

Original Article

Doxorubicin-induced vasomotion and $[Ca^{2+}]_i$ elevation in vascular smooth muscle cells from C57BL/6 mice

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Aim: To explore the action of doxorubicin on vascular smooth muscle cells.

Methods: Isometric tension of denuded or intact thoracic aortic vessels was recorded and $[Ca^{2+}]_i$ in isolated aortic smooth muscle cells was measured by using Fluo-3.

Results: Doxorubicin induced phasic and tonic contractions in denuded vessels and increased levels of $[Ca^{2+}]_i$ in single muscle cells. Treatment with 10 $\mu\text{mol/L}$ ryanodine had no effect on basal tension, but it did abolish doxorubicin-induced phasic contraction. Treatment with 10 mmol/L caffeine induced a transient phasic contraction only, and the effect was not significantly altered by ryanodine, the omission of extracellular Ca^{2+} or both. Phenylephrine induced rhythmic contraction (RC) in intact vessels. Treatment with 100 $\mu\text{mol/L}$ doxorubicin enhanced RC amplitude, but 1 mmol/L doxorubicin abolished RC, with an increase in maximal tension. Caffeine at 100 $\mu\text{mol/L}$ increased the frequency of the RC only. In the presence of 100 $\mu\text{mol/L}$ caffeine, however, 100 $\mu\text{mol/L}$ doxorubicin abolished the RC and decreased its maximal tension. Treatment with 10 $\mu\text{mol/L}$ ryanodine abolished the RC, with an increase in the maximal tension. In Ca^{2+} -free solution, doxorubicin induced a transient $[Ca^{2+}]_i$ increase that could be abolished by ryanodine pretreatment in single muscle cells. The doxorubicin-induced increase in $[Ca^{2+}]_i$ was suppressed by nifedipine and potentiated by ryanodine and thapsigargin.

Conclusion: Doxorubicin not only releases Ca^{2+} from the sarcoplasmic reticulum but also promotes the entry of extracellular Ca^{2+} into vascular smooth muscle cells.

Keywords: doxorubicin; rhythmic contraction; sarcoplasmic reticulum; vascular smooth muscle

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Introduction

Doxorubicin, an anticancer drug, has been proven to be able to bind to Ca^{2+} release channels (ryanodine receptors, RyRs) of the sarcoplasmic reticulum (SR) of myocardium and to cause dual (stimulatory or inhibitory) effects on the contraction and transient $[Ca^{2+}]_i$ of cardiac myocytes depending on the $[Ca^{2+}]_i$ loading of the myocyte, the frequency of electrical stimulation, the temperature, and the concentration of doxorubicin^[1–7]. In vascular smooth muscle cells, a study performed with skinned rabbit mesenteric smooth muscle fibers has demonstrated that doxorubicin in the micromolar range can elicit a release of Ca^{2+} from the SR and a concomitant vasoconstriction, essentially similar to its effect on skinned skeletal muscle cells^[8]. Moreover, an experimental study with aortas taken from rats chronically treated with low doses of doxorubicin has dem-

onstrated that the contractile response to norepinephrine of thoracic aortic vessel is significantly attenuated, suggesting that doxorubicin at clinical dosage can affect vascular smooth muscle function^[9]. Therefore, further investigating the effects of doxorubicin on vessel contraction and $[Ca^{2+}]_i$ change in vascular smooth muscle cells will help to understand its toxicity in the cardiovascular system.

Rhythmic contraction (RC) in isolated vessels has been observed in physiological and pathophysiological conditions, although clarification of the functional roles in physiology and pathophysiology still remains a challenge. Studies on RC have been undertaken for more than 150 years^[10–13], after the first report by Jones regarding the veins of bats' wings in 1852^[11]. To date, the mechanism of RC is still not very clear, and plenty of cellular components of the vascular smooth muscle cell have been reported to participate in generating RC; these include RyR and the Ca^{2+} -pump of SR, the L-type Ca^{2+} channel, the K^+ channel, Na^+ , K^+ -ATPase and the nitric oxide (NO)-cGMP system. Moreover, the mechanism of RC generation displays slight variations in different vascular beds

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or species^[12-15]. In a previous study, Jiang *et al* found that an α -adrenergic receptor agonist, phenylephrine (PE), can evoke spontaneous RC in isolated intact aortas of C57BL/6 mice, and that this PE-induced RC is related to RyR of the SR of vascular smooth muscle cells^[10].

In the present study, we used fresh isolated aortic vessel rings and single cells to investigate the effects of doxorubicin on vessel tension and $[Ca^{2+}]_i$ elevation. PE-induced RC was also compared to address the effects of doxorubicin on RyR of the SR of vascular smooth muscle cells.

Materials and methods

Animals and tissue preparation

Male C57BL/6 mice were anaesthetized with an intraperitoneal injection of sodium pentobarbital (150 mg/kg) together with sodium heparin (100 IU/kg, ip), to prevent intravascular coagulation. The thoracic aorta was quickly dissected free and placed in Krebs Henseleit solution, which consisted of (in mmol/L): NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ (7 H₂O) 1.2, NaHCO₃ 25.2 and glucose 11.1. By means of a dissecting microscope, adhering perivascular tissue was carefully removed, and the descending thoracic aorta was cut into 2 mm-long rings. All experiments complied with national guidelines and were approved by the Institutional Animal Care and Use Committee.

Tension measurement

The vessel rings with or without endothelium were mounted onto two thin stainless steel holders, one of which was connected to a force displacement transducer and the other to a movable device that allowed the application of passive tension from 550 to 600 mg, a range that was determined to be the optimal resting tension for obtaining maximal active tension induced by a 60 mmol/L K⁺ solution. The mounted rings were kept in 2-mL organ baths containing the Krebs Henseleit solution, kept at 37 °C and continuously bubbled with a gas mixture of 95% O₂ and 5% CO₂ to maintain a pH of 7.4. The isometric tension was recorded and analyzed by a data acquisition and analysis system (BL-420E+, Chengdu Technology & Market Corp). After an equilibration period of 60 min, the contractile function of the vessel was tested twice by replacing the Krebs Henseleit solution with 60 mmol/L K⁺ solution that was prepared by exchanging NaCl with an equimolar amount of KCl, and the second contraction was taken as the reference contraction. Prolonged exposure to 10 μ mol/L phenylephrine (PE) evoked spontaneous rhythmic contraction (RC), which was recorded at approximately 15 min. The amplitude of RC was measured as the mean of the last three oscillations and expressed as a percentage of the maximal tension. Each drug was studied on thoracic aortic segments from at least 4 mice.

Vascular smooth muscle cell preparation

The thoracic aortic segments obtained by the above method were placed in ice-cold modified Ca²⁺-free physiological salt solution (PSS) containing (in mmol/L) NaCl 55, sodium glutamate 80, KCl 5.6, HEPES 10, MgCl₂ 2, and glucose 10. The

vessel was opened longitudinally, and the smooth muscle layer was obtained by tearing out adherent adventitia under a dissection microscope. Then the smooth muscle layer was cut into many scraps and incubated in a plastic tube with Ca²⁺-free PSS containing type IA collagenase 2 g/L, papain 0.5 g/L, ovalbumin 5 g/L, and 1,4-dithiothreitol 1.75 g/L, at 37 °C for 35 min. After being washed twice in Ca²⁺-free PSS for 10 min, the tissue masses were dispersed with a Pasteur pipette using gentle up-down force, and the cell suspension was kept at 4 °C.

Measurement of $[Ca^{2+}]_i$

In order to measure $[Ca^{2+}]_i$, the cells were seeded onto the coverslip of the 2-mL chamber, and 20 min later they were incubated in Ringer's solution (in mmol/L) NaCl 135, KCl 5.6, MgCl₂ 1, CaCl₂ 2, HEPES 10, and glucose 10, pH 7.4 with NaOH containing 5 μ mol/L acetoxymethyl ester of Fluo-3 and 2.5 mg/L pluronic acid for 20 min^[16]. Consequently, the fluorescence was scanned with a Leica TCS SP2 laser-scanning confocal microscope (Leica Microsystems AG, Germany), and the average fractional fluorescence (F/F_0) of the acquisition area (5 s) was measured ratiometrically (488:520 nm) using an LCS quantitative fluorescence measurement program (Leica Confocal Software, Germany) at room temperature (22–23 °C). Only the spiral-shaped and well-attached cells were selected for the experiment.

Drugs

Type IA collagenase, papain, ovalbumin, phenylephrine, doxorubicin, caffeine, nifedipine, charybdotoxin (Sigma Chemical, St Louis, MO, USA), ryanodine (Wako Pure Chemical Industries, Japan), acetoxymethyl ester of Fluo-3, and pluronic acid (Molecular Probes, Eugene, USA) were dissolved in distilled water or Ca²⁺-free PSS. All subsequent dilutions were made with Krebs Henseleit solution. Similar dilutions of the solvents into the Krebs Henseleit solution were used as controls and had no effect on either the basal tension or the evoked tension of the vessel. All concentrations given are the final molar concentrations in the organ chambers.

Statistics

Data were expressed as means \pm SEM. The two-tailed paired-samples *t*-test was used to compare results in treated and untreated aortas, and the two-tailed independent samples *t*-test was used to compare the results in different groups. Differences were considered significant with a value of $P < 0.05$. In $[Ca^{2+}]_i$ measurements, *n* represents the number of experiments.

Results

Effects of doxorubicin, ryanodine, and caffeine on vasomotion in denuded aortic vessels

Doxorubicin 10 μ mol/L induced a small contraction (8 \pm 1.0 mg, *n*=4 from 4 mice), but 100 μ mol/L doxorubicin induced a large two-phase contraction, *ie*, an initial phasic contraction (21.2 \pm 2.1 mg, *n*=6 from 5 mice) and a later tonic contraction (40 \pm 5.6 mg, *n*=6 from 5 mice, Figure 1B) in denuded aortic

vessels. In a different experimental group, in which the denuded vessels were pretreated with 10 $\mu\text{mol/L}$ ryanodine for 10 min, there was no significant induction of contraction *per se*; the initial phasic contraction was abolished but the later tonic contraction that was induced by 100 $\mu\text{mol/L}$ doxorubicin increased robustly (406 ± 38 mg, $n=6$ from 6 mice, $P>0.01$ vs 100 $\mu\text{mol/L}$ doxorubicin alone, Figure 1C). However, replacement of the physiological bath solution with Ca^{2+} -free bath solution had no significant effect on the initial phasic contraction (19 ± 2.4 mg, $n=6$ from 5 mice, $P>0.05$), but it did abolish the later tonic contraction induced by 100 $\mu\text{mol/L}$ doxorubicin (Figure 1D). Furthermore, pretreatment of the denuded vessels with 10 $\mu\text{mol/L}$ ryanodine in Ca^{2+} -free bath solution abolished both the phasic contraction and the tonic contraction induced by 100 $\mu\text{mol/L}$ doxorubicin (Figure 1E).

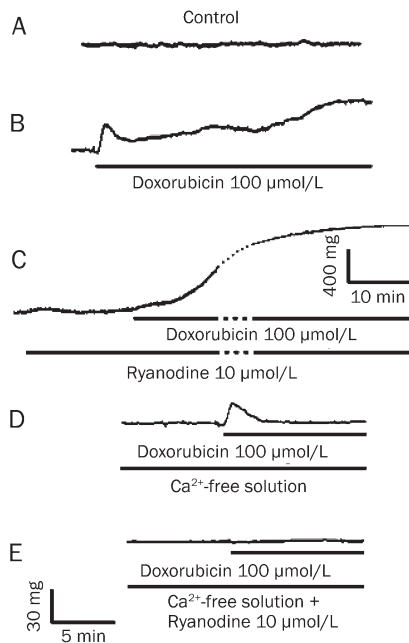


Figure 1. The contraction induced by doxorubicin in denuded thoracic aortic vessels. (A) control without any treatment; (B) 100 $\mu\text{mol/L}$ doxorubicin induced two-phase contraction; (C) pretreatment of vessels with 10 $\mu\text{mol/L}$ ryanodine for 10 min abolished initial phasic contraction but strongly increased later tonic contraction induced by 100 $\mu\text{mol/L}$ doxorubicin. The scale in (C) is special for the later contraction behind the dashed line; (D) in Ca^{2+} -free bath solution, 100 $\mu\text{mol/L}$ doxorubicin induced only the initial phasic contraction; (E) pretreatment of vessels with 10 $\mu\text{mol/L}$ ryanodine in Ca^{2+} -free bath solution cancelled doxorubicin-induced two-phase contraction.

Treatment with 10 mmol/L caffeine induced a transient contraction only (223 ± 18 mg, $n=6$ from 6 mice), and this effect was not significantly affected by removal of Ca^{2+} from the bath solution (230 ± 21 mg, $n=6$ from 6 mice), pretreatment of the denuded vessels with 10 $\mu\text{mol/L}$ ryanodine (236 ± 24 mg, $n=6$ from 6 mice), or both (225 ± 19 mg, $n=6$ from 5 mice; Figure 2).

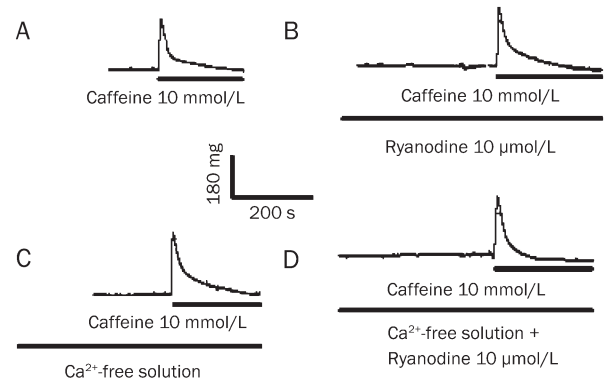


Figure 2. The contraction induced by caffeine in denuded thoracic aortic vessels. (A) 10 mmol/L caffeine induced a transient phasic contraction; (B) pretreatment of vessels with 10 $\mu\text{mol/L}$ ryanodine for 10 min or (C) Ca^{2+} -free bath solution or both (D) did not significantly affect caffeine-induced transient phasic contraction.

Effects of doxorubicin, caffeine, and ryanodine on PE-induced RC in intact aortic vessels

Spontaneous RC occurred 3–5 min after exposure to 10 $\mu\text{mol/L}$ PE in intact aortas. Approximately 10 min after PE-induced RC, administration of 100 $\mu\text{mol/L}$ doxorubicin significantly enhanced the amplitude of PE-induced RC and also resulted in a transient decrease in maximal tension (Figure 3A and Table 1). Furthermore, this increased amplitude of PE-induced RC was below the maximal tension before doxorubicin treatment. Therefore, the vessel tension spent more time below the maximal tension. In other words, doxorubicin at a 100 $\mu\text{mol/L}$ concentration just enhanced vessel relaxation. In another group, 1 mmol/L doxorubicin abolished PE-induced RC while notably enhancing the maximal tension (Figure 3B, Table 1).

In caffeine group experiments, approximately 10 min after the occurrence of PE-induced RC in the intact aortic vessels, administration of 100 $\mu\text{mol/L}$ caffeine did not significantly affect the amplitude of PE-induced RC, and it slightly increased the maximal tension, possibly due to the release of Ca^{2+} from the SR; however, it increased its frequency nota-

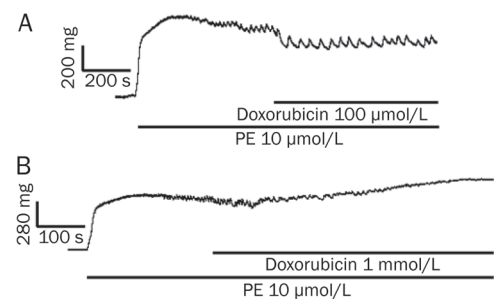


Figure 3. The effects of doxorubicin on PE-induced RC in intact thoracic aortic vessels. (A) 100 $\mu\text{mol/L}$ doxorubicin increased the amplitude of the relaxation of PE-induced RC, with a transient decrease in maximal tension; (B) 1 mmol/L doxorubicin abolished PE-induced RC while enhancing the maximal tension.

Table 1. Effects of doxorubicin, caffeine and ryanodine on the maximal tension and the amplitude of PE-induced rhythmic contraction (RC). The rhythmic contraction was induced by 10 mol/L phenylephrine (PE). $n=6-8$. Values are mean \pm SEM of n experiments from different mice. ^b $P<0.05$, ^c $P<0.01$ vs control,

Groups	n	Maximal tension (MT, mg)	Amplitude of RC (% of control MT)
Control	6	577 \pm 28	9.9 \pm 0.9
Doxorubicin (100 μ mol/L)	6	515 \pm 26 ^b	14 \pm 1.1 ^b
Control	6	585 \pm 42	9.7 \pm 1.3
Doxorubicin (1 mmol/L)	6	774 \pm 27 ^b	0 \pm 0 ^c
Control	6	589 \pm 25	10.2 \pm 1.5
Caffeine (100 μ mol/L) + doxorubicin (100 μ mol/L)	6	210 \pm 29 ^c	0 \pm 0 ^c
Control	8	557 \pm 18	9.6 \pm 1.1
Ryanodine (10 μ mol/L)	8	676 \pm 45 ^b	0 \pm 0 ^c
Washout of ryanodine (10 μ mol/L)	8	780 \pm 78 ^b	23.5 \pm 2.1 ^c

bly (from 1.01 \pm 0.17 to 1.58 \pm 0.15 per minute, $n=6$ from 6 mice; Figure 4A). Thirty minutes after washing out the caffeine, repeated application of PE elicited RC similar to that before the administration of caffeine (Figure 4B). In another experimental group, pretreatment of the intact vessels with 100 μ mol/L caffeine for 10 min accelerated the occurrence of PE-induced RC, and subsequent administration of 100 μ mol/L doxorubicin abolished PE-induced RC and significantly diminished its maximal tension (Figure 4C, Table 1).

In the ryanodine group experiments, approximately 10 min after the occurrence of PE-induced RC in the intact aortic vessels, administration of 10 μ mol/L ryanodine induced dual effects on PE-induced RC: the amplitude of PE-induced RC, a measure of capacity to improve vessel relaxation, was temporarily enhanced at first (from 11.6 \pm 1.2 to 17.3 \pm 2, $n=6$ from 6

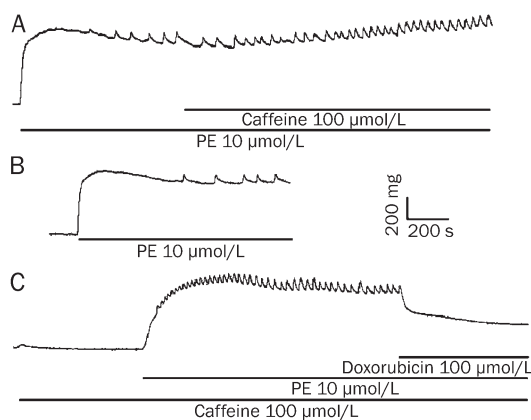


Figure 4. The effects of caffeine on PE-induced RC in intact thoracic aortic vessels. (A) 100 μ mol/L caffeine increased the frequency of PE-induced RC; (B) PE-induced RC after washout of caffeine described in (A) in the same vessel; (C) 100 μ mol/L doxorubicin abolished PE-induced RC and significantly inhibited its maximal tension.

mice, $P<0.05$), and then PE-induced RC was abolished (Figure 5A and Table 1). Furthermore, in another group, pretreatment with 10 μ mol/L ryanodine also abolished PE-induced RC (dashed line shown in Figure 5C). Thirty minutes after washout, the vessel tension returned to baseline, and repeated application of PE significantly augmented the amplitude of PE-induced RC; however, this enhanced RC was not found in the parallel control group (Figure 5B). This enhanced RC resulted in the vessel tension spending more time below maximal tension, therefore enhancing vessel relaxation (Figure 5C and Table 1).

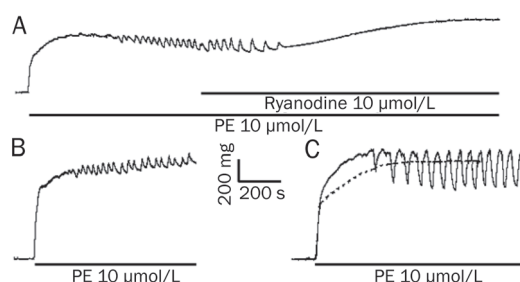


Figure 5. The effects of ryanodine on PE-induced RC in intact thoracic aortic vessels. (A) 10 μ mol/L ryanodine initially enhanced the amplitude of PE-induced RC and then abolished it; (B) PE-induced RC of the parallel control for (C) in a different vessel; (C) the dashed line shows that 10 μ mol/L ryanodine abolished PE-induced RC. Thirty minutes after washout of ryanodine, repeated application of PE strikingly augmented the amplitude of PE-induced RC.

Doxorubicin increases cell $[Ca^{2+}]_i$

To further investigate the effect of doxorubicin on vascular smooth muscle cells, we employed $[Ca^{2+}]_i$ measurement with fast Ca^{2+} indicator Fluo-3/AM in freshly isolated aortic smooth muscle cells of mice. Figures 6A and B show that 10 μ mol/L doxorubicin induced a small $[Ca^{2+}]_i$ elevation, and 100 μ mol/L doxorubicin significantly enhanced $[Ca^{2+}]_i$ in the aortic smooth muscle cells. Exposure of the cells to 10 μ mol/L ryanodine for 10 min strongly enhanced doxorubicin-induced Ca^{2+} influx (Figure 6C). When the cells were presented in Ca^{2+} -free solution, doxorubicin only induced a transient elevation of $[Ca^{2+}]_i$ (Figure 6D). With pretreatment of the cells with 10 μ mol/L ryanodine in Ca^{2+} -free solution, the doxorubicin-induced increase in $[Ca^{2+}]_i$ was abolished (Figure 6E).

Considering the linkage between RyR, Ca^{2+} -activated K^+ (K_{Ca}) channels, and voltage-dependent Ca^{2+} channels, the corresponding inhibitors were used to verify involvement in the doxorubicin-induced increase in $[Ca^{2+}]_i$. The cells were pretreated with the voltage-dependent Ca^{2+} channel inhibitor nifedipine (1 μ mol/L) for 10 min. Treatment with 100 μ mol/L doxorubicin was still capable of inducing an initial transient and later sustained increases in $[Ca^{2+}]_i$, but the later maximal $[Ca^{2+}]_i$ increase was notably suppressed compared with the increase with doxorubicin treatment alone (Figure 7A). More interestingly, in another group, pretreatment with K_{Ca} chan-

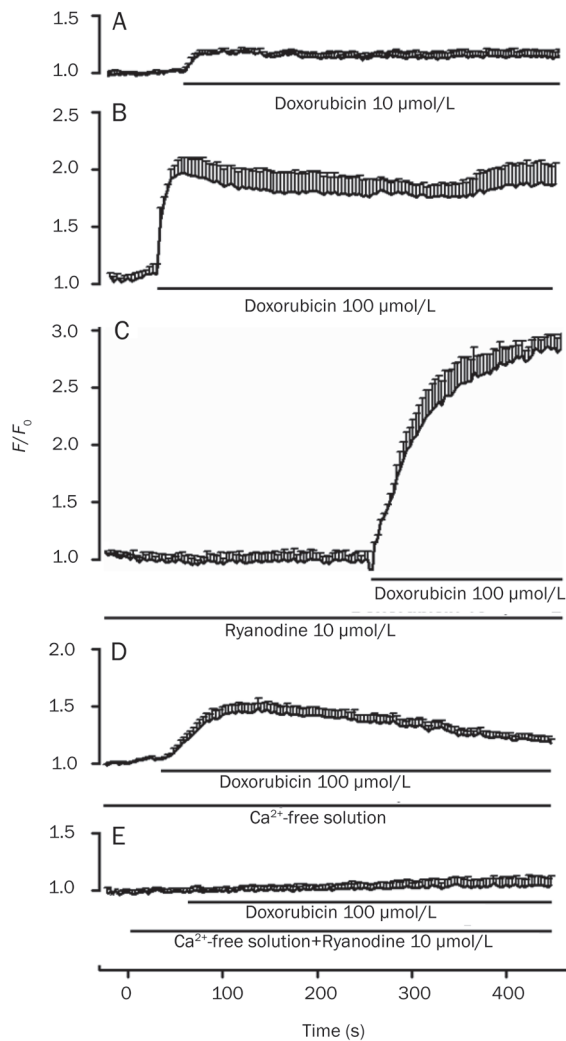


Figure 6. The effects of doxorubicin on $[Ca^{2+}]_i$ of isolated aortic vascular smooth muscle cells. (A and B) 10 and 100 $\mu\text{mol/L}$ doxorubicin induced sustained $[Ca^{2+}]_i$ increases; (C) with pretreatment of the cells with 10 $\mu\text{mol/L}$ ryanodine for 10 min, initial transient $[Ca^{2+}]_i$ was inhibited and the later increase in $[Ca^{2+}]_i$ was enhanced; (D) in Ca^{2+} -free bath solution, 100 $\mu\text{mol/L}$ doxorubicin only induced an initial increase in transient $[Ca^{2+}]_i$; (E) pretreatment of the cells with 10 $\mu\text{mol/L}$ ryanodine in Ca^{2+} -free bath solution abolished the increase in $[Ca^{2+}]_i$ induced by 100 $\mu\text{mol/L}$ doxorubicin. Data are shown as means \pm SEM (on average five experiments, with each one including 10–20 cells from 4–5 mice).

nel inhibitor charybdotoxin (ChTX, 100 nmol/L) markedly enhanced the doxorubicin-induced increase in $[Ca^{2+}]_i$ in vascular smooth muscle cells (Figure 7B).

Discussion

$[Ca^{2+}]_i$ concentration is an important factor for the initiation and control of the contraction of vascular smooth muscle, and it is regulated by both the entry of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels and Ca^{2+} -permeable non-selective cation channels such as transient receptor potential (TRP) channels on the cell membrane and the release of Ca^{2+}

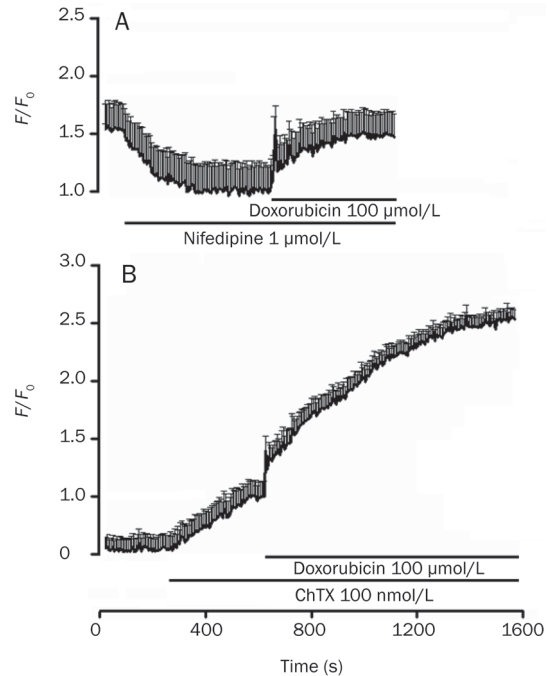


Figure 7. The effects of nifedipine and charybdotoxin on doxorubicin-induced $[Ca^{2+}]_i$ increases in isolated aortic vascular smooth muscle cells. (A) Pretreatment with 1 $\mu\text{mol/L}$ nifedipine for 10 min suppressed the increase in $[Ca^{2+}]_i$ induced by 100 $\mu\text{mol/L}$ doxorubicin; (B) 100 nmol/L charybdotoxin (ChTX) enhanced the increase in $[Ca^{2+}]_i$ induced by 100 $\mu\text{mol/L}$ doxorubicin. Data are shown as means \pm SEM (on average four experiments, with each one including 10–20 cells from 4 mice).

from SR. The Ca^{2+} released from SR seems to be mainly contributed to initial phasic contraction, while the Ca^{2+} that enters through voltage-dependent Ca^{2+} channels and/or Ca^{2+} -permeable non-selective cation channels on the cell membrane contributes to later tonic contraction^[17–19].

In the present study, 100 $\mu\text{mol/L}$ doxorubicin was shown to induce both phasic and tonic contractions in the denuded vessels. Treatment with 100 $\mu\text{mol/L}$ doxorubicin was also shown to increase the $[Ca^{2+}]_i$ of the aortic smooth muscle cells as measured by the application of Fluo-3, which is consistent with the results from the vasomotion experiments. Furthermore, doxorubicin-induced two-phase contractions and $[Ca^{2+}]_i$ elevation were notably blocked by ryanodine and omission of Ca^{2+} from the bath solution, respectively. In addition, doxorubicin-induced $[Ca^{2+}]_i$ elevation was also suppressed by a voltage-dependent Ca^{2+} channel inhibitor and reversely potentiated by blocking RyR and K_{Ca} channels. Taken together, these results indicate that doxorubicin is capable of inducing Ca^{2+} release from SR and promoting extracellular Ca^{2+} influx. Interestingly, the later effect of doxorubicin has also been observed in myocardium^[2].

Unlike treatment with doxorubicin (100 $\mu\text{mol/L}$), 10 mmol/L caffeine induced only a transient contraction in the denuded vessels, either in normal bath solution or in Ca^{2+} -free bath solution, and its effects were not significantly affected by

ryanodine (10 $\mu\text{mol/L}$). These phenomena imply that caffeine does not directly influence extracellular Ca^{2+} influx.

Among the three agents examined in the present study, 10 $\mu\text{mol/L}$ ryanodine failed to induce both vessel contraction and an increase in the transient $[\text{Ca}^{2+}]_i$ of the cells. The data from previous studies indicate that the effects of ryanodine on vessel contraction and transient $[\text{Ca}^{2+}]_i$ are determined by at least three factors, *ie*, the type of vessel, the basal tension of the vessel, and the concentration of ryanodine. Significant ryanodine-induced vessel contraction and/or increase in transient $[\text{Ca}^{2+}]_i$ have been observed in dog cerebral arteries with basal tone at 1–100 $\mu\text{mol/L}$ ^[20], in fetal rabbit distal (but not proximal) pulmonary artery smooth muscle cells at 50 $\mu\text{mol/L}$ ^[21], and in rat femoral arteries at 30 nmol/L –30 $\mu\text{mol/L}$ ^[22]; the absence of an effect of ryanodine on vessel contraction and/or transient $[\text{Ca}^{2+}]_i$ has been shown in the ferret portal vein at a dose of 10 $\mu\text{mol/L}$ ^[17], in the aorta of C57BL/6 mice at 10 $\mu\text{mol/L}$ ^[10], and in rat aortas at 10 $\mu\text{mol/L}$ ^[23]. Therefore, one should be very cautious to compare the effects of ryanodine on the vasomotion and/or transient $[\text{Ca}^{2+}]_i$ of cells and to explain the interaction between ryanodine and other inhibitors of RyRs in different vascular smooth muscles.

In vascular smooth muscle cells, three types of RyR isoforms, RyR₁, RyR₂, and RyR₃, have been identified so far^[24], and experimental data show that RyR₁ and RyR₂ contribute to Ca^{2+} release underlying a single spark, whereas RyR₃ down-regulates the activity of RyR₁ and RyR₂ by controlling the basal Ca^{2+} frequency^[24, 25]. Moreover, growing evidence demonstrates that $[\text{Ca}^{2+}]_i$ released from the SR of vascular smooth muscle cells contributes not only to the regulation of contraction but also to the regulation of relaxation. Ca^{2+} -dependent relaxation is mediated by discretely localized Ca^{2+} release events through RyR channels in the SR of the cells. These local increases in Ca^{2+} concentration in close proximity to the cell membrane, termed sparks, stimulate nearby K_{Ca} channels, causing spontaneous transient outward K^+ currents, the hyperpolarizing currents that close voltage-dependent Ca^{2+} channels and decrease global $[\text{Ca}^{2+}]_i$ and vasoconstriction^[26, 27]. An earlier study demonstrates that PE-induced RC is a good model for indirect observation of Ca^{2+} -dependent relaxation^[10]. Therefore, in this study, we compared the effects of doxorubicin on PE-induced RC with those of ryanodine and caffeine.

Depending on concentration, ryanodine exhibits two opposite effects on RyR channels. In the range of nanomolar to micromolar, ryanodine activates RyR channels, inducing Ca^{2+} release from SR; however, at higher concentrations, (micromolar to millimolar), ryanodine may block RyR channels, inhibiting Ca^{2+} release^[28]. The data from this study showed that 10 $\mu\text{mol/L}$ ryanodine abolished PE-induced RC, as observed in earlier studies^[10, 13]. Surprisingly, after washout of ryanodine, repeated administration of PE elicited much greater RC to enhance vessel relaxation than that of the previous application of PE in the absence of ryanodine. These phenomena imply that: (1) ryanodine, at very high concentrations of 10 $\mu\text{mol/L}$, blocks all subtypes of RyRs equally so that the Ca^{2+} sparks in the cells and RC of the vessel do not occur in the presence of

ryanodine^[24]; (2) after washout of ryanodine with normal solution, a residual effect of ryanodine modifies RyR₁₋₂ to be more sensitive to Ca^{2+} . Repeated application of PE after washout of ryanodine therefore induces more Ca^{2+} release from the SR and subsequently enhances the opening of K_{Ca} channels and relaxation of the vessel. A recent study has demonstrated that ryanodine is able to increase the sensitivity of RyR₂ to Ca^{2+} activation by approximately 1000-fold^[28].

As observed in rat aortas^[13], our results showed that 100 $\mu\text{mol/L}$ caffeine did not influence the amplitude of PE-induced RC but increased its frequency and that, after washing out the caffeine, repeated administration of PE elicited RC similar to that of the previous application of PE in the absence of caffeine; this suggests that caffeine, unlike ryanodine, may only activate RyR₁₋₂ but does not change their sensitivity to Ca^{2+} . An earlier study targeting RyR₃ by the application of the antisense oligonucleotides has demonstrated that the deletion of RyR₃ does not affect caffeine-induced responses in the vascular smooth muscle cells^[29].

Because PE-induced RC is directly related to Ca^{2+} release from SR, the finding that 100 $\mu\text{mol/L}$ doxorubicin enhanced the amplitude of PE-induced RC to improve vessel relaxation in association with a transient decrease in the maximal tension suggests that 100 $\mu\text{mol/L}$ doxorubicin mainly promotes Ca^{2+} release from SR to regulate PE-induced RC. By contrast, 1 mmol/L doxorubicin abolished PE-induced RC, with an increase in the maximal tension, implying that 1 mmol/L doxorubicin perhaps induced extracellular Ca^{2+} entry strongly and inhibited PE-induced RC owing to a higher vessel contraction effect. Furthermore, the finding that 100 $\mu\text{mol/L}$ doxorubicin abolished PE-induced RC and inhibited the maximal tension in the presence of 100 $\mu\text{mol/L}$ caffeine indicates that doxorubicin has a synergic effect with caffeine to impair/deplete Ca^{2+} stores. Together, these data imply that doxorubicin may only activate RyR₁₋₂ with caffeine. It is definitely necessary to probe the differences in receptor type and affinity for the three agents in future studies

The results of our study show that the mechanism by which doxorubicin moderates $[\text{Ca}^{2+}]_i$ is complicated. Even when voltage-dependent Ca^{2+} channels were inhibited with nifedipine, 100 $\mu\text{mol/L}$ doxorubicin was still able to stimulate an initial transient and to sustain later increases in $[\text{Ca}^{2+}]_i$. Moreover, if the Ca^{2+} store was depleted by thapsigargin, an inhibitor of SR Ca^{2+} ATPase, doxorubicin could still induce an increase in $[\text{Ca}^{2+}]_i$ (data not shown). These findings indicate that doxorubicin can also induce Ca^{2+} influx by activating Ca^{2+} -permeable non-selective cation channels besides voltage-dependent Ca^{2+} channels. On the one hand, it is well known that non-selective cation channels can also permeate monovalent Na^+ ions to depolarize the membrane. On the other hand, to date, there is no evidence showing that doxorubicin can directly activate voltage-dependent Ca^{2+} channels. Thus, it is possible that doxorubicin indirectly activates voltage-dependent Ca^{2+} channels by enhancing non-selective cation channels, leading to membrane depolarization. Our data also show that doxorubicin-induced $[\text{Ca}^{2+}]_i$ increases were markedly enhanced if the

cells were pretreated with 10 $\mu\text{mol/L}$ ryanodine or ChTX. The results suggest that 100 $\mu\text{mol/L}$ doxorubicin may stimulate RyR channels to activate K_{Ca} channels, inducing membrane repolarization via close voltage-dependent Ca^{2+} channels. This pathway forms a negative feedback loop to avoid higher $[\text{Ca}^{2+}]_i$ loading. However, at very high concentrations of doxorubicin, Ca^{2+} influx will have a dominant effect, leading to a huge increase in $[\text{Ca}^{2+}]_i$. Indeed, further work is needed to clarify the mechanism of doxorubicin's effects on $[\text{Ca}^{2+}]_i$ in vascular smooth muscle cells.

In conclusion, 100 $\mu\text{mol/L}$ doxorubicin induced both phasic and tonic contractions of the vessels by enhancing initial and later $[\text{Ca}^{2+}]_i$; these effects were abolished by ryanodine and the deletion of Ca^{2+} from the bath solution, respectively. These results indicate that doxorubicin is able to induce Ca^{2+} release from the SR of vascular smooth muscle cells and promote the entry of extracellular Ca^{2+} via voltage-dependent Ca^{2+} channels and non-selective cation channels. The mechanism by which doxorubicin triggers Ca^{2+} release from the SR of vascular smooth muscle cells is completely different from that of ryanodine or caffeine because both ryanodine and caffeine only act on the SR, without inducing Ca^{2+} entry, and ryanodine has dual effects on RyR channels. In addition, the present study indicates that vasoconstrictor-induced RC is a useful model for the indirect observation of changes in the activity of the SR.

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Author contribution

Bing SHEN, Kai-he YE, Chun-ling YE, and Jia-hua JIANG designed the study; Bing SHEN, Kai-he YE, and Lan ZHUANG performed the experiments; Bing SHEN, Kai-he YE, Chun-ling YE analyzed the data; Bing SHEN and Jia-hua JIANG wrote the manuscript.

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