and myocytes grown on glass coverslips as described above. After 0, 3, 6 and 10 h of doxorubicin exposure, the myocytes were loaded with 1 μ M-rh-123 for 1 h at 37 °C. The cells were then washed three times with R-KRHI buffer and mounted in a chamber for subsequent DFI. Rh-123 was excited at 470 nm. A 510 nm dichroic long-pass filter was used to reflect the excitation wavelength to the sample, whereas a 520 nm long-pass filter was used to help select the emission wavelength. In addition, a 490–530 nm band-pass filter was placed after the 520 nm long-pass emission filter to reduce further the fluorescent emission from doxorubicin (550–625 nm) and increase the selective emission of rh-123 (525 nm).

Measurement of cellular ATP

Cellular ATP content was determined by h.p.l.c. on cell extracts taken from cell monolayers which had been plated in 35 mm dishes. After appropriate treatments, the cell monolayers were rinsed twice with ice-cold 0.1 M-potassium phosphate buffer, pH 7.4, and placed on ice. An aliquot of ice-cold 0.1 M- HClO_4

Fig. 2 (above). Effects of loading conditions on compartmentalization of fura-2 (FURA)

The photograph represents ratioed images (350 nm/380 nm) of fura-2. Image A, cytosolic fura-2 from myocytes loaded with 1 μ M-fura-2 AM for 30 min at 25 °C. Image B, myocytes shown in image A after permeabilization of the plasma membrane with 25 μ M-digtonin (DIG). Image C, total cellular fura-2 from myocytes loaded with 2.5 μ M-fura-2 AM for 1 h at 37 °C. Image D was recorded after permeabilization of myocytes shown in image C, which represents fura-2 localized within the mitochondria.

Fig. 3 (opposite, top). Effect of doxorubicin (DOX) on cytoplasmic Ca^{2+}

The images represent pseudocolour assignments of intensity-weighted ratios of fura-2 (350 nm/380 nm). Ratios of 1 for control cells were set by slit width adjustments described under the Experimental section. Images A, B, C and D, cardiac cells loaded with 1 μ M-fura-2 AM for 30 min at 25 °C after 0, 3, 6 and 10 h of exposure to 50 μ M-doxorubicin respectively.

Fig. 4 (opposite, bottom). Effect of doxorubicin (DOX) on mitochondrial (MITO) and total cellular Ca^{2+}

The images represent intensity-weighted ratios of fura-2 (350 nm/380 nm). Images A, C, E and G represent total cellular Ca^{2+} from intact cells. Images B, D, F and H (mitochondrial Ca^{2+}) are respectively images A, C, E and G after selective permeabilization of the plasma membrane with 25 μ M-digtonin.

(1 ml) was added to each dish to extract the ATP. The monolayers were scraped off and collected in 2 ml centrifuge vials. The solution was adjusted to pH 5 with 1.0 M-KOH (0.5 ml) dissolved in mobile phase, at which time a white precipitate of KClO_4 formed. The resultant suspension was centrifuged at 11 000 g for 1 min. The supernatant was passed through a 0.45 μ m-pore-size filter and immediately injected into the h.p.l.c. apparatus for subsequent analysis. Chromatography was performed using Waters h.p.l.c. equipment. Detection of ATP was achieved using a variable-wavelength detector set at 254 nm. The stationary phase consisted of a Waters 4 μ m Radial PAK C_{18} cartridge. The mobile phase (1 ml/min) consisted of an isocratic elution using 5% acetonitrile/0.1 M- KH_2PO_4 , adjusted to pH 5, and 0.001 M-tetrabutylammonium hydrogen sulphate as an ion-pairing agent. A standard curve was constructed using solutions containing known concentrations of ATP which had been treated similarly to the extracted cell samples. ATP content was normalized for the protein content of the dish and expressed per mg of cell

protein. Protein was determined by the microbiuret method (Bailey, 1967).

RESULTS AND DISCUSSION

Recently we reported that the production of intracellular reactive oxygen species by doxorubicin may result from changes in mitochondrial calcium homeostasis (as measured by indirect techniques) (Chacon & Acosta, 1991). Thus the purpose of the present study was to evaluate the direct effects of doxorubicin on mitochondrial Ca^{2+} . To estimate mitochondrial Ca^{2+} , we took advantage of fura-2's ability to localize within the mitochondria of fura-2-loaded cells. By dual-loading the myocytes with fura-2 and rh-123, we could selectively image the intensity-emission signals from either probe. For fura-2 emission intensities, we chose the Ca^{2+} -sensitive excitation wavelength of 350 nm. Fura-2 displays a diffuse labelling of the cytosol (Fig. 1, image A), whereas rh-123 shows a selective localization of the dye to the

mitochondria (Fig. 1, image D). After selective permeabilization of the plasma membrane with 25 μM -digitonin (Fig. 1, images B and E) we found that both images (fura-2 and rh-123 respectively) were essentially superimposable. Our permeabilizing experiments with digitonin, as shown in Fig. 1, images B and E, included the incorporation of EGTA to chelate Ca^{2+} present in the incubation buffer and the addition of malate, glutamate, and succinate to provide essential respiratory substrates. The exclusion of these additives resulted in a collapse of the mitochondrial membrane potential immediately upon permeabilization of the plasma membrane (results not shown). To release both Ca^{2+} and rh-123 from the mitochondria, we added the protonophore DNP to collapse the mitochondrial membrane potential (Fig. 1, images C and F respectively). These data support mitochondrial localization of fura-2.

Loading conditions (1 μM -fura-2 AM for 30 min at 25 °C) were determined to keep the majority of the fura-2 within the cytosolic compartment (Fig. 2, image A). After permeabilization of the plasma membrane with digitonin (Fig. 2, image B), we found that the majority of the fura-2 signal was lost, suggesting that the fura-2 was localized mainly to the cytosolic compartment. When the myocytes were incubated with 2.5 μM -fura-2 AM for 1 h at 37 °C (Fig. 2, image C), we found that, after permeabilization of the plasma membrane, some of the fura-2 had sequestered within the mitochondria (Fig. 2, image D). We added Ruthenium Red, a mitochondrial Ca^{2+} -uptake blocker (Nichols & Akerman, 1982), and diltiazem, an inhibitor of mitochondrial Ca^{2+} release (Rizzuto *et al.*, 1987), to the permeabilizing solution to inhibit mitochondrial Ca^{2+} uptake and release, thus minimizing mitochondrial Ca^{2+} artefacts upon permeabilization. Therefore Fig. 2, image D, represents mitochondrial Ca^{2+} (Fig. 2, image C, total cellular Ca^{2+} ; Fig. 2, image A, cytosolic Ca^{2+}).

Cytosolic free Ca^{2+} in our myocardial-cell preparation was essentially unaffected after 3–6 h of 50 μM -doxorubicin (Fig. 3) in 100 % of the cells examined (Table 1). A greater-than-2-fold increase in cytosolic free Ca^{2+} was observed in 12 % of the cells examined after 10 h of exposure to 50 μM -doxorubicin. This rise in Ca^{2+} paralleled the release of the cytosolic marker enzyme lactate dehydrogenase, which was detectable after 12 h (Chacon & Acosta, 1991). Considering that the cells were loaded with fura-2 AM for 30 min after a 10 h exposure to doxorubicin, one cannot rule out the possibility that some of the cells in Fig. 3, image D, represent cells with leaky plasma membranes.

Control cells (100 %) showed a ratio of approx. 1.0 for total cellular Ca^{2+} (Fig. 4, image A). Permeabilization of the control cells with digitonin revealed that 98 % of the mitochondria also retained a ratio of 1.0 for Ca^{2+} (Fig. 4, image B). After 3 h of doxorubicin treatment, 85 % of the myocytes showed a ratio of 1 for total cellular Ca^{2+} (Fig. 4, image C). Permeabilization showed that 14 % of the mitochondria exhibited a greater-than-2-fold increase in Ca^{2+} (Fig. 4, image D). After 6 h of doxorubicin treatment, 23 % of the cells exhibited greater-than-2-fold increases in total cellular Ca^{2+} , and with 50 % of their mitochondria also showing greater-than-2-fold increases (Fig. 4, images E and F respectively). After 10 h of doxorubicin treatment, 53 % of the cells showed greater-than-2-fold increases in total cellular Ca^{2+} and with 66 % of their mitochondria demonstrating Ca^{2+} ratios greater than 2.0 (Fig. 4, images G and H respectively). Interestingly, we observed some cells that experienced greater-than-2-fold increases in mitochondrial Ca^{2+} (Fig. 4, image E), whereas total cellular Ca^{2+} remained similar to controls (Fig. 4, image C versus image A). In Fig. 4, images D and F, the intensity-weighted ratio presentation did not indicate the mitochondrial fraction of the respective cells shown in Fig. 4, images C and E, to be higher than a ratio of 1. An explanation

Table 1. Effect of doxorubicin on the percentage of cells and mitochondria exhibiting greater-than-2-fold increases in Ca^{2+}

Cytosolic Ca^{2+} was estimated using ratioed images (350 nm/380 nm) of myocytes loaded with 1 μM -fura-2 AM for 30 min at 25 °C. Total cellular Ca^{2+} represents myocytes loaded with 2.5 μM -fura-2 AM for 1 h at 37 °C. Mitochondrial Ca^{2+} was determined after total cellular Ca^{2+} recordings by permeabilization of the plasma membrane with 25 μM -digitonin. Values are from three independent DFI experiments with each percentage calculated from a total of 150 ± 50 fura-2-loaded cells.

Time (h)	Percentage of cells/mitochondria exhibiting > 2-fold increase in Ca^{2+}		
	Cytosolic	Total cellular	Mitochondrial
0	0	0	2
3	0	15	14
6	0	23	56
10	12	53	66

may be that the ratioed signals from the mitochondrial regions in the intact cells did not constitute a significant change when compared with the total cellular fura-2 signal (M. Poenie, personal communication). Despite the ambiguity, it is apparent that doxorubicin induced a time-dependent increase in mitochondrial Ca^{2+} (Table 1). Considering the elaborate systems contained in cells which regulate the concentration of Ca^{2+} , it is not surprising that the cytosolic Ca^{2+} levels in cells treated with doxorubicin for 3–6 h were similar to control values (Fig. 3, images B and C) and that mitochondrial Ca^{2+} may increase 2-fold (Fig. 4, images D and F). These data suggest that the mitochondria may be involved in buffering cytoplasmic Ca^{2+} .

The doxorubicin-induced time-dependent increase in mitochondrial Ca^{2+} at both the 3 and 6 h time points (Table 1) paralleled the intracellular production of reactive oxygen species (Chacon & Acosta, 1991). We had postulated that the doxorubicin-induced formation of superoxide radical anions might be due to a cycling of mitochondrial Ca^{2+} . Concomitant with the energy-dependent uptake of Ca^{2+} is the electroneutral release of Ca^{2+} that provides a continuous cycling of the cation across the mitochondrial inner membrane (Crompton, 1985). Because doxorubicin may cause mitochondrial Ca^{2+} accumulation (Singal *et al.*, 1987) and at the same time an inability to sequester accumulated calcium (Sokolove & Shinaberry, 1988), it is conceivable that the observed increases in mitochondrial calcium. Fig. 4, images D, F and H, may indirectly represent mitochondrial Ca^{2+} cycling.

Mitochondrial Ca^{2+} accumulation is a process made energetically possible by either respiratory substrate oxidation or ATP hydrolysis (Scarpa, 1979; Gunter & Pfeiffer, 1990). Hence, Ca^{2+} transport driven by either respiration or ATP hydrolysis occurs at the expense of a common pool of energy maintained by the electrochemical proton gradient. Thus futile mitochondrial Ca^{2+} cycling would result in a dissipation in the mitochondrial membrane potential. DFI of rh-123, a specific fluorescent probe used to label and monitor mitochondrial membrane potential (Chen, 1988), served as a measure of the relative mitochondrial membrane potential in cardiac cells exposed to doxorubicin. Chemical agents that dissipate the mitochondrial membrane potential, such as 2,4-DNP, prevent rh-123 uptake (Chen, 1988). Fig. 5 shows intensity images of rh-123 and the time-dependent

Fig. 5. Effect of doxorubicin (DOX) on the mitochondrial membrane potential

The images represent fluorescent intensities of accumulated rh-123 observed by digitized fluorescence imaging. Images A, B, C and D are after 0, 3, 6 and 10 h of exposure to 50 μ M-doxorubicin respectively.

collapse of the mitochondrial membrane potential in cardiac cells exposed to 50 μ M-doxorubicin. Fig. 5, image A, illustrates a group of control myocytes with regions of intense staining by rh-123. Decreased rh-123 fluorescence was observed in myocytes exposed to 50 μ M-doxorubicin for 3 h (Fig. 5, image B). After 6 h of doxorubicin treatment there appears to be a greater collapse in the mitochondrial membrane potential relative to the 3 h time point, as indicated by a reduction in fluorescent intensity of rh-123 (Fig. 5, image C versus image B). After 10 h of doxorubicin treatment there is almost a complete collapse in the mitochondrial membrane potential, as noted by faint fluorescence of rh-123 (Fig. 5, image D). Our results showed a doxorubicin-induced dissipation in the mitochondrial membrane potential which paralleled an increase in mitochondrial Ca^{2+} (Figs. 5 and 4 respectively). These data suggest that doxorubicin appears to induce a cycling of mitochondrial Ca^{2+} that results in a dissipation of the mitochondrial membrane potential.

Exposure of cardiac myocytes to 50 μ M-doxorubicin resulted in a time-dependent decrease in cellular ATP content. Cellular ATP was decreased significantly from 27.5 ± 1.1 nmol/mg in controls ($P < 0.01$) to 22.0 ± 0.2 after 6 h and to 11.0 ± 2 after 10 h ($P < 0.001$) of exposure to 50 μ M-doxorubicin. The delay in onset of ATP depletion, relative to increased mitochondrial Ca^{2+} , is reflected in the rate of dissipation of the mitochondrial membrane potential and suggests that ATP depletion is secondary to a decreased membrane potential. It is noteworthy that cell death ensues after 12 h of exposure to 50 μ M-doxorubicin (Chacon & Acosta, 1991). The underlying

mechanism responsible for irreversible cell injury remains to be determined.

Our hypothesis, based on our previous report (Chacon & Acosta, 1990) and presented herein, is that doxorubicin produces an increase in mitochondrial Ca^{2+} that is associated with the production of reactive oxygen species and a dissipation in the mitochondrial membrane potential. In addition, doxorubicin-induced mitochondrial dysfunction may result in a depletion in cellular ATP. Previous reports have indicated that mitochondrial Ca^{2+} overload may be a mechanism for cellular injury by doxorubicin (Olson *et al.*, 1974). On the other hand, reports by Jensen (1986) challenged the idea of a Ca^{2+} -mediated onset of cell injury by showing a decreased myocardial contractility of rats treated with doxorubicin, suggestive of decreased cytoplasmic calcium. In addition, Ito *et al.* (1990) have shown an early onset of selective inhibition in cardiac myofibrillar gene expression *in vivo* and *in vitro* by low concentrations of doxorubicin. Such an early onset of myofibrillar dysfunction would present itself as a decrease in contractility, despite high or low cytoplasmic Ca^{2+} . It is therefore conceivable that the cardiospecific toxicity of doxorubicin may be explained by its selective inhibition of cardiac gene expression in conjunction with its ability to target and derange mitochondrial Ca^{2+} homeostasis.

We are especially grateful to Dr. Martin Poenie, University of Texas at Austin, for his time and helpful suggestions. E.C. is supported by a Procter and Gamble Toxicology Fellowship. D.A. is a Burroughs Wellcome Scholar in Toxicology. A grant from the Upjohn Co. also helped to support this study.

REFERENCES

- Bailey, J. L. (1967) in *Techniques in Protein Chemistry* (Laggett, J., ed.), pp. 341–346, Elsevier, New York
- Chacon, E. & Acosta, D. (1991) *Toxicol. Appl. Pharmacol.* **107**, 117–128
- Chen, L. B. (1988) *Annu. Rev. Cell. Biol.* **4**, 155–181
- Crompton, M. (1985) *Curr. Top. Membr. Transp.* **25**, 231–276
- Davis, M. H., Atschuld, R. A., Jung, D. W. & Brierley, G. P. (1987) *Biochem. Biophys. Res. Commun.* **149**, 40–45
- DiMarco, A. (1975) *Cancer Chemother. Rep.* **6**, 91–106
- Gores, J. J., Nieminen, A.-L., Wray, B. E., Herman, B. & Lemasters, J. J. (1989) *J. Clin. Invest.* **83**, 386–396
- Gryniewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Gunter, T. E. & Pfeiffer, D. R. (1990) *Am. J. Physiol.* **258**, C755–C786
- Ito, H., Miller, S. C., Billingham, M. E., Akimoto, H., Torti, S. V., Wade, R., Gahlmann, R., Lyons, G., Kedes, L. & Torti, F. M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4275–4279
- Jensen, R. A. (1986) *J. Pharmacol. Exp. Ther.* **236**, 197–203
- Johnson, R. K., Zee Cheng, R. K. Y., Lee, W. W., Acton, E. M., Henry, D. W. & Cheng, C. C. (1979) *Cancer Treat. Rep.* **63**, 425–439
- Kapuscinski, J. & Darynkiewicz, Z. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6302–6306
- Krishan, A., Israel, M., Modest, E. J. & Frei, E. (1976) *Cancer Res.* **36**, 2108–2109
- Lipscombe, D., Madison, D. V., Poenie, M., Reuter, H., Tsien, R. W. & Tsien, R. Y. (1988) *Neuron* **1**, 355–365
- Mbuga, P. M., Welder, A. A. & Acosta, D. (1988) *Toxicology* **52**, 187–207
- Nichols, D. G. & Akerman, K. (1982) *Biochim. Biophys. Acta* **683**, 47–88
- Olson, R. D. & Mushlin, P. S. (1990) *FASEB J.* **4**, 3076–3086
- Olson, H. M., Young, D. M., Prieur, D. J., LeRong, A. F. & Regan, R. L. (1974) *Am. J. Pathol.* **77**, 439–454
- Powis, P. K. (1989) *Free Radical. Biol. Med.* **6**, 63–101
- Rizzuto, R., Bernardi, P., Favaron, M. & Azzone, G. F. (1987) *Biochem. J.* **246**, 271–277
- Scarpa, A. (1979) in *Membrane Transport in Biology* (Giebisch, G., Tosteson, D. C. & Ussing, H. H., eds.), pp. 263–355, Springer, New York
- Singal, P. K., Deally, C. M. & Weinberg, L. E. (1987) *J. Mol. Cell. Cardiol.* **19**, 817–828
- Sokolove, P. M. & Shinaberry, R. G. (1988) *Biochemical Pharmacol.* **37**, 803–812
- Steinberg, S. F., Bilezikian, J. P. & Al-Awqati, Q. (1987) *Am. J. Physiol.* **253**, C744–C747
- Tsien, R. Y. & Harootunian, A. T. (1990) *Cell Calcium* **11**, 93–109
- Wenzel, D. G., Wheatly, J. W. & Byrd, G. D. (1970) *Appl. Pharmacol.* **17**, 774–785

Received 2 April 1991/5 July 1991; accepted 22 July 1991