FULL PAPER *Toxicology*

Mechanisms Responsible for Reduced Cardiotoxicity of Mitoxantrone Compared to Doxorubicin Examined in Isolated Guinea-Pig Heart Preparations

Akihito CHUGUN[1)]**, Tsuyoshi UCHIDE[2)], Chieko TSURIMAKI[2)], Hajime NAGASAWA[2)], Takushi SASAKI[2)], Shunji UENO[3)], Kiyohiko TAKAGISHI[4)], Yukio HARA[1)] and Kyosuke TEMMA[2)]*

[1)]Laboratories of Veterinary Pharmacology, [2)]Toxicology, [3)]Veterinary Public Health and [4)]Cell and Molecular Biology, School of Veterinary Medicine, Kitasato University, Towada-shi, Aomori 034–8628, Japan

(Received 18 September 2007/Accepted 21 November 2007)

ABSTRACT. We reported previously that doxorubicin, an anticancer agent that has an anthracycline structure, alters Ca^{2+} releasing and uptake mechanisms in the sarcoplasmic reticulum of myocardial cells. These effects of doxorubicin are apparently related to its cardiotoxicity. Mitoxantrone is a similar anticancer agent with an anthracenedion structure that has been shown to be significantly less cardiotoxic. In the present study, the effects of mitoxantrone on the functions of the sarcoplasmic reticulum were examined in isolated muscle preparations obtained from the guinea-pig heart. In electrically-stimulated left atrial muscle preparations, incubation *in vitro* for 4 hr with 30 or 100 μM mitoxantrone significantly prolonged the time to the peak of twitch tension, markedly increased the developed tension observed at lower stimulation frequencies, thereby attenuating the slope of positive force-frequency relationships, and increased the postrest contraction observed after a 60-sec quiescent period. In myocytes isolated from ventricular muscles, 30 μM mitoxantrone increased the peak and the size of intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$), and prolonged the time to peak $[Ca^{2+}]i$. In skinned muscle fiber preparations obtained from the left ventricular muscle, 30 μM mitoxantrone significantly increased the caffeine-induced contraction without affecting the Ca^{2+} sensitivity of contractile proteins. These results suggest that mitoxantrone enhances Ca^{2+} release from the sarcoplasmic reticulum in isolated atrial muscle preparations obtained from the guinea-pig heart. Apparent enhancement of the sarcoplasmic reticulum functions, in contrast to anthracyclines that has been shown to suppress these functions, seems to explain the relative lack of marked cardiotoxicity of mitoxantrone.

KEY WORDS: caffeine-induced contraction, Ca^{2+} release and uptake by sarcoplasmic reticulum, inotropic effects, intracellular Ca^{2+} concentration, mitoxantrone.

J. Vet. Med. Sci. 70(3): 255–264, 2008

Anthracyclines are efficacious anti-tumor agents; however, their clinical usefulness is limited by serious cardiotoxic effects. Mitoxantrone, an anthracenedion derivative, has been synthesized in an attempt to develop an agent that has a reliable anticancer activity without the compromising cardiotoxic effects [9, 35].

Human as well as animal studies demonstrated that the cardiotoxic effects of mitoxantrone are significantly less severe at clinically equivalent anticancer doses compared to those observed with anthracyclines [2, 9, 11, 13, 20, 22, 33].

We reported previously [7] that *in vivo* treatment of rats with doxorubicin results in a prolongation of the time to the peak of twitch tension, a reduction of the developed tension observed at lower stimulation frequencies and an attenuation of the postrest contraction (contraction that is evoked by the first stimulus after a brief quiescent period) observed in electrically-stimulated heart muscle preparations obtained from the drug-treated animals. These changes developed slowly with time after intravenous injections of doxorubicin. The results show that doxorubicin causes an irreversible myocardial dysfunction. The impairment of the Ca^{2+} handling mechanism of the sarcoplasmic reticulum is apparently the cause of myocardial dysfunction and hence the doxorubicin-induced late (chronic) cardiotoxicity.

It has been reported that the time to the peak of twitch tension depends primarily on the rate of Ca^{2+}-uptake by the sarcoplasmic reticulum [5], whereas the size of muscle contractions observed at lower stimulation frequencies [1, 17, 21, 24, 25, 27] or the postrest contraction [4, 27] depends on the Ca^{2+} release from the sarcoplasmic reticulum. Consistent with these concepts, we observed that doxorubicin-treatment of the rat results in a significant reduction of the caffeine-induced contraction in skinned muscle fiber preparations, although Ca^{2+} sensitivity was unchanged [7].

Similar changes, namely the prolongation of the time to the peak of twitch tension and the reduction of postrest contraction were observed in heart muscle preparations isolated from non-drug-treated rats or guinea pigs, and exposed a relatively long period to doxorubicin *in vitro* [10, 18, 29]. These results indicate that cardiac dysfunctions produced by doxorubicin *in vitro* resemble those induced by the treatment of animals *in vivo*.

In the present study, the effects of mitoxantrone on the Ca^{2+} releasing mechanism of the sarcoplasmic reticulum were examined from changes in the force-frequency relationships and the postrest contractions using isolated guinea-pig heart preparations exposed *in vitro* to mitox-

* CORRESPONDENCE TO: TEMMA, K., Laboratory of Toxicology, School of Veterinary Medicine, Kitasato University, 35–1, Higashi 23-bancho, Towada-shi, Aomori 034–8628, Japan.
e-mail: temma@vmas.kitasato-u.ac.jp

** PRESENT ADDRESS: CHUGUN, A., Department of Pharmacology, School of Medicine, Juntendo University, Tokyo 113–8421, Japan.

antrone for a relatively long period (4 hr). In addition, the effects of mitoxantrone on the Ca^{2+}-sensitivity of the contractile proteins and on the Ca^{2+}-releasing mechanism of the sarcoplasmic reticulum were examined using skinned fiber preparations. Further, in order to directly examine whether mitoxantrone increase intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$), the $[Ca^{2+}]i$ were estimated in fura-2 loaded ventricular myocytes.

MATERIALS AND METHODS

Animals: Male Hartly guinea pigs obtained from Japan SLC Inc., Hamamatsu, Japan, were used. The animals were housed 3 per cage and maintained in a room with a constant temperature (at 25 ± 1°C), humidity (60 ± 10%) and lighting conditions (the lights were on between 8 a.m and 8 p.m), and were fed a standard laboratory chow.

All protocols in this study were approved by the Institutional Animal Care and Use Committee at Kitasato University and in accordance with the Guide for the Use of Laboratory Animals issued by the United State Department of Health and Human Services.

Cell membrane-intact atrial muscle preparations: Male Hartly guinea pigs (350–450 g) were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneal injection). The hearts were rapidly removed and perfused with a modified Krebs-Henseleit bicarbonate buffer solution (NaCl, 118 mM; $NaHCO_2$, 27.2 mM; KCl, 4.8 mM; KH_2PO_4, 1.0 mM; $CaCl_2$, 1.2 mM; $MgSO_4$, 1.2 mM; glucose, 11.1 mM) through the aorta using a Langendorff apparatus for 5 min to remove visible blood. The buffer solution was saturated with a 95% O_2/5% CO_2 gas mixture and maintained at 30°C (pH 7.4). After all visible blood was removed from the heart, strips of the left atrial muscle were excised, and vertically suspended in a chamber containing 20 m*l* of the above oxygenated buffer solution. The atrial muscle preparations were electrically stimulated at 2 Hz with square-wave pulses of 3-msec duration at a voltage approximately 30% above the threshold. A pair of platinum electrodes was used for electrical-field stimulation. Isometric force of contraction was continuously recorded using a force displacement transducer (TB-651: Nihon Kohden, Tokyo, Japan) and a pen recorder (WI621G: Nihon Kohden, Tokyo, Japan) with the resting tension adjusted to 1.0 g [7, 29]. After a 90- to 120-min equilibration period with 2-Hz stimulation, the stimulator was turned off for 60 sec. The first contraction, observed when the 2-Hz electrical stimulation was resumed, was recorded as the postrest contraction, and the force-frequency relationship was examined 20 min later. Subsequently, mitoxantrone or 20 μl of double distilled water for the vehicle was added to a 20-m*l* incubation solution when the developed tension observed at 2-Hz stimulation was stabilized about 10 min later. The postrest contraction was examined again following 4-hr incubation with mitoxantrone, and the force-frequency relationship was examined 20 min later. The effects of doxorubicin on the force of contraction were observed at 2-Hz stimulation by adding 100 μM doxorubicin (final concentration) to the incubation medium after a 90- to 120-min equilibration period.

Measurements of $[Ca^{2+}]i$ concentrations

Single cell isolations: Myocytes were isolated from ventricular muscle of guinea-pig hearts using collagenase (0.66 mg/m*l*) as described previously [30, 31]. The myocytes were loaded with fura-2 by incubating at 37°C for 30 min in a HEPES buffer solution containing 1.5 μM fura-2/AM and bovine serum albumin (20 mg/m*l*). The myocytes were washed 3 times to remove extracellular fura-2/AM, and stored at room temperature (18–23°C) under a continuous stream of 100% O_2 until use.

Recording of $[Ca^{2+}]i$: The methods used to record and analyze $[Ca^{2+}]i$ in fura-2 loaded myocytes were almost the same as those previously described [32, 33]. In this study, a CCD camera with the picture elements vertically reduced to one fourth (slit-scan video camera; C-2400-77, Hamamatsu Photonics, Hamamatsu, Japan). This camera is capable of recording fluorescence images at 4-msec intervals. Myocytes were placed in an open chamber with a thin quartz-glass bottom (0.2 m*l* in volume) on the stage of an inverted epifluorescence microscope (Nikon TMD, Nihon Kogaku, Tokyo, Japan), and were superfused at a flow rate of 0.1 m*l*/min with a modified Krebs-Henseleit bicarbonate buffer solution (described above) with 2.5 mM sodium pyruvate. Myocytes were electrically stimulated at 1.5 Hz at 30°C by means of a pair of platinum electrodes for electrical field stimulation.

The ratio of fluorescence intensities observed at 340 and 380 nm excitation (340/380 nm) in an entire myocyte was analyzed using an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan) after subtracting the background. In this recording system, 30 μM mitoxantrone did not change the fluorescence ratio.

The $[Ca^{2+}]i$ in myocytes was estimated from the calibration curves for Ca^{2+} concentration constructed using a Ca^{2+}-EGTA buffer solution containing 22 nM to 2.36 μM Ca^{2+} (pCa 7.66 to 5.63) plus 10 μM fura-2 pentapotassium salt, as described earlier [30, 31].

Skinned fiber preparations

Skinned fiber preparations for caffeine-induced contraction studies: Skinned fibers were prepared from the left ventricular muscle of the guinea-pig heart using the method described by Chugun *et al.* [7]. Briefly, muscle fibers (2–3 mm long, 100–200 μm wide) were dissected from the left ventricular muscle. One end of the muscle fiber was fixed to a tungsten rod (diameter 300 μm) and the other end was attached to an L-shaped tungsten bar connected to an isometric transducer (TB-651T: Nihon Khoden, Tokyo, Japan). The muscle fiber preparations were set horizontally in a 100-μl incubation chamber which was placed on the stage of an inverted microscope (IX 70: Olympus, Tokyo, Japan), and were incubated with a low concentration of saponin (50 μg/m*l*) for 30 min at 24°C in the relaxing solution containing 20 mM 3-(N-morpholino)-2-hydroxypropanesulfonic

acid (MOPS), 2 mM (b-aminoethyl-ether)-N, N, N', N'-tetraacetic acid (EGTA), 4.3 mM Na_2ATP and 5 mM Mg-methanesulfonate. This procedure completely disrupts cell membranes without altering membrane functions of the sarcoplasmic reticulum or the mitochondria [7, 16].

After the skinning procedure, skinned fiber preparations were incubated in the presence or absence of 30 μM mitoxantrone for 15 min in the relaxing solution containing 20 mM MOPS, 2 mM EGTA, 4.3 mM Na_2ATP, 5 mM Mg-methanesulfonate, and incubated in a Ca^{2+}-EGTA buffer solution containing 20 mM MOPS, 10 mM EGTA, 4.3 mM Na_2ATP, 5 mM Mg-methanesulfonate and 6.0 mM Ca^{2+} (pCa 6.19; free Ca^{2+}, 0.65 μM) for 2.5 min to load the sarcoplasmic reticulum with Ca^{2+}. Then the muscle fiber preparations were exposed to 20 mM caffeine in a low-EGTA relaxing solution (EGTA concentration was 0.05 mM instead of 2 mM) to observe the caffeine-induced contraction. The caffeine-induced contraction was examined in the presence or absence of mitoxantrone. The magnitude of the caffeine-induced contraction was expressed as a percentage of the maximum tension observed with Ca^{2+} concentration at pCa 4.37.

Skinned fiber preparations for Ca^{2+} concentration-force relationship studies: In these studies, we used muscle fiber preparations in which membrane functions of the sarcoplasmic reticulum and mitochondria were completely disrupted. Muscle fibers dissected from left ventricular muscles were set horizontally in a 100 μl chamber placed on the stage of an inverted microscope as described above, and incubated for 30 min in the relaxing solution containing a high concentration of saponin (1 mg/m*l*). This treatment completely abolished the effect of 20 mM caffeine to induce a contraction [6, 7, 30] showing the loss of functions of the sarcoplasmic reticulum. The preparations were exposed to a series of Ca^{2+} EGTA buffer solutions having Ca^{2+} concentrations ranging from pCa of 7.32 to 4.37 (free Ca^{2+}, 47.7 nM to 42.1 μM) to generate the control Ca^{2+} concentration-response curves. After 60 min incubation in the absence or presence of mitoxantrone, the Ca^{2+} concentration-response curves were obtained again. Values are shown as the percentage of maximum contraction observed at Ca^{2+} concentration at pCa of 4.37.

In these skinned fiber studies, buffer solutions were adjusted to pH 7.0 and the ionic strength of 0.17 (at 24°C) using KOH and K^+-methanesulfonate.

The free calcium concentrations were determined using the method described by Bers [3].

Chemicals and statistical analyses: Mitoxantrone, collagenase and fura-2 AM were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.), Roche Diagnostics Co. (Indianapolis, IN, U.S.A.) and Dojindo (Kumamono, Japan), respectively. All other chemicals used were of reagent grade. The data were analyzed by one way analysis of variance (ANOVA) followed by Tukey's or Dunnett's multiple comparison test. When appropriate, Student's paired *t*-test or unpaired *t*-test was used. The criterion for statistical significance is a *P* value of less than 0.05. All values are expressed as the mean ± S.E.

RESULTS

Force of contraction and the time to peak twitch tension in cell membrane-intact atrial muscle preparations: After 90 to 120 min equilibration period at 2-Hz stimulation, the postrest contraction was examined first, and the force-frequency relationship was observed 20 min later. Subsequently, mitoxantrone or double distilled water was added. The postrest contraction was examined again 4 hr after the addition of mitoxantrone or the vehicle. The force-frequency relationship was examined 20 min after the postrest contraction study.

Developed tension observed with 2-Hz stimulation at the end of the equilibration period (initial developed tension) was 9.98 ± 0.63 mN (n=21), and time to peak twitch tension was 91.4 ± 3.31 msec (n=21, Fig. 1 A and Table 1). In the drug-free solution, the developed tension gradually decreased with time, and reached 46.6 ± 7.5 % (n=5) of the initial value at the end of the 4-hr experimental period (Fig. 1 B and Table 1). The addition of 30 or 100 μM mitoxantrone (final concentration) increased the developed tension gradually. The developed tension was 10.4 ± 6.4% (n=5) or 23.1 ± 4.4% (n=7), respectively, above the initial value at 4 hr after the addition of 30 or 100 μM mitoxantrone (Fig. 1B and Table 1). Therefore, the developed tension was 63.8 % and 76.5% higher in the presence of 30 and 100 μM mitoxantrone, respectively, compared to those observed in the drug-free solution.

In these atrial muscle preparations, either 30 or 100 μM mitoxantrone prolonged the time to the peak of twitch tension in a concentration dependent manner (Figs. 1A and 1C, and Table 1).

Doxorubicin, 100 μM, also increased the developed tension and the time to peak twitch tension (Figs. 1B and 1C, Table 1). The increase in the developed tension was smaller, whereas the prolongation of the time to peak twitch tension was significantly greater, compared to those observed with 100 μM mitoxantrone (Table 1).

Control values for the postrest contraction was 19.6 ± 1.4 mN (n=6). Mitoxantrone, at 30 or 100 μM, significantly increased the size of the postrest contraction (Figs. 2A and 2B).

Cumulative increases in the stimulation frequency from 0.5 to 3 Hz at approximately 2.5-min intervals markedly increased the developed tension in control preparations (Fig. 3, control and Fig. 4A). Thus, the left atrial muscle preparations of guinea-pig hearts show a typical positive force-frequency relationship (positive staircase phenomenon) under the present experimental conditions.

The force-frequency relationship was reproducible 260 min later, although the developed tension observed at each stimulation frequency was approximately 33 to 74% of the corresponding values observed at the first trial (Fig. 4B, control). Under these conditions, 30 or 100 μM mitoxantrone caused a significant increase in developed tension

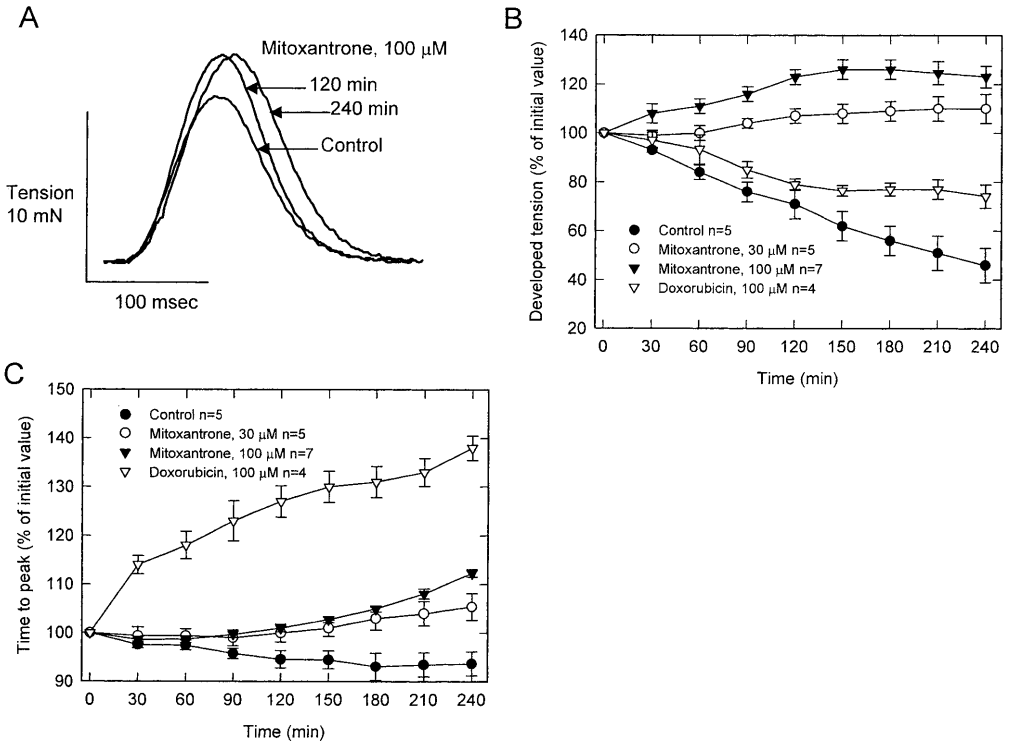

Fig. 1. Positive inotropic effects and prolongation of the time to peak twitch tension caused by mitoxantrone in isolated left atrial muscle preparations. Atrial muscle preparations were incubated at 30°C with 2-Hz electrical field stimulation for 90 to 120 min. Subsequently, mitoxantrone, either 30 or 100 μM, or 20 μl of double distilled water for control was added to the incubation solution. A, typical tracings of an increase in twitch tension and a prolongation of the time to peak twitch tension caused by 100 μM mitoxantrone. B and C, summarized results for the positive inotropic effects and the prolongation of the time to peak twitch tension by mitoxantrone. Values are expressed as percentage of the initial values observed at the end of the equilibration period. ●, control; ○, 30 μM mitoxantrone; ▼,100 μM mitoxantrone; ▽, 100 μM doxorubicin. Each point represents the mean of 4 to 7 experiments and vertical lines indicate the standard error of the mean.

Table 1. Effects of mitoxantrone or doxorubicin on the developed tension and the time to the peak of twitch tension observed in isolated left atrial muscle preparations of the guinea-pig heart

	Developed tension	Time to peak tension
Initial value	9.98 ± 0.63 mN (21) (100%)	91.4 ± 3.32 msec (21) (100%)
After 4 hr		
Control	46.6 ± 7.5 % (5)[a)]	93.7 ± 2.5 % (5)[a)]
Mitoxantrone, 30 μM	110.4 ± 6.4 % (5)[a,b)]	105.4 ± 2.7 % (5)[a,b)]
Mitoxantrone, 100 μM	123.1 ± 4.4 % (7)[a,b)]	112.3 ± 1.9 % (7)[a,b)]
Doxorubicin, 100 μM	74.2 ± 4.7 % (4)[a,b,c)]	134.2 ± 2.1 % (4)[a,b,c)]

See legend to Fig. 1. After an equilibration period, left atrial muscle preparations obtained from the guinea-pig heart were incubated for 4 hr in the absence or the presence of mitoxantrone or doxorubicin.
The numbers in parentheses indicate the number of experiments.
a) Values represent the percentage of initial values (mean ± SE).
Significantly different b) from the control value , or c) from the corresponding value observed in the presence of 100 μM mitoxantrone (Tukey, $P<0.05$).

observed at all frequencies (Fig. 4B). The increase was greater at slower stimulation frequencies compared with those observed at higher frequencies. In the presence of 100 μM mitoxantrone, the increase in developed tension elicited by 0.5- or 1-Hz stimulation was remarkable (Fig. 3, mitoxantrone, 100 μM and 4B).

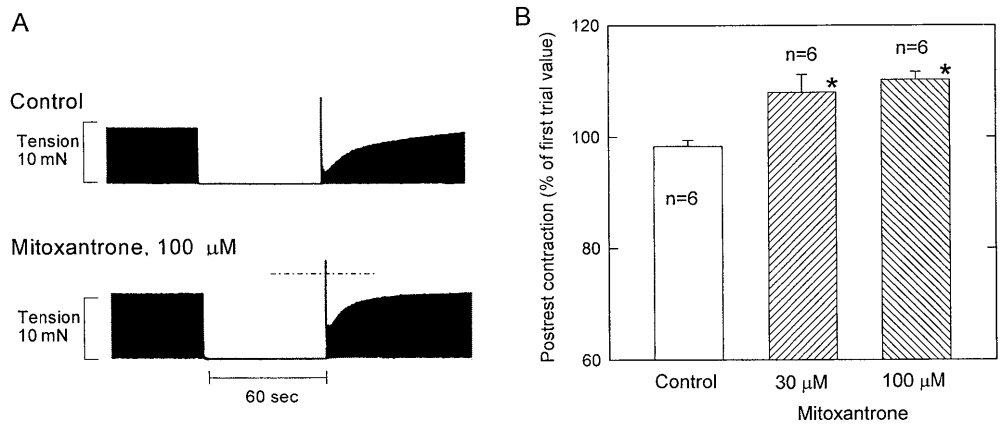

Fig. 2. Effects of mitoxantrone on postrest contraction in isolated left atrial muscle preparations. After a 90 to 120 min equilibration period at 2-Hz stimulation, postrest contraction was examined following a 60-sec quiescent period (first trial). Subsequently, mitoxantrone, either 30 or 100 μM, or 20 μl of double disitilled water for control was added to the incubation medium, and the postrest contraction was examined again 4 hr later. A, typical tracings of the increase in postrest contraction by 100 μM mitoxantrone (Control, upper panel; 100 μM, Mitoxantrone, lower panel). A dotted line at the lower panel indicates a level of control postrest contraction. B, summarized results for the increase in the postrest contraction by 30 or 100 μM mitoxantrone. The values are expressed as percentage of values observed at the first trial. Each bar represents the mean of 6 experiments. * Significantly different from the control values (Dunnett, $P<0.05$).

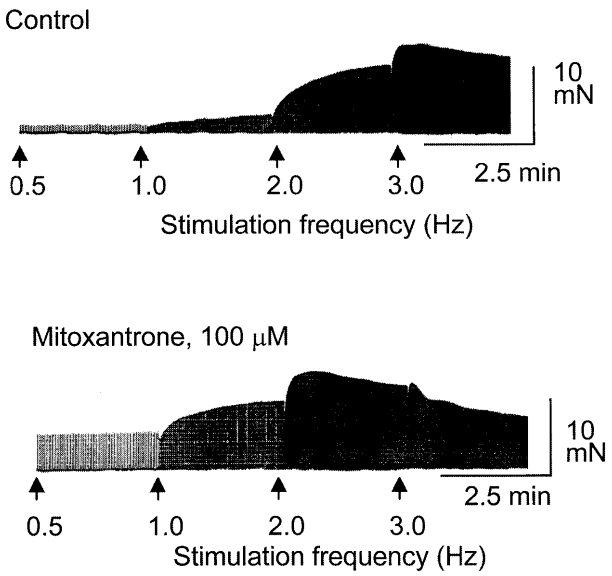

Fig. 3. Typical tracings of the force-frequency relationship observed in isolated left atrial preparations. After a 90 to 120 min equilibration period at 2-Hz stimulation, the postrest contration was examined first, and force-frequency relationships were examined 20 min after the postrest contraction study at stimulation frequencies ranging from 0.5 Hz to 3 Hz (Control). Subsequently, 100 μM mitoxantrone (final concentration) was added to the incubation medium, and the force-frequency relationships were examined again 260 min later (Mitoxantrone, 100 mM). The stimulation frequency was increased stepwise at approximately 2.5 min intervals.

Thus, mitoxantrone significantly increased the developed tension observed at lower stimulation frequencies attenuating the slope of the force-frequency relationship.

$[Ca^{2+}]i$ in single cells: Whether mitoxantrone increases the $[Ca^{2+}]i$, was studied in fura-2 loaded ventricular myocytes.

Under the present conditions (1.5 Hz, 30°C), diastolic $[Ca^{2+}]i$ was 204 ± 64 nM (n=8, Table 2). The $[Ca^{2+}]i$ rapidly increased responding to an electrical stimulation, and reached a peak at 81.9 ± 5.0 msec (n=8) after the electrical stimulation (Fig. 5 and Table 2). The peak $[Ca^{2+}]i$ was 686 ± 142 nM. These values did not change during a 45-min experimental period. Mitoxantrone, 30 μM, caused an increase in the peak $[Ca^{2+}]i$, initially (Fig. 5, 25 min), and then caused a prolongation of the time to peak $[Ca^{2+}]i$ (Fig. 5, 45 min) in addition to the increase in peak $[Ca^{2+}]i$. The size of $[Ca^{2+}]i$ (peak $[Ca^{2+}]i$ minus diastolic $[Ca^{2+}]i$) at the 25 min incubation with 30 μM mitoxantrone was greater than that of the control value. The diastolic $[Ca^{2+}]i$ has a tendency to slightly increase with time although changes were statistically not significant (Table 2).

Caffeine-induced contractions in skinned fiber preparations: Effects of mitoxantrone on the Ca^{2+} releasing mechanism of the sarcoplasmic reticulum were examined from the caffeine-induced contraction using skinned fiber preparations prepared with a low concentration of saponin. In these preparations, cell membranes were disrupted, but membrane functions of the sarcoplasmic reticulum and mitochondria were intact. The caffeine-induced contraction was transient reaching the peak within 3 sec (Fig. 6A, and see also Chugun *et al.* [7]). Because 20 mM caffeine produced the maximum contraction in skinned fiber preparations under the

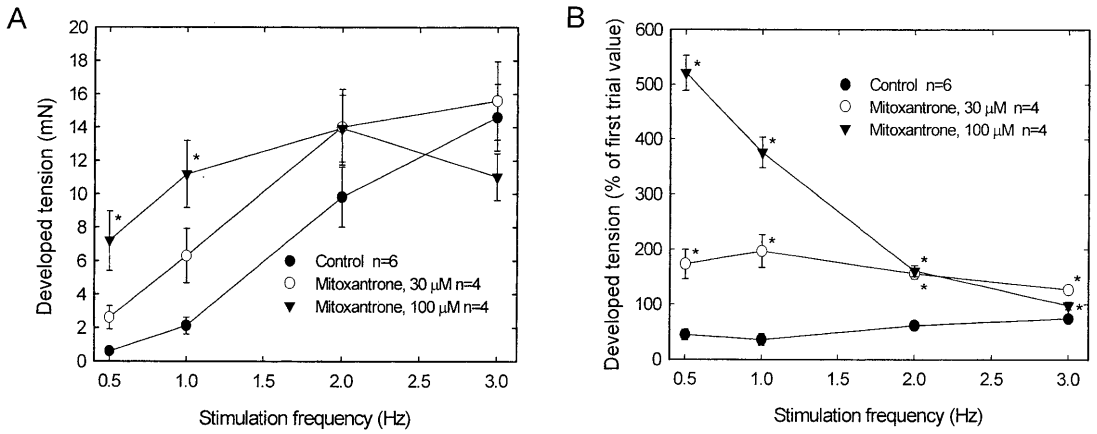

Fig. 4. Effects of mitoxantrone on force-frequency relationships in isolated left atrial muscle preparations. See legend to Fig. 3. After an equilibration period, the first postrest contraction study was performed and the force-frequency relationship was examined 20 min later (first trial). Subsequently, 30 (○) or 100 (▼) μM mitoxantrone, or 20 μl double distilled water for control (●) was added to the incubation medium. The force-frequency relationships were was examined again 260 min later (2nd trial). A, the force-frequency relationship data observed at the 2nd trial in the presence of 30 or 100 μM mitoxantrone. B, values of the 2nd trial are expressed as the percentage of the values observed at the first trial. Each value at A and B represents the mean of 4 to 6 experiments. * Significantly different from the control values (Dunnett, $P<0.05$).

Table 2. Effects of mitoxantrone on $[Ca^{2+}]i$ in myocytes isolated from ventricular muscles

Time of mitoxantrone exposure (min)	Diastolic $[Ca^{2+}]i$ (nM)	Peak $[Ca^{2+}]i$ (nM)	Size $[Ca^{2+}]i$ (%)	Time to peak (msec)
0	204 ± 64	686 ± 142	100	81.9 ± 5.0
25	212 ± 75	790 ± 165[a)]	123 ± 9[a)]	82.2 ± 4.3
45	241 ± 53	783 ± 143[a)]	112 ± 5	138.1 ± 12.0[a)]

After myocytes were equilibrated at 30°C for 30 min at 1.5 Hz stimulation, fluorescent images were taken at time zero (immediately before the addition of mitoxantrone), 25 min and 45 min later from the addition of 30 μM mitoxantrone.
Values are the mean ± SE. of 8 experiments.
a) Significantly different from the values observed at time zero (Paired *t*-test, $P<0.05$).

present conditions (data not shown), the contraction induced by 20 mM caffeine is considered to represent the maximum Ca^{2+} storage capacity corresponding to the maximal Ca^{2+} release.

The caffeine-induced contraction observed in a drug-free solution was 0.039 ± 0.002 mN (n=5). This value was 24.2 ± 0.9% of the maximum contraction (0.163 ± 0.017 mN, n=5) induced by a Ca^{2+}-EGTA buffer solution at pCa of 4.37. The exposure to 30 μM mitoxantrone for 15 min significantly increased the maximum contraction induced by caffeine (Fig. 6B) without affecting the maximum contraction caused by pCa of 4.37.

Thus, mitoxantrone enhances caffeine-induced contractions without affecting the maximal contraction produced by a high concentration of Ca^{2+} in skinned fiber preparations in which cytoplasmic membranes are disrupted.

Concentration-response curves for Ca^{2+} in skinned fiber preparations: The effect of mitoxantrone on the Ca^{2+} sensitivity of contractile proteins was examined using skinned fiber preparations that were exposed to a high concentration of saponin to disrupt functions of the sarcoplasmic reticulum and mitochondrial membranes as well as those of cell membranes. In these preparations, 20 mM caffeine failed to cause a contraction, indicating that the functions of Ca^{2+} uptake and storage mechanisms of the sarcoplasmic reticulum were completely destroyed and hence Ca^{2+} of the sarcoplasmic reticulum was totally drained [6, 7, 30]. Concentrations of Ca^{2+} ranging from pCa 7.32 to 4.37 (free Ca^{2+}, 47.7 nM to 42.1 μM) caused typical sigmoidal-shaped concentration-response curves (Fig. 7). The ED_{50} value (pCa) observed with control preparations was 6.10 ± 0.03 (n=13, Table 3). Under these conditions, 20 mM caffeine, a positive control, shifted the concentration-response curve for Ca^{2+} to the left. Mitoxantrone, however, failed to alter the ED_{50} value at either 30 or 100 μM.

These results show that Ca^{2+} sensitivity of the contractile proteins is not altered by mitoxantrone.

DISCUSSION

One prominent action of mitoxantrone observed in the present study is the prolongation of the time to the peak of

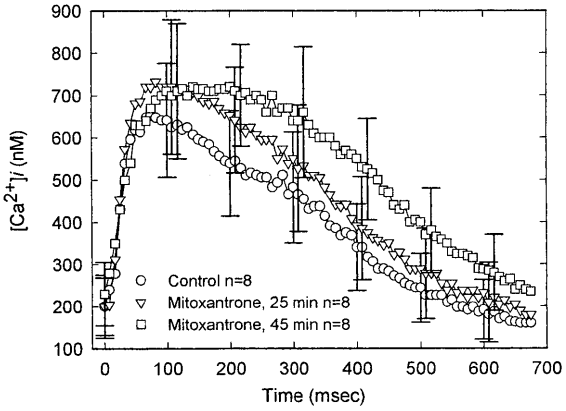

Fig. 5. Effects of mitoxantrone on the time-course of $[Ca^{2+}]i$ in myocytes isolated from ventricular muscles. Myocytes obtained from ventricular muscles were loaded with Fura-2, and equilibrated for 30 min at 30°C at 1.5 Hz electrical stimulation. Subsequently, mitoxantrone (30 μM) was added to the incubation solution. Fluorescent images were taken at time zero (○, immediately before the addition of mitoxantrone), 25 (▽) and 45 (□) min later from the addition of mitoxantrone. The values for $[Ca^{2+}]i$ were ploted against time after electrical stimulation. Each points represents mean of 8 experiments and vertical lines indicate the standard error of the mean.

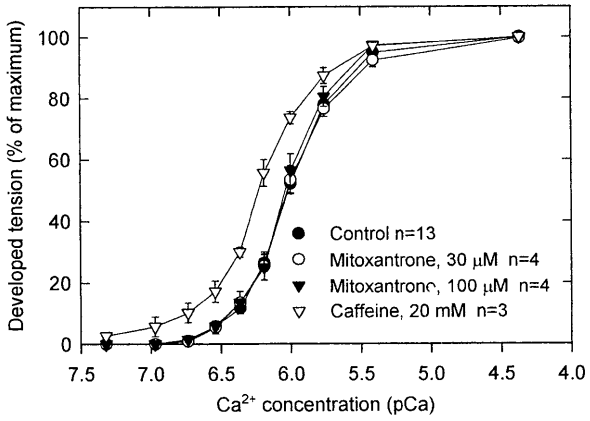

Fig. 7. Effects of mitoxantrone or caffeine on Ca^{2+} concentration-contraction curves observed in skinned fiber preparations. The muscle fiber preparations were isolated from left ventricular muscles, and exposed to a high concentration of saponin (1 mg/ml). Ca^{2+} concentration-response curves were generated after the skinned fiber preparations were incubated for 60 min in the absence or presence of mitoxantrone. For the positive control, the skinned fiber preparations were incubated in the presence of 20 mM caffeine instead of mitoxantrone for 60 min. Values are expressed as the percentage of the maximum contraction observed with Ca^{2+} concentrations at pCa 4.37. Each point represents the mean of 3 to 13 experiments. The vertical line indicates the standard error of the mean.

twitch tension, or the delay of muscle relaxation, observed at 2-Hz stimulation. These results confirm the observation by Wang *et al.* [37] in isolated ventricular papillary muscles preparations and in ventricular myocytes.

Bers [5] proposed that the rate of extrusion of intracellular Ca^{2+} via the Na^{+}- Ca^{2+} exchange system plays an important role in the rate of muscle relaxation. Inhibition of this exchange system, and an ensuing delay in the Ca^{2+} extrusion from the cells, causes the prolongation of action potential duration, prolongation of the time to the peak of twitch tension, and an increase in developed tension [5]. Wang *et al.*

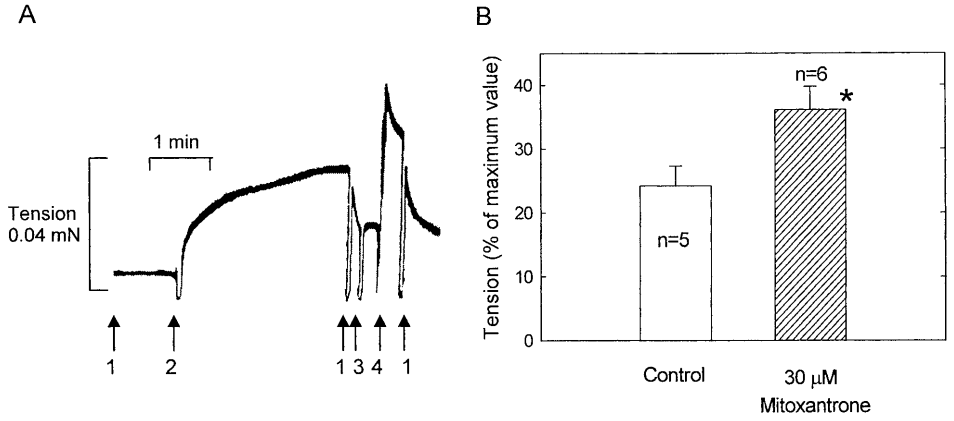

Fig. 6. Effects of mitoxantrone on caffeine-induced contraction in skinned fiber preparations. The muscle fiber preparations were isolated from left ventricular muscles, and exposed to saponin (50 μg/ml) for 30 min to disrupt sarcolemmal cell membranes. A, typical tracings of caffeine-induced contraction. 1: relaxing solution , 2: Ca^{2+} EGTA buffer solution containing 6.0 mM Ca^{2+} (pCa 6.19), 3: low EGTA relaxing solution, 4: low EGTA relaxing solution containing 20 mM caffeine. B, summarized results for the effects of 30 mM mitoxantrone on the caffeine-induced contraction. Values are expressed as the percentage of the maximum contraction observed with Ca^{2+} concentrations at pCa 4.37. Each bar represents the mean of 5 or 6 experiments. * Significantly different from the control values (Unpaired *t*-test, $P<0.05$).

Table 3. Effects of mitoxantrone or caffeine on ED_{50} values for Ca^{2+} concentration-response curves observed in skinned fiber preparations treated with a high concentration of saponin

	ED_{50} value (pCa)[a)]
Control	6.10 ± 0.03 (13)
Mitoxantrone, 30 μM	6.12 ± 0.03 (4)
Mitoxantrone, 100 μM	6.10 ± 0.04 (4)
Caffeine, 20 mM	6.25 ± 0.02 (3)[b)]

The numbers in parentheses indicate the number of experiments.
a) ED_{50} values are calculated by using SigmaPlot software version 8.0 (Systat Software, Point Richmond, CA, U.S.A.).
b) Significantly different from the values observed in control preparations (Control *vs* caffeine, unpaired *t*-test, $P<0.05$).

[37] reported these three effects of mitoxantrone observed in single cells, and in isolated heart muscle preparations. Consistent with the hypothesis, 30 μM mitoxantrone increased the time to peak $[Ca^{2+}]i$ and slightly elevated the diastolic $[Ca^{2+}]i$ in the present study observed in fura-2 loaded myocytes.

Mitoxantrone, however, produced stronger increases in developed tension at lower stimulation frequencies in the present study, whereas a known inhibitor of the Na^+– Ca^{2+} exchange system, SEA0400, has been shown to produce a stronger increase in developed tension at higher stimulation frequencies in isolated ventricular muscle preparations obtained from mouse hearts [26]. These effects are expected because an inhibition of the Na^+–Ca^{2+} exchange system would elevate intracellular Ca^{2+} concentrations more at higher stimulation frequencies when greater amounts of Ca^{2+} enter the cells through the slow Ca^{2+} channels [5, 34]. Therefore, an inhibition of the Na^+– Ca^{2+} exchange system is unlikely to be the main mechanism responsible for the delay of muscle relaxation and the positive inotropic effects observed with mitoxantrone.

Another mechanism to remove intracellular Ca^{2+} during the daistolic phase is Ca^{2+} uptake by the sarcoplasmic reticulum. In fact, Ca^{2+} removal by the Ca^{2+} uptake mechanism accounts for approximately 70% of the total removal [5]. Thus, such a mechanism is thought to be the most important one, and the inhibition of the rate of Ca^{2+} uptake is inferred to be the greatest cause of the delay in muscle relaxation [5]. A decrease in Ca^{2+} uptake is likely to prolong the time to the peak and also to increase diastolic $[Ca^{2+}]i$ similar to an inhibition of the Na^+–Ca^{2+} exchange system that decreases Ca^{2+} extrusion. The prolongation caused by doxorubicin has been shown to be mediated by the impairment of this Ca^{2+} uptake mechanism [14, 29, 31, 32]. Therefore, it would appear most likely that the prolongation of the time to peak twitch tension is caused by a decrease in the rate of Ca^{2+} uptake by the sarcoplasmic reticulum.

Prolongation of the time to the peak of twitch tension caused by 100 μM mitoxantrone was significantly smaller than that produced by 100 μM doxorubicin. Moreover, we observed in earlier studies that doxorubicin causes a marked elevation of the diastolic $[Ca^{2+}]i$ [30, 31], but in the present study the elevation of $[Ca^{2+}]i$ by mitoxantrone was only modest. Similarly, Wang *et al.* [37] failed to notice any changes in the level of diastolic $[Ca^{2+}]i$ caused by mitoxantrone although they observed a significant increase in peak $[Ca^{2+}]i$, in myocytes isolated from guinea-pig ventricular muscles. Therefore, it appears that the effect of mitoxantrone to inhibit Ca^{2+} uptake by the sarcoplasmic reticulum is moderate.

Postrest contractions have been reported to be totally dependent on the amount of Ca^{2+} released from the sarcoplasmic reticulum through Ca^{2+} release channels [4, 23, 27]. Myocardial contractions observed at lower stimulation frequencies also depend primarily on the Ca^{2+} release from the sarcoplasmic reticulum, whereas contractions observed at higher frequencies depend more on Ca^{2+} entry through the cell membrane [1, 4, 17, 21, 24, 25]. The positive inotropic effect of mitoxantrone was greater when preparations were stimulated at lower frequencies. Moreover, the postrest contraction was significantly enhanced by mitoxantrone. These effects of mitoxantrone are opposite to those of ryanodine that significantly decreases developed tension at lower stimulation frequencies and markedly suppresses the postrest contraction observed in isolated guinea-pig heart muscle preparations [24, 27, 28]. Furthermore, mitoxantrone enhanced caffeine-induced transient contractions in skinned muscle fiber preparations without affecting the Ca^{2+} sensitivity of the contractile proteins. These results support the hypothesis that mitoxantrone increases Ca^{2+} release through Ca^{2+} release channels in the sarcoplasmic reticulum.

Holmberg and Williams [12] reported that mitoxantrone increases open probability of Ca^{2+} release channels in artificial lipid bilayer membrane preparations. Increased probability of Ca^{2+} release channel openings, if it occurs in the present preparations, can explain increases in Ca^{2+} release through Ca^{2+} release channels in the sarcoplasmic reticulum. This hypothesis may be argued to be inconsistent with the observed prolongation of the time to the peak of $[Ca^{2+}]i$ and of twitch tention. The primary determinant of the time to the peak of twitch tension, however, is the rate of Ca^{2+} uptake by the sarcoplasmic reticulum [5]. The relationship between enhanced open probability of Ca^{2+} release channels and the time to the peak of twitch tension or $[Ca^{2+}]i$ is presently unknown.

Alternatively, enhanced Ca^{2+} loading of the sarcoplasmic reticulum may account for the increases in Ca^{2+} release through Ca^{2+} release channels. Enhanced Ca^{2+} loading of the sarcoplasmic reticulum can explain the augmentation of caffeine-induced contractions, postrest contractions and the peak $[Ca^{2+}]i$. Wang *et al.* [37] has proposed that the prolongation of transmembrane action potential duration by mitoxantrone enhances Ca^{2+} influx, causing the increase in Ca^{2+} content of the sarcoplasmic reticulum in cell membrane-intact atrial muscle preparations and in isolated myocytes.

The effect of mitoxantrone observed in the present study were opposite to those of doxorubicin observed in left arterial muscle preparations isolated from doxorubicin-treated

rats, or in atrial muscle preparations exposed relatively long time to doxorubicin *in vitro*. In contrast to mitoxantrone, doxorubicin decreased postrest contractions (*in vitro* exposure), developed tension observed at lower stimulation frequencies and also caffeine-induced contractions (*in vivo* treatment) [7, 10, 18, 29].

It seems both mitoxantrone and doxorubicin delay the Ca^{2+} uptake by the sarcoplasmic reticulum. However, doxorubicin inhibits and mitoxantrone stimulates the Ca^{2+} release through the Ca^{2+} release channels in the sarcoplasmic reticulum. This latter action of mitoxantrone must be the major mechanism for its positive inotropic effects. The action of doxorubicin on the Ca^{2+} release channels of the sacroplasmic reticulum is biphasic. Initially, it stimulates the Ca^{2+} release channels. Subsequently, doxorubicin inhibits the Ca^{2+} release channels. These two actions have been reported to be mediated by two separate processes [19]. Mitoxantrone has either a very weak or no action on the process that leads to inhibition of the Ca^{2+} release channels.

In patients, the cardiotoxicity caused by mitoxantrone is significantly less severe than that observed with doxorubicin [2, 9, 11, 22]. Therefore, the differences in actions, or the lack of an inhibitory action of mitoxantrone on the Ca^{2+} release channels in the sarcoplasmic reticulum, can explain the relative lack of the clinical cardiotoxicity of mitoxantrone.

In conclusion, mitoxantrone enhanced the magnitude of the postrest contraction, increased the developed tension especially at lower stimulation frequencies, and prolonged the time to the peak of twitch contractions. In ventricular myocytes, mitoxantrone increased the peak $[Ca^{2+}]i$ and prolonged the time to peak $[Ca^{2+}]i$. In skinned fiber preparations, mitoxantrone enhanced caffeine-induced transient contractions without affecting the Ca^{2+} sensitivity. These results show that mitoxantrone enhances Ca^{2+} release through the ryanodine-sensitive Ca^{2+} release channels in the sarcoplasmic reticulum. In addition, mitoxantrone seems to alter Ca^{2+} uptake by the mechanism common with doxorubicin.

ACKNOWLEDGMENTS. We gratefully acknowledge Dr. Tai Akera, Adjunct Professor, School of Medicine, Keio University, for his helpful advice and suggestions. This work was supported by general funds, and by a special research grants from the School of Veterinary Medicine, Kitasato University.

REFERENCES

1. Bayer, R., Hennekes, R., Kaufmann, R. and Mannhold, R. 1975. Inotropic and electrophysiological actions of verapamil and D600 in mammalian myocardium. *Naunya-Schmiedberg's Arch. Pharmacol.* **290**: 49–68.
2. Benjamin, R.S. 1995. Rationale for the use of mitoxantrone in the older patient: cardiac toxicity. *Seminars in Oncol. 22 (Suppl.)*: 11–13.
3. Bers, D. M. 1982. A simple method for the accurate determination of free [Ca] in Ca-EGTA solutions. *Am. J. Physiol.* **242**: C404–C408.
4. Bers, D. M. 1985. Ca influx and sarcoplasmic reticulum Ca release in cardiac muscle activation during postrest recovery. *Am. J. Physiol.* **248**: H366–H381.
5. Bers, D. M. 2001. Na/Ca exchange and the sarcolemmal Ca-pump. pp. 133–160. *In*: Excitation-Contraction Coupling and Cardiac Contractile Force, 2nd ed., Kluwer Academic Publishers, Dordrecht.
6. Chugun, A., Temma, K., Kondo, H. and Kurebayashi, N. 1996. Ca^{2+} sensitivity and caffeine-induced changes in skinned cardiac muscle fibers of the carp, Cyprinus carpio. *J. Comp. Physiol. (B)* **166**: 412–417.
7. Chugun, A., Temma, K., Oyamada, T., Suzuki, N., Kamiya, Y., Hara, Y., Sasaki, T., Kondo, H. and Akera, T. 2000a. Doxorubicin-induced late cardiotoxicity: delayed impairment of Ca^{2+}-handling mechanisms in the sarcoplasmic reticulum in the rat. *Can. J. Physiol. Pharmacol.* **78**: 329–338.
8. Chugun, A., Uchide, T., Fujimori, Y., Temma, K., Hara, Y., Sasaki, T. and Akera, T. 2000b. Anti-muscarinic action of mitoxantrone in isolated heart muscles of guine pigs. *Eur. J. Pharmacol.* **407**: 183–189.
9. Faulds, D., Balfour, J.A., Chrisp, P. and Langtry, H.D. 1991. Mitoxantrone, a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in the chemotherapy of cancer. *Drugs* **3**: 400-449.
10. Hagane, K., Akera, T. and Berlin, R. J. 1988. Doxorubicin:mechanism of cardiodepressant actions in guinea pigs. *J. Pharmacol. Exp. Ther.* **246**: 655-661.
11. Henderson, I.C., Allegra, J.C., Woodcock, T., Wolff, S., Bryan, S., Cartwright, K., Dukart, G. and Henry, D. 1989. Randomized clinical trial comparing mitoxantrone with doxorubicin in previously treated patients with metastatic breast cancer. *J. Clin. Oncol.* **7**: 560-571.
12. Holmberg, S. R. M. and Williams, A. J. 1990. Patterns of interaction between anthraquinone drugs and the calcium-release channel from cardiac sarcoplasmic reticulum. *Circ. Res.* **67**: 272–283.
13. Iatropoulos, M.J. 1984. Anthracycline cardiomyopathy: predictive value of animal models. *Cancer. Treat. Sym.* **3**: 3-17.
14. Jensen, R. A. 1986. Doxorubicin cardiotoxicity: contractile changes after long-term treatment in the rat. *J. Pharmacol. Exp. Ther.* **236**: 197–203.
15. Kim, E., Giri, S. N. and Pessah, I. N. 1994. Antithetical actions of mitoxantrone and doxorubicin on ryanodine-sensitive Ca^{++} release channels of rat cardiac sarcoplasmic reticulum: evidence for a competitive mechanism. *J. Pharmacol. Exp. Ther.* **268**: 1212–1221.
16. Kitazawa, T., 1988. Caffeine contracture in guinea-pig ventricular muscle and the effect of extracelluar sodium ions. *J. Physiol. (Lond.)* **402**: 703–729.
17. McCans, J. L., Lindenmayer, G. E., Munson, R. G., Evans, R. W. and Schwartz, A. 1974. A dissociation of positive staircase (Bowditch) from ouabain-induced positive inotropism. *Circ. Res.* **35**: 439–447.
18. Minotti, G., Parlani, M., Salvatorelli, E., Menna, P., Cipollone, A., Animati, F., Maggi, C. A. and Manzini, S. 2001. Impairment of myocardial contractility by anticancer anthracyclines: role of secondary alcohol metabolites and evidence of reduced toxicity by a novel disaccharide analogue. *Br. J. Pharmacol.* **134**: 1271-1278.
19. Ondrias, K., Borgatta, L., Kim, D.H. and Ehrlich, B.E. 1990. Biphasic effects of doxorubicin on the calcium release channel from sarcoplasmic reticulum of cardiac muscle. *Circ. Res.* **67**:

1167–1174.

20. Pekins, W., Schroeder, R. L., Carrano, R. A. and Imondi, A. R. 1984. Myocardial effects of mitoxantrone and doxorubicin in the mouse and guinea pig. *Cancer Treat. Rep.* **68**: 841–847.
21. Schouten, V. J. A. and ter Keurs, H. E. D. J. 1991. Role of I_{ca} and Na^+/Ca^{2+} exchange in the force-frequency relationship of rat heart muscle. *J. Mol. Cell. Cardiol.* **23**: 1039–1050.
22. Shenkenberg, T. D. and von Hoff, D. D. 1986. Mitoxantrone: a new anticancer drug with significant clinical activity. 1986. *Annals Inter. Med.* **105**: 67–81.
23. Sleator, W., Furchgott, R. F., De Gubareff, T. and Krespi, V. 1964. Action potentials of guinea pig atria under conditions which alter contraction. *Am. J. Physiol.* **206**: 270–282.
24. Stemmer, P. and Akera, T. 1986. Concealed positive force-frequency relationships in rat and mouse cardiac muscle revealed by ryanodine. *J. Am. Physiol.* **251**: H1106–H1110.
25. Stuyvers, B. D., McCulloch, A. D., Guo, J., Duff, H. J. and ter Keurs, H. E. D. J. 2002. Effects of stimulation rate, sarcomere length and Ca^{2+} on force generation by mouse cardiac muscle. *J. Physiol. (Lond).* **544**: 817–830.
26. Tanaka, H., Namekata, I., Takada, K., Kazama, A., Shimizu, Y., Moriwaki, R., Hirayama, W., Sato, A., Kawanichi, T. and Shigenobu, K. 2005. Unique excitation-contraction characteristics of mouse myocardium as revealed by SEA0400, a specific inhibitor of Na^+-Ca^{2+} exchanger. *Naunyen-Schmiedeberg's Arch. Pharmacol.* **371**: 526–534.
27. Temma, K., Akera, T. and Brody, T. M. 1981. Inotropic effects of digitoxin in isolated guinea-pig heart under conditions which alter contraction. *Eur. J. Pharmacol.* **76**: 361–370.
28. Temma, K., Akera, T. and Ng, Y.C. 1986. Effects of inotropic agents on isolated guinea pig heart under conditions that modify calcium pools involved in contractile activation. *Can. J. Physiol. Pharamacol.* **64**: 947–953.
29. Temma, K., Akera, T., Chugun, A., Kondo, H., Hagane, K. and Hirano, S. 1993. Comparison of cardiac actions of doxorubicin, pirarubicin and aclarubicin in isolated guinea-pig heart. *Eur. J. Pharmacol.* **243**: 173-181.
30. Temma, K., Chugun, A., Akera, T., Kondo H. and Kurebayashi, N. 1996. Doxorubicin alters Ca^{2+} transients but fails to change Ca^{2+} sensitivity of contractile proteins. *Environ. Toxicol. Pharmacol.* **1**: 131–139.
31. Temma, K., Chugun, A., Akera, T., Hara, Y., Sasaki, T. and Kondo, H. 1997. Ca^{2+} overloading causes the negative inotropic effects of doxorubicin in myocytes isolated from guinea-pig hearts. *Eur. J. Pharamcol.* **332**: 235–242.
32. Van Boxtel, C. J., Olson, R. D., Boerth, R. C. and Oates, J. A. 1978. Doxorubicin: inotropic effects and inhibitory action of ouabain. *J. Pharmacol. Exp. Ther.* **207**: 277-283.
33. Viglione, P. N., Praprotnik, A. and Pinto, J. E. B. 1993. *In vitro* evaluation of acute effects of mitoxantrone (Novantrone) in rat and guinea pig atria. *Pharmacol. Toxicol.* **72**: 208–212.
34. Vila Petroff, M, G., Palomeque, J. and Mattiazzi, A. R. 2003. Na^+-Ca^{2+} exchange function underlying contraction frequency inotropy in the cat myocardium. *J. Physiol. (Lond).* **550**: 801–817.
35. Wallace, R. E., Murdock, K. C., Angier, R. B. and Durr, F. E. 1979. Activity of a novel anthracenedione, 1, 4-dihydroxy-5, 8-bis{{{2-[(2-hydroxyethyl)amino]ethyl}amino}}-9, 10-anthracenedione dihydrochloride, against experimental tumors in mice. *Cancer Res.* **39**: 1570–1574.
36. Wang, G. X., Zhou, X. B., Eschenhagen, T. and Korth, M. 1999. Effects of mitoxantrone on action potential and membrane currents in isolated cardiac myocytes. *Brit. J. Pharmacol.* **127**: 321–330.
37. Wang, G. X., Zhou, X. B. and Korth, M. 2000. Effects of mitoxantrone on excitation-contraction coupling in guinea pig. *J. Pharamacol. Exp. Ther.* **293**: 501–508.