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Ca^{2+} overloading causes the negative inotropic effect of doxorubicin in myocytes isolated from guinea-pig hearts

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Abstract

We reported previously that conditions shown to increase Ca^{2+} loading augment the negative inotropic effect of doxorubicin. To examine if the negative inotropic effect is caused by Ca^{2+} overloading, doxorubicin-induced changes in diastolic and systolic Ca^{2+} concentrations and twitch contractions were studied under altered Ca^{2+} loading. Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were monitored in fura-2-loaded myocytes isolated from the ventricular muscle of guinea-pig hearts. Twitch contractions were estimated from the shortening of myocytes. In myocytes incubated at 34°C in a medium containing 1.2 mM $CaCl_2$ (standard conditions), doxorubicin (100 μM) caused a significant decrease in diastolic length, and an increase in the amplitude of contraction. The positive inotropic effect of doxorubicin was followed by a negative effect. Concomitant with these changes in myocyte contractions, a monophasic increase in diastolic Ca^{2+}, and an increase and a subsequent decrease in the amplitude of Ca^{2+} transients (peak $[Ca^{2+}]_i$ minus diastolic $[Ca^{2+}]_i$) were observed. When the Ca^{2+} load of myocytes was increased by an incubation at a low temperature (25°C) or in the presence of high Ca^{2+} in the incubation medium (2.4 mM $CaCl_2$), diastolic $[Ca^{2+}]_i$ was elevated. Doxorubicin further increased diastolic $[Ca^{2+}]_i$. Under these conditions, the doxorubicin-induced increase in the twitch contraction lasted a shorter period of time and the subsequent decrease in contraction was significantly enhanced. The peak $[Ca^{2+}]_i$ initially increased slightly and then decreased. Thus, the decrease in the amplitude of Ca^{2+} transients, as well as myocyte contraction, was greater compared to the corresponding values observed under the standard conditions. These changes in the contraction and Ca^{2+} transient developed with the same time course. The effects of the low-temperature incubation were antagonized by verapamil. These results indicate that the negative inotropic effect of doxorubicin results from a decrease in the amplitude of Ca^{2+} transients caused by an increased diastolic $[Ca^{2+}]_i$ and a decreased peak $[Ca^{2+}]_i$. These changes are likely to be caused by myocardial Ca^{2+} overload. © 1997 Elsevier Science B.V. All rights reserved.

Keywords: Doxorubicin; Cardiotoxicity; Cell contraction; Ca^{2+} concentration, intracellular; Myocardial cell, single

1. Introduction

Doxorubicin produces a dose-dependent cardiotoxicity leading to heart failure. This side effect limits the clinical usefulness of this highly effective anticancer chemotherapeutic agent (Henderson and Frei, 1979; Singal et al., 1987; Olson and Mushlin, 1990; Doroshow, 1991).

Olson et al. (1974) reported that chronic treatment of rabbits with doxorubicin causes a significant increase in Ca^{2+} concentration in the heart muscle. They concluded that the cause of doxorubicin-induced heart failure is intracellular Ca^{2+} overloading. Consistent with this concept, doxorubicin increased Ca^{2+} influx and caused Ca^{2+} accumulation in isolated heart cells (Azuma et al., 1981; Combs et al., 1985; Kusuoka et al., 1991; Earm et al., 1994).

Evidence contrary to the above concept has also been reported. Jensen (1986) reported a decrease, instead of an increase, in the rate of development and the peak twitch tension in papillary muscle preparations isolated from doxorubicin-treated rats. An increase in Ca^{2+} concentration in the incubation medium ($[Ca^{2+}]_o$) produced a greater increase in developed tension in doxorubicin-treated prepara-

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tions compared to that observed in control preparations. In addition, Bernardini et al. (1986) and Bossa et al. (1988) reported that amrinone, a positive inotropic agent that increases Ca^{2+} influx, reduces doxorubicin cardiotoxicity. Dodd et al. (1993) reported that a chronic doxorubicin treatment reduces functions of Ca^{2+} release channels in the sarcoplasmic reticulum of rabbit hearts. These results suggest that the primary cause of doxorubicin cardiotoxicity is the decrease in $[Ca^{2+}]_i$ rather than an increase.

We reported that doxorubicin causes a transient increase and a subsequent decrease in developed tension in left atrial muscle preparations isolated from guinea-pig heart (Temma et al., 1993; Temma et al., 1994). The decrease in developed tension observed in these preparations was significantly enhanced under the conditions that increase myocardial Ca^{2+} loading, i.e., incubation at a low temperature or in the presence of high $[Ca^{2+}]_o$ (Temma et al., 1994). Ca^{2+} overloading, caused by an exposure to an extremely high concentration of $CaCl_2$ or digitalis, decreases the developed tension of isolated heart muscle preparations (Vassalle and Lin, 1979; Lakatta and Lappe, 1981; Valdeolmillos and Eisner, 1985). These results suggest that the negative inotropic effects of doxorubicin result from myocardial Ca^{2+} overloading. We have recently shown that a relatively high concentration of doxorubicin decreases the peak and the amplitude of Ca^{2+} transients in fura-2-loaded myocytes isolated from guinea-pig hearts (Jiang et al., 1994). The study reported by us is the first attempt to directly measure doxorubicin-induced changes in Ca^{2+} transients.

To examine whether the negative inotropic effects of doxorubicin result from the Ca^{2+} overloading of myocardial cells, effects of Ca^{2+} loading on doxorubicin-induced changes in $[Ca^{2+}]_i$ and twitch contractions were examined. Ca^{2+} loading was increased by an incubation at a low temperature (Bers, 1991) or in a medium containing a high $CaCl_2$ concentration (Langer, 1990; Frampton et al., 1991). Additionally, Ca^{2+} loading was reduced using verapamil in preparations incubated at a low temperature.

2. Materials and methods

2.1. Myocyte preparation

Ventricular myocytes were isolated from guinea-pig hearts using collagenase as previously described (Koyama et al., 1991; Jiang et al., 1994; Temma et al., 1996). Hearts of male Hartly guinea pigs, weighing approximately 400 g, were perfused for 20 min at 37°C with a 'regular' HEPES buffer solution containing (mM): 130 NaCl, 5.8 KCl, 1.0 Na_2HPO_4, 1.0 $MgCl_2$, 1.2 $CaCl_2$, 10 glucose, 2.5 sodium pyruvate and 5.0 HEPES. Subsequently, the hearts were perfused with a low Ca^{2+} HEPES buffer solution containing (mM): 130 NaCl, 5.8 KCl, 1.0 Na_2HPO_4, 1.0 $MgCl_2$, 0.01 $CaCl_2$, 10 glucose, 5.0 sodium pyruvate, 5.0 taurine, 2.5 D-mannitol and 5.0 HEPES. After 10 min, collagenase (final concentration 0.66 mg/ml) was added, and the hearts were perfused further. After a 30-min perfusion with collagenase, the ventricular muscle was minced. Dispersed myocytes were separated from large tissue debris by filtering through a nylon mesh.

Myocytes were incubated for 30 min at 37°C for fura-2 loading in a regular HEPES buffer solution containing 1.5 μM fura-2 acetoxymethyl ester (fura-2/AM) and bovine serum albumin (20 mg/ml). Subsequently, myocytes were washed 3 times with the regular HEPES buffer solution containing neither fura-2/AM nor bovine serum albumin, and stored at room temperature (18–23°C) for up to 4 h before use. The regular and low Ca^{2+} HEPES buffer solutions were adjusted to a pH value of 7.4 using NaOH, and equilibrated with 100% O_2.

2.2. Recording of $[Ca^{2+}]_i$

The light signals of intracellular fura-2 were recorded and analyzed using methods similar to those previously reported (Koyama et al., 1991; Jiang et al., 1994; Temma et al., 1996). In this study, a charge-coupled device camera with the vertical picture elements reduced to one-fourth was used (slit-scan video camera; C-2400-77, Hamamatsu Photonics, Hamamatsu, Japan). This camera is capable of recording fluorescence images at 4-ms intervals. Fura-2-loaded myocytes were placed in an open chamber (approximately 0.3-ml capacity) with a thin quartz-glass bottom and placed on the stage of an inverted epifluorescence microscope (Nikon, Nihon Kogaku, Tokyo, Japan). Cells were superfused at a flow rate of 0.1 ml/min with a Krebs-Henseleit bicarbonate buffer solution containing (mM): 118 NaCl, 27.2 $NaHCO_3$, 4.8 KCl, 1.2 $MgSO_4$, 1.2 $CaCl_2$, 1.0 KH_2PO_4, 11.1 glucose and 5.0 sodium pyruvate. Myocytes were stimulated at 2 Hz with square wave pulses at a voltage 30% above the threshold using a pair of platinum electrodes for field stimulation. To study the effects of low temperature, the temperature of the incubation medium was lowered from 34°C to 25°C. The Ca^{2+} concentration in the incubation medium was increased from 1.2 mM to 2.4 mM to examine the effect of a high extracellular Ca^{2+} concentration.

The myocytes were illuminated with ultraviolet light having a wavelength of either 340 or 380 nm using a 100-watt xenon arc lamp. The fluorescence images at 500 nm were recorded at 4-ms intervals for 533 ms (from 33 ms before the electrical stimulation to 500 ms after the stimulation), and stored in a computer. The ratio of fluorescence intensities observed at 340 and 380 nm excitation after subtracting the background values was analyzed at each pixel over an entire myocyte using an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan). The $[Ca^{2+}]_i$ in myocytes was estimated from the calibration curves for Ca^{2+} concentration constructed using Ca^{2+}-EGTA buffer solution containing 22 nM to 2.36 mM Ca^{2+}

plus 10 mM fura-2 pentapotassium salt as described earlier (Koyama et al., 1991; Jiang et al., 1994).

The magnitude of myocyte contraction was determined by estimating the length of fluorescence ratio images on the monitor as previously described (Jiang et al., 1994). The index of contraction value was calculated using the following equation:

$$[1 - (\text{peak myocyte length} / \text{diastolic myocyte length})] \times 100$$

2.3. Miscellaneous

Statistical analysis was performed using Student's *t*-test. The criterion for statistical significance was a P value of less than 0.05. Doxorubicin (Adriacin), fura-2/AM and collagenase (type A, isolated from *Clostridium histolyticum*) were purchased from Kyowa Hakkou Kogyo (Tokyo, Japan), Dojindo (Kumamoto, Japan) and Boehringer-Mannheim (Mannheim, Germany), respectively. All other chemicals used were of reagent grade.

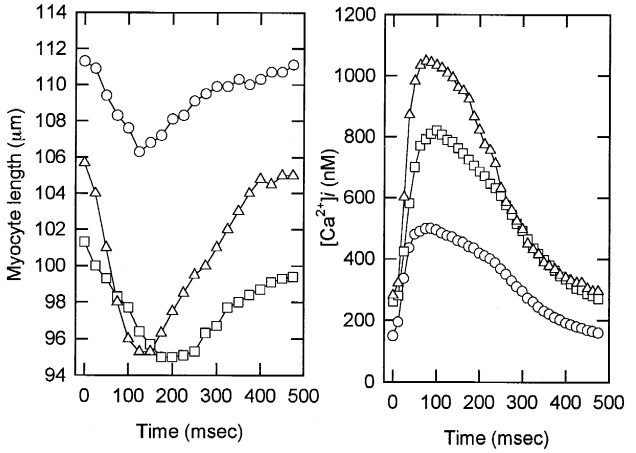

Fig. 1. Effects of Ca^{2+} loading on the time-course of myocyte contraction and Ca^{2+} transients. Fura-2-loaded myocytes were stimulated at 2 Hz under standard conditions (○: 34°C, 1.2 mM $CaCl_2$), low temperature (□: 25°C, 1.2 mM $CaCl_2$) or high $[Ca^{2+}]_o$ (△: 34°C, 2.4 mM $CaCl_2$). Fluorescence images were recorded after a 30-min equilibration period. Left and right panels show myocyte contraction and Ca^{2+} transients, respectively. Myocyte length was estimated from the fluorescence ratio images on the monitor. Each point represents the mean of 7 or 8 experiments.

3. Results

3.1. Effects of increased Ca^{2+} loading

At the end of 30-min equilibration under the standard conditions (2-Hz stimulation, 34°C, 1.2 mM $CaCl_2$), diastolic myocyte length of fura-2-loaded myocytes and the time to peak twitch contraction were 111 ± 4 μm and 131 ± 10 ms ($n = 7$, mean ± S.E.), respectively (Fig. 1, left panel and Table 1). The index of contraction value was 5.18 ± 0.47 ($n = 7$). Decreasing the temperature of the incubation medium from 34°C to 25°C in the presence of 1.2 mM $CaCl_2$ caused an increase in the index of contraction value (standard conditions, 5.18 ± 0.4, $n = 7$; low temperature, 7.4 ± 1.2, $n = 7$) and a significant prolongation of the time to peak twitch contractions (Fig. 1, left panel and Table 1). The index of contraction value was also increased by a high $[Ca^{2+}]_o$ at 34°C (12.0 ± 2.5,

Table 1
Influence of increased Ca^{2+} loading on doxorubicin-induced changes in myocyte contractions

Time	34°C, 1.2 mM $CaCl_2$	25°C, 1.2 mM $CaCl_2$	34°C, 2.4 mM $CaCl_2$
Myocyte length at diastole (μm)			
0 min	111.3 ± 4.3 [a]	101.3 ± 4.8	105.7 ± 5.7
8 min	110.3 ± 4.4	100.9 ± 4.9	105.0 ± 5.7 [b]
30 min	109.6 ± 4.2 [b]	99.4 ± 5.3	103.1 ± 6.3 [b]
60 min	107.7 ± 4.4 [b]	98.3 ± 5.8 [b]	99.3 ± 6.5 [b]
90 min	105.2 ± 4.5 [b]	96.6 ± 6.3 [b]	98.5 ± 6.4 [b]
Myocyte length at peak twitch contraction (μm)			
0 min	105.6 ± 4.3	93.9 ± 5.0	93.6 ± 6.6
8 min	104.3 ± 4.2	93.3 ± 4.5	92.7 ± 6.9
30 min	103.0 ± 4.4 [b]	93.0 ± 4.9	92.5 ± 7.2
60 min	102.2 ± 4.4 [b]	93.4 ± 5.6	93.1 ± 6.9
90 min	100.1 ± 4.7 [b]	93.1 ± 6.5	93.1 ± 6.6
Time to peak twitch contraction (ms)			
0 min	131 ± 10	186 ± 12	124 ± 8
8 min	158 ± 7	194 ± 12	150 ± 11 [b]
30 min	222 ± 10 [b]	238 ± 16 [b]	190 ± 11 [b]
60 min	231 ± 4 [b]	245 ± 14 [b]	223 ± 10 [b]
90 min	260 ± 10 [b]	271 ± 5 [b]	252 ± 10 [b]

Myocytes were incubated under the conditions of standard, low temperature or high $[Ca^{2+}]_o$. The fluorescence images were recorded immediately before (0 min) and after the addition of doxorubicin (final concentration, 100 μM) at the indicated times. Myocyte length was estimated from fluorescence ratio images on the monitor. [a] Values are the mean ± S.E. of 7–8 experiments. [b] Significantly different from values observed at time zero.

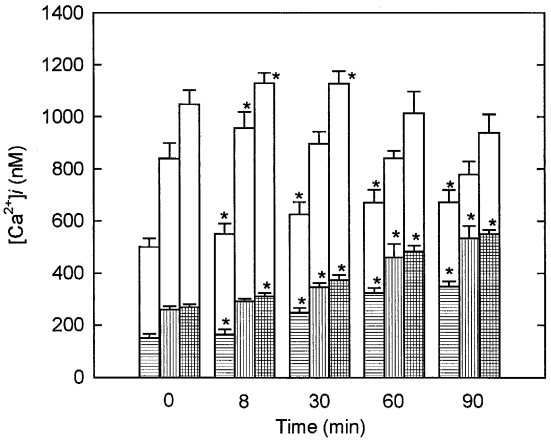

Fig. 2. The effects of doxorubicin on diastolic $[Ca^{2+}]_i$ and peak Ca^{2+} transients under the conditions of standard, low temperature or high $[Ca^{2+}]_o$. Doxorubicin (final concentration, 100 μM) was added to the incubation medium after a 30-min equilibration period under the indicated conditions. Fluorescence images were recorded before, and 8, 30, 60 or 90 min after the addition of 100 μM doxorubicin (final concentration). Horizontally striped, vertically striped and hatched bars represent the diastolic $[Ca^{2+}]_i$ for standard, low temperature and high $[Ca^{2+}]_o$, respectively. The total bars and open bars represent peak Ca^{2+} transients and the size of Ca^{2+} transients, respectively. Vertical lines indicate standard error of the mean. Each bar represents the mean of 7 or 8 experiments. * Significantly different from values observed at time zero.

$n = 7$), but there was no significant change in the time to peak twitch contraction (Fig. 1, left panel and Table 1).

Under the standard conditions, diastolic $[Ca^{2+}]_i$ was 149 ± 16 nM ($n = 8$), and rapidly increased responding to an electrical stimulation. The peak $[Ca^{2+}]_i$ of 500 ± 33 nM was reached 64.4 ± 3.5 ms ($n = 8$) after an electrical stimulation (Fig. 1, right panel and Fig. 2). Lowering the incubation temperature from 34°C to 25°C elevated the diastolic (259 ± 14 nM) and the peak $[Ca^{2+}]_i$ (840 ± 61 nM, $n = 7$), and prolonged the time to peak Ca^{2+} transients (93 ± 7 ms). The magnitude of the elevation of $[Ca^{2+}]_i$ was greater at the peak than at the diastole (Fig. 1, right panel and Fig. 2). The size of the Ca^{2+} transients (peak $[Ca^{2+}]_i$ minus diastolic $[Ca^{2+}]_i$) was therefore significantly greater at the low temperature (581 ± 55 nM, $n = 7$) than the corresponding value observed under the standard conditions (351 ± 33 nM, $n = 8$).

An increase in $[Ca^{2+}]_o$ at 34°C elevated diastolic $[Ca^{2+}]_i$ (268 ± 13 nM, $n = 7$) to a similar level caused by lowering the temperature to 25°C in the presence of 1.2 mM $CaCl_2$ (Fig. 1, right panel and Fig. 2). The peak $[Ca^{2+}]_i$, however, was significantly higher than that observed at the low temperature (1048 ± 54 nM, $n = 7$). The time to peak $[Ca^{2+}]_i$ was unchanged (71 ± 3 ms, $n = 7$). The size of the Ca^{2+} transients was 780 ± 43 nM ($n = 7$). These results are consistent with earlier reports (Bers, 1991; Frampton et al., 1991) that lowering the temperature or elevating the $[Ca^{2+}]_o$ increases the size of Ca^{2+} transients concomitant with an increase in twitch contractions. In the present study, the size of Ca^{2+} transients was largest at high $[Ca^{2+}]_o$, intermediate at the low temperature and smallest under the standard conditions.

After 30-min equilibration, Ca^{2+} transients and twitch contractions of myocytes were stable for the next 90-min period under these three conditions (data not shown).

3.2. Effects of doxorubicin under increased Ca^{2+} loading

We have previously shown that doxorubicin alters twitch contractions of myocytes and Ca^{2+} transients in parallel under the standard conditions of the present study (Temma et al., 1996). In the present study, doxorubicin-induced changes in myocyte contractions and Ca^{2+} transients were examined under altered Ca^{2+} loading of myocytes. As reported previously, 100 μM doxorubicin decreased the diastolic and systolic myocyte length under the standard conditions (Table 1). The index of contraction value was higher during the first 60 min following the addition of doxorubicin (Fig. 3). Moreover, 100 μM doxorubicin prolonged the time to peak twitch contraction (Table 1). In these myocytes, doxorubicin produced a time-dependent increase in the diastolic $[Ca^{2+}]_i$, and an initially large but subsequently small increases in the peak Ca^{2+} transients (Fig. 2). The size of the Ca^{2+} transients, therefore, was slightly greater during the first 60 min and then decreased (Fig. 4). These results confirm our earlier observations (Temma et al., 1996).

At the low incubation temperature (25°C, 1.2 mM $CaCl_2$) or in the presence of high $[Ca^{2+}]_o$ (2.4 mM $CaCl_2$, 34°C), 100 μM doxorubicin shortened the diastolic myocyte length (Table 1). The shortening of the diastolic length observed 90 min after the addition of 100 μM

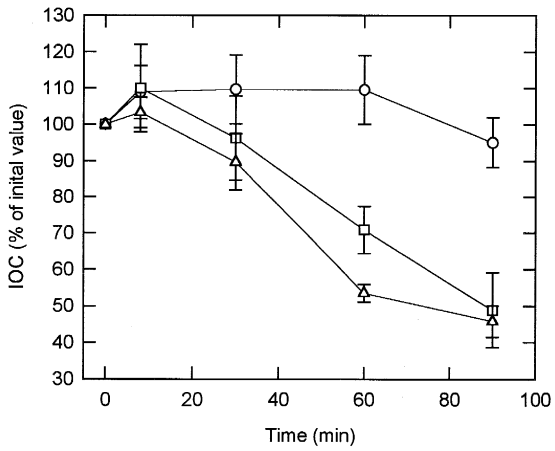

Fig. 3. Effects of doxorubicin on the index of contraction values under the conditions of standard (○), low temperature (□) or high $[Ca^{2+}]_o$ (△). See legend to Fig. 1. The fluorescence images were recorded before (0 min), and 8, 30, 60 or 90 min after the addition of doxorubicin (final concentration, 100 μM). Myocyte length was estimated from the fluorescence ratio image on the monitor. The index of contraction (IOC) value observed at time zero (immediately before the addition of doxorubicin) in each preparation was set at 100%. Vertical lines indicate standard error of the mean. Each point represents the mean of 7 experiments.

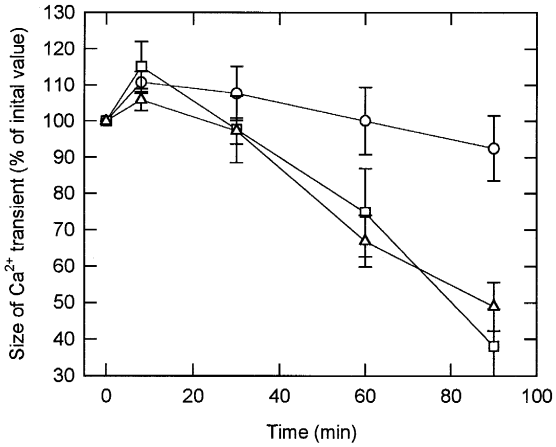

Fig. 4. Effects of doxorubicin on the size of Ca^{2+} transients under the conditions of standard (○), low temperature (□) or high $[Ca^{2+}]_o$ (△). See legend to Fig. 1. Fluorescence images were recorded before (0 min), and 8, 30, 60 or 90 min after the addition of 100 μM doxorubicin (final concentration). The size of Ca^{2+} transients observed immediately before the addition of doxorubicin in each preparation was set at 100%. Vertical lines indicate standard error of the mean. Each point represents the mean of 7 or 8 experiments.

doxorubicin was 14.0 ± 3.2% (standard conditions, $n = 8$), 13.6 ± 3.9% (low temperature, $n = 7$) or 15.1 ± 1.7% (high $[Ca^{2+}]_o$, $n = 7$). Doxorubicin failed to affect the myocyte length at peak twitch contraction at either low temperature or high $[Ca^{2+}]_o$ (Table 1). Under these conditions, the index of contraction value was increased at 8 min, and then decreased significantly with time (Fig. 3).

The prolongation of the time to peak twitch contraction observed 90 min after the addition of 100 μM doxorubicin was smaller at low temperature than that observed under the standard conditions or at high $[Ca^{2+}]_o$ (standard conditions, 104.4 ± 15.0%, $n = 8$; low temperature, 48.7 ± 7.2%, $n = 7$; high $[Ca^{2+}]_o$, 106.5 ± 11.1%, $n = 7$). The effect of doxorubicin expressed in absolute values, however, was significantly greater at the low temperature compared to corresponding values observed under the other two conditions (Table 1).

At a low temperature or in the presence of high $[Ca^{2+}]_o$, 100 μM doxorubicin increased diastolic $[Ca^{2+}]_i$ (Fig. 2). The percent increase observed at a low temperature or at high $[Ca^{2+}]_o$ was smaller than that observed under the standard conditions. At 90 min after the addition, doxorubicin caused 107 ± 17% (low temperature, $n = 7$), 107 ± 6% (high $[Ca^{2+}]_o$, $n = 7$) and 136 ± 19% increases (standard conditions, $n = 8$) in diastolic $[Ca^{2+}]_i$. Absolute values of diastolic $[Ca^{2+}]_i$ observed at 90 min after the addition of doxorubicin, however, were greater at a low temperature or at high $[Ca^{2+}]_o$ compared to that observed under the standard conditions (Fig. 2).

The doxorubicin-induced increase in peak $[Ca^{2+}]_i$ observed at a low temperature or at high $[Ca^{2+}]_o$ lasted only a shorter period of time. The peak $[Ca^{2+}]_i$ reached the highest value 8 min after doxorubicin addition, and then decreased gradually (Fig. 2). Increase in the size of Ca^{2+} transients was observed also only during a short period of time after the addition of doxorubicin at a low temperature or at high $[Ca^{2+}]_o$, in contrast to that observed under the standard conditions (Fig. 4).

Doxorubicin prolonged the time to peak Ca^{2+} transients at a low temperature or at high $[Ca^{2+}]_o$ similar to that observed under the standard conditions (Fig. 5). The time to peak Ca^{2+} transients observed at 90 min after the drug addition were 162 ± 9 ms (standard conditions, $n = 8$); 190 ± 9 ms (low temperature, $n = 7$) and 170 ± 6 ms (high $[Ca^{2+}]_o$, $n = 7$).

3.3. Effects of verapamil

We examined whether the Ca^{2+} channel antagonist, verapamil, reverses the influence of increased Ca^{2+} loading. In preparations incubated at a low temperature (25°C), verapamil caused a slight decrease in the diastolic $[Ca^{2+}]_i$ and a marked decrease in the peak $[Ca^{2+}]_i$, resulting in a significant decrease in the size of Ca^{2+} transients (Table 2). Verapamil increased the time to peak Ca^{2+} transients confirming our previous results (Jiang et al., 1994). At 25°C, the doxorubicin-induced increase in diastolic $[Ca^{2+}]_i$ observed in the presence of verapamil (Table 2) was smaller than the corresponding value observed at 25°C in the absence of verapamil: only a 41 ± 16% ($n = 7$) increase was observed, instead of the 104 ± 17% ($n = 8$) increase observed in the absence of verapamil. The decrease in peak $[Ca^{2+}]_i$ observed after an initial increase was almost abolished. Verapamil failed to affect the initial increase in the size of Ca^{2+} transients caused by doxorubicin and attenuated the subsequent time-dependent de-

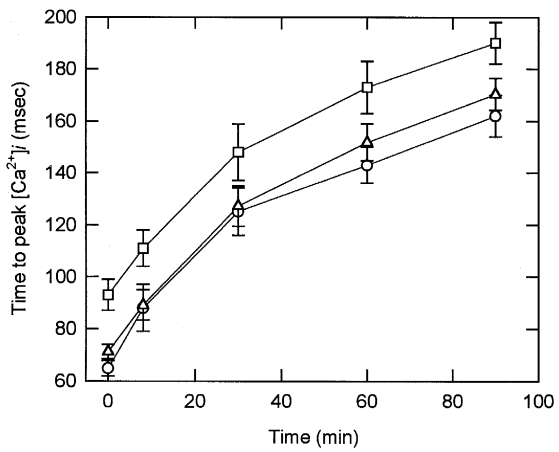

Fig. 5. Effects of doxorubicin on the time to peak Ca^{2+} transients under the conditions of standard (○), low temperature (□) or high $[Ca^{2+}]_o$ (△). See legend to Fig. 1. Doxorubicin (final concentration, 100 μM) was added to the incubation medium after a 30-min equilibration period. Fluorescence images were recorded before, and 8, 30, 60 or 90 min after the addition of 100 μM doxorubicin (final concentration). Vertical lines indicate standard error of the mean. Each point represents the mean of 7 or 8 experiments.

Table 2
Effects of verapamil on doxorubicin-induced changes in $[Ca^{2+}]_i$ observed at a low incubation temperature

	Diastolic $[Ca^{2+}]_i$ (nM)	Peak $[Ca^{2+}]_i$ (nM)	Size of Ca^{2+} transients (%)	(%)	Time to peak $[Ca^{2+}]_i$ (ms)
25°C	272 ± 49 [a]	656 ± 75	100 [b]		93 ± 11
Verapamil	237 ± 35	462 ± 44 [c]	62 ± 11 [c]	100 [d]	131 ± 18 [c]
Doxorubicin					
8 min	234 ± 35	461 ± 58		107 ± 2 [e]	166 ± 14 [e]
30 min	254 ± 35	472 ± 43		97 ± 4	190 ± 9 [e]
60 min	288 ± 33	504 ± 28		94 ± 13	202 ± 11 [e]
90 min	302 ± 32	493 ± 26		83 ± 11	215 ± 11 [e]

After myocytes were incubated at low temperature (25°C) for 30 min, verapamil (final concentration, 0.15 μM) was added to the incubation medium. The myocytes were incubated for 30 min in the presence of verapamil, and then doxorubicin (final concentration, 100 μM) was added. Fluorescence images were recorded before the addition of verapamil (labeled '25°C'), and before (labeled 'Verapamil') or after the addition of doxorubicin at the indicated times. [a] Values are the mean ± S.E. of 7 experiments. [b] Size of Ca^{2+} transients observed at the end of the equilibration period was set at 100%. [c] Significantly different from control values. [d] Sizes of Ca^{2+} transients observed immediately before the addition of doxorubicin (30 min after the addition of verapamil) were set at 100%. [e] Significantly different from values observed immediately before the addition of doxorubicin.

crease in the size of Ca^{2+} transients. The time to peak Ca^{2+} transient observed in the presence of verapamil (Table 2) was extended to a level similar to that observed in the absence of verapamil. These results suggest that verapamil attenuated Ca^{2+} overloading and reversed the influence of low temperature on doxorubicin-induced changes in twitch contractions and diastolic and systolic $[Ca^{2+}]_i$.

4. Discussion

Incubation of myocytes at a low temperature or in the presence of high $[Ca^{2+}]_o$ has been shown to increase Ca^{2+} loading of myocytes (Langer, 1990; Bers, 1991; Frampton et al., 1991). These conditions increased diastolic and peak $[Ca^{2+}]_i$ in the present study indicating myocytes are in fact in a state of Ca^{2+} overloading. At the low temperature, Ca^{2+} overloading is caused by a reduced removal of intracellular Ca^{2+} by the Ca^{2+} uptake mechanisms in the sarcoplasmic reticulum and by the Na^+/Ca^{2+} exchanger in the sarcolemma (Bers, 1991). In the presence of high $[Ca^{2+}]_o$, the Ca^{2+} overloading is apparently caused by an increase in Ca^{2+} influx (Langer, 1990). Thus, the two methods used in the present study to increase Ca^{2+} loading of myocytes have subtle differences in their influence on $[Ca^{2+}]_i$ regulation.

Incubation of myocytes either at a low temperature or in the presence of high $[Ca^{2+}]_o$ increased the peak Ca^{2+} transients more than diastolic $[Ca^{2+}]_i$ resulting in an increase in the size of Ca^{2+} transients. These results confirm earlier reports (Bers, 1991; Frampton et al., 1991). Doxorubicin caused similar changes in $[Ca^{2+}]_i$ suggesting that doxorubicin causes myocardial Ca^{2+} overloading; however, the positive inotropic effect of doxorubicin or the initial enhancement of the size of Ca^{2+} transients lasted for about 60 min under the standard conditions of the present study, and about 8 min when myocytes were incubated at a low temperature or in the presence of high $[Ca^{2+}]_o$. After 8 min, a gradual decrease in the size of Ca^{2+} transients was observed making the negative inotropic effect predominant. Changes in twitch contractions were parallel with the above changes in the size of Ca^{2+} transients. These results are consistent with the concept that a moderate Ca^{2+} loading increases the size of Ca^{2+} transients and myocardial contractions, whereas marked Ca^{2+} overloading decreases the size of Ca^{2+} transients and produces the negative inotropic effects. After a long exposure to a high concentration of doxorubicin, myocytes would appear to be severely overloaded with Ca^{2+}.

Our previous study in atrial muscle preparations isolated from guinea-pig hearts (Temma et al., 1994) showed that a relatively high concentration of doxorubicin (200 μM) produces a gradual decrease in developed tension following a brief initial increase. Doxorubicin-induced decrease in developed tension was enhanced when the incubation temperature was lowered or $[Ca^{2+}]_o$ was increased. When the changes in twitch contractions were estimated from the shortening of myocytes in the present study, similar results were obtained. Moreover, changes in the characteristics of twitch contractions observed in myocyte preparations were similar to those observed in isolated heart muscle preparations. These results indicate that the inotropic effects of doxorubicin estimated from shortening of fura-2-loaded myocytes in the present study are essentially the same as those observed in isolated heart muscle preparations.

Either an incubation at a low temperature or an exposure to doxorubicin prolonged the time to peak twitch contractions or the time to peak Ca^{2+} transients. These effects are apparently not the direct consequence of the Ca^{2+} overloading because an incubation in the presence of high $[Ca^{2+}]_o$ failed to cause similar changes.

It has been reported that doxorubicin inhibits Ca^{2+} uptake by the sarcoplasmic reticulum (Liu and Vassalle,

1991), and inhibits the Na^+/Ca^{2+} exchange system in cell membranes (Caroni et al., 1981). In this regard, the effects of doxorubicin resemble that of a low-temperature incubation. Moreover, it was recently reported that doxorubicin causes a prolongation of action potential duration resulting from decreased I_k current (Wang and Korth, 1995). The magnitude of the doxorubicin-induced prolongation of the time to peak Ca^{2+} transient was not affected by an incubation at a low temperature or in the presence of high $[Ca^{2+}]_o$, indicating that the effect of doxorubicin to prolong the time to peak Ca^{2+} transients is independent of the Ca^{2+}-loading state of the myocardium. This is in contrast to the observation that the negative inotropic effect of doxorubicin was enhanced by an incubation at a low temperature or in the presence of high $[Ca^{2+}]_o$.

The working hypothesis that the negative inotropic effect of doxorubicin results from myocardial Ca^{2+} overloading is supported by the finding that the negative inotropic effect is augmented when myocytes were incubated at a low temperature or in the presence of high $[Ca^{2+}]_o$. The enhancement is caused by an augmentation of the decrease in the size of Ca^{2+} transients. Moreover, the doxorubicin-induced increase in diastolic $[Ca^{2+}]_i$, expressed as an increase in absolute value of $[Ca^{2+}]_i$, was greater at a low incubation temperature or in the presence of high $[Ca^{2+}]_o$. The results indicate that doxorubicin acts additively with a low-temperature or high $[Ca^{2+}]_o$ incubation to enhance myocardial Ca^{2+} loading.

After a prolonged incubation in the presence of doxorubicin, the size of the Ca^{2+} transients was markedly decreased under the conditions that cause Ca^{2+} overloading, associated with the augmented negative inotropic effects. The high diastolic $[Ca^{2+}]_i$ observed under these conditions has been shown to inhibit Ca^{2+} release from the sarcoplasmic reticulum (Fabiato, 1983) or Ca^{2+} influx through the cell membranes (Campbell and Giles 1990). Inhibition of Ca^{2+} release from the sarcoplasmic reticulum or Ca^{2+} influx through the cell membranes may be responsible for the doxorubicin-induced prolongation of the time to peak Ca^{2+} transients and the time to peak twitch contractions.

The hypothesis that the negative inotropic effect of doxorubicin is caused by Ca^{2+} overloading of myocytes is further supported by the finding that the Ca^{2+} channel blocker, verapamil, anatagonized the influence of Ca^{2+} overloading caused by a low-temperature incubation. Verapamil by itself reversed the changes in diastolic and systolic $[Ca^{2+}]_i$ caused by an incubation of myocytes at a low temperature. In the presence of verapamil, modification of the effects of doxorubicin on $[Ca^{2+}]_i$ caused by a low-temperature incubation was generally attenuated or reversed. In particular, the doxorubicin-induced decrease in peak $[Ca^{2+}]_i$ was modest when the diastolic $[Ca^{2+}]_i$ was decreased by verapamil. Consistent with these findings, the enhancement of the negative inotropic effect of doxorubicin observed in myocytes incubated at a low temperature was reversed by verapamil. These results indicate that the negative inotropic effect of doxorubicin is secondary to increased Ca^{2+} loading of myocytes.

In conclusion, an incubation at a low temperature or in the presence of high $[Ca^{2+}]_o$, i.e., the conditions that would increase Ca^{2+} loading of myocytes, augments the negative inotropic effect of doxorubicin. The enhanced decrease in developed tension is associated with an augmented decrease in the size of Ca^{2+} transients. These results indicate that the negative inotropic effect of doxorubicin is caused by Ca^{2+} overloading of myocytes.

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