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# Mechanisms in Inhibitory Action of Aclarubicin on Contractility of Rat Aorta

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## Abstract

The effects of aclarubicin on vasocontractile response and $^{45}Ca^{2+}$ influx were investigated using rat isolated aorta.

KCl-induced contractile force in medium containing 2·5 mM calcium and calcium-induced contractile force in high $K^+$ (60 mM)-depolarized aorta were both markedly attenuated by aclarubicin (70 $\mu$M) pretreatment. $^{45}Ca^{2+}$ influx stimulated by 60 mM KCl was significantly lower in the aclarubicin (70 $\mu$M)-pretreated aorta compared with the control. Aclarubicin pretreatment attenuated phorbol 12, 13-dibutyrate (1 $\mu$M)-induced contraction both in the presence and absence of calcium in the medium. Aclarubicin pretreatment also attenuated caffeine (20 mM)-induced transient contraction.

These results suggest that aclarubicin attenuates vasoconstriction by inhibiting both $Ca^{2+}$ entry through the voltage-dependent calcium channel and the intracellular contractile pathway after elevation of intracellular free calcium in vascular smooth muscle, in addition to the known mechanism of inhibition of phosphoinositides hydrolysis.

Anthracycline antibiotics show the common side effect of cardiotoxicity (Kantrowitz & Bristow 1984). Recent studies have revealed that some anthracycline antibiotics, e.g. daunorubicin, doxorubicin, aclarubicin and pirarubicin, affect the tone of not only cardiac muscle but also vascular smooth muscle, although their vascular actions are different in the different drugs (Wakabayashi et al 1989a, b; Hirano et al 1991). We previously reported that aclarubicin decreased the vasocontractility of rat aortic smooth muscle from the results of in-vitro and ex-vivo experiments (Wakabayashi et al 1989b). Also, aclarubicin is reported to induce relaxation of rat aortic strips precontracted with noradrenaline (Hirano et al 1991). As one possible mechanism of the aclarubicin action, inhibition of phosphoinositide hydrolysis in plasma membrane has recently been proposed (Wakabayashi et al 1994). To investigate other possible mechanisms of the inhibitory action of aclarubicin on vasocontractility, we used rat isolated aortic smooth muscle to examine the effects of aclarubicin on the types of contraction not involving phosphatidylinositol turnover and also on transmembraneous calcium influx.

## Materials and Methods

*Measurement of isometric tension*

Male Wistar rats, 13–16 weeks old, were anaesthetized by intraperitoneal injection of sodium pentobarbitone (50 mg kg$^{-1}$) and killed by exsanguination. The thoracic aorta was rapidly excised and immediately placed in fresh Krebs-Ringer bicarbonate solution (mM): NaCl 118, KCl 4·7, $CaCl_2$ 2·5, $KH_2PO_4$ 1·2, $MgSO_4$ 1·2, glucose 10 and $NaHCO_3$ 25. After removal of excess fat and connective tissue, helical strips (2 mm × 12 mm) were prepared. Since aclarubicin strongly inhibits the endothelium-dependent relaxing response in rat aorta (Wakabayashi et al 1991b), the endothelium of all strips was removed by gentle abrasion of the intimal surface with ultrafine sandpaper (Nippon Coated Abrasive C-1000). Endothelial removal was confirmed functionally by the disappearance of the 10 $\mu$M acetylcholine-induced relaxing response of the 0·1 $\mu$M nor adrenaline-precontracted aorta (Wakabayashi et al 1987). Each strip was suspended vertically in a 10-mL organ chamber filled with Krebs-Ringer solution maintained at 37°C and aerated with a mixture of 95% $O_2$ and 5% $CO_2$, which gave a pH of 7·4. A force-displacement transducer (Nippon Kohden Kohgyo Co., Tokyo, Japan) was attached to each strip. After 1-h equilibrium at a resting tension of 9·8 mN, changes in the isometric force were recorded. During the equilibrium period, the medium in the organ bath was replaced every 20 min. First, the vessels were contracted with 60 mM KCl. After washout of KCl with the fresh medium, the strips were incubated with aclarubicin or vehicle (saline), and then contractile response by each stimulant was obtained. Only one kind of stimulant was applied per strip. The contractile force was expressed in terms of mN tension (mg wet tissue weight)$^{-1}$. EC50, the concentration required to induce a half-maximal response, for calcium-induced contraction in the strips depolarized with 60 mM KCl was determined graphically after calculating the linear regression of the 20–80% region of each log concentration–response curve.

*Measurement of $^{45}Ca^{2+}$ uptake*

The helical strips were equilibrated for 1 h in 5 mL physiological solution of the following composition (mM): NaCl

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140, KCl 4·7, $CaCl_2$ 2·5, $MgCl_2$ 1, glucose 10 and HEPES buffer 5 (pH 7·4 at 37°C). The solution was bubbled with 100% $O_2$ and maintained at 37°C. After 1-h preincubation with saline, aclarubicin or nifedipine, the strips were transferred to another tube containing 0·4 $\mu$Ci mL$^{-1}$ $^{45}CaCl_2$ in 5 mL normal or high $K^+$ solution, together with saline, aclarubicin or nifedipine. After 5-min incubation, the strips were taken out and washed for 45 min with ice-cold $Ca^{2+}$-free EGTA solution (composition, mM: NaCl 140, KCl 4·7, $MgCl_2$ 1, glucose 10, EGTA 2 and HEPES 5; pH 7·4 at 4°C). The tissues were then blotted, weighed and digested with the addition of 400 $\mu$L tissue solubilizer (Soluene-350 from Packard Instrument B.V., Groningen, The Netherlands), the radioactivity remaining in the tissue was detected with a liquid scintillation counter (Packard Tricarb 2500 TR, Packard Instrument Company, Meriden, CT, USA). The rate of Ca uptake was calculated as counts per 5 min g$^{-1}$ of the EGTA-resistant $^{45}Ca^{2+}$ fraction divided by counts nmol$^{-1}$ Ca of the $^{45}Ca^{2+}$-containing medium.

*Drugs*

Drugs used in this study were: aclarubicin hydrochloride (Yamanouchi Seiyaku Co., Ltd, Tokyo, Japan), nifedipine, noradrenaline hydrochloride and phorbol 12, 13-dibutyrate (Sigma Chemical Co., Ltd, St Louis, MO, USA), caffeine (Wako Pure Chemical Co., Ltd, Osaka, Japan) and $^{45}CaCl_2$ (Amersham International plc, Bucks., UK). Aclarubicin and nifedipine were dissolved in physiological saline and ethanol to make up each stock solution of 4·72 and 1 mM, respectively, and kept at 4°C in the dark. Phorbol 12, 13-dibutyrate was dissolved in dimethylsulphoxide to make up a stock solution of 1 mM and was kept frozen. Caffeine was dissolved in heated Krebs-Ringer solution not containing $KH_2PO_4$ and $CaCl_2$ to prepare a solution of 0·2 M at each time immediately before use, incubated at 37°C and then added to the organ chamber at the ratio of 1 (the caffeine solution) to 9 (Krebs-Ringer solution). High $K^+$ (60 mM KCl) solution was constituted by substituting the additive amount of KCl for an equal amount of NaCl. The concentration of each drug is expressed as the final concentration in the organ chamber.

*Statistical analysis*

The data were expressed as means with standard error. Statistical analysis of the contraction data, except for caffeine-induced contraction, was performed with analysis of variance and subsequent Scheffe F-test. For the caffeine-induced contraction and $^{45}Ca$ uptake data, Student's *t*-test was used. *P* values less than 0·05 were considered significant.

## Results

*Effect of aclarubicin on contractile response to KCl*

Figure 1A shows the time course of the KCl-induced contractile response. In the control aortae, KCl produced a rapid increase in tone, followed by a sustained contraction. In the presence of aclarubicin, both the initial phasic and the subsequent tonic components of the KCl-induced contraction were markedly attenuated, and the tension displayed a gradual decrease after reaching a plateau. Nifedipine (1 $\mu$M) pretreatment almost completely abolished the KCl-induced contraction. Fig. 1B shows calcium-induced contraction in the aortae depolarized with 60 mM KCl. The aclarubicin pretreatment markedly inhibited calcium-induced contractile force at all calcium concentrations tested (0·1–7·5 mM). The EC50 values of the calcium-induced contraction were similar for the control and aclarubicin-pretreated strips (0·27 ± 0·03 and 0·28 ± 0·10 mM, respectively).

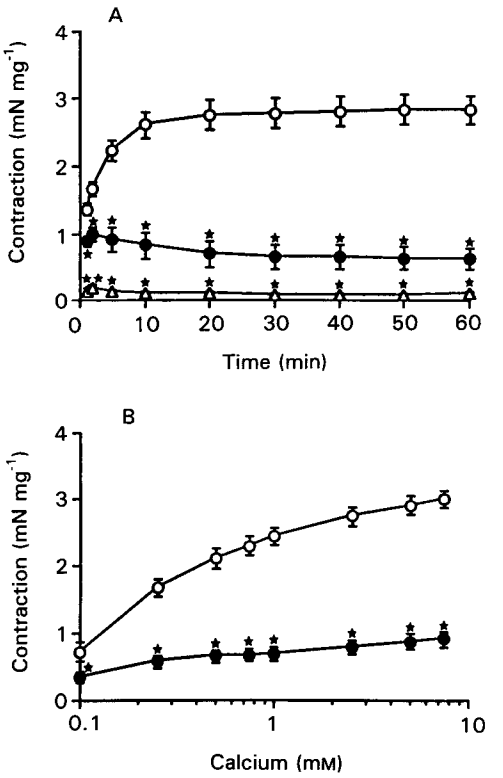


FIG. 1. Effects of preincubation with aclarubicin on KCl-induced contraction in rat aortic strips. A. Time course of 60 mM KCl-induced contraction. The strips were preincubated for 60 min with ● aclarubicin (70 $\mu$M), △ nifedipine (1 $\mu$M) or ○ vehicle (saline) before KCl stimulation. B. Concentration-force relationship of calcium-induced contraction in high $K^+$ (60 mM) solution. The strips were washed three times with calcium-free Krebs-Ringer solution and subsequently incubated with ● aclarubicin (70 $\mu$M) or ○ vehicle (saline) for 30 min in calcium-free Krebs-Ringer solution. Next, the solution was exchanged for calcium-free high $K^+$ (60 mM) solution, and $CaCl_2$ (0·1–7·5 mM at final concentrations) was added to the organ chamber in a cumulative manner. $^*P < 0·01$ (n = 7).

*Effects of aclarubicin on contractile responses to phorbol 12, 13-dibutyrate (PDB) and caffeine*

Figure 2 shows the time course of PDB-induced contraction in the presence (A) and absence (B) of calcium in the medium. Since PDB induced a slowly developing contractile response, the time course of the contraction by PDB at a concentration of 1 $\mu$M, which can elicit the maximal force, was compared between the control and aclarubicin-pretreated aortae. Aclarubicin pretreatment significantly attenuated the PDB-induced contractile force both in the presence and in the absence of calcium in the medium.

Caffeine (20 mM) induced a transient and phasic contraction (Fig. 3), which was also significantly attenuated in

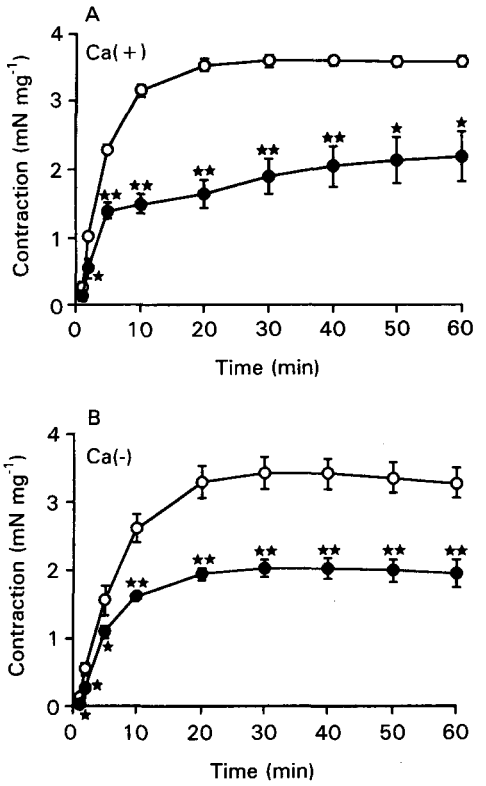


FIG. 2. Effect of preincubation with aclarubicin on PDB-induced contraction in the presence (A) and absence (B) of calcium in the medium. A. Rat aortic strips were preincubated with ● aclarubicin (70 $\mu$M) or ○ vehicle (saline) for 60 min, and then contracted with PDB (1 $\mu$M). B. Rat aortic strips were washed three times with calcium-free Krebs-Ringer solution and subsequently incubated with ● aclarubicin (70 $\mu$M) or ○ vehicle (saline) for 30 min in the calcium-free Krebs-Ringer solution. Next, PDB (1 $\mu$M) was added to the organ chamber. $*P<0.05$, $**P<0.01$ (n = 7).

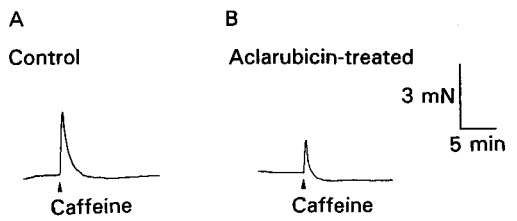


FIG. 3. Representative tension recordings of rat aortic strips. The strips were preincubated with 70 $\mu$M aclarubicin or vehicle for 60 min, and then stimulated with caffeine (20 mM).

Table 1. Effect of preincubation with aclarubicin on $^{45}Ca^{2+}$ uptake in non-stimulated (basal) and high $K^+$ (60 mM)-stimulated rat aortic strips. Aortic strips were incubated with aclarubicin (70 $\mu$M), nifedipine (1 $\mu$M) or vehicle (saline) before the addition of $^{45}Ca^{2+}$. The tissue $^{45}Ca^{2+}$ uptake was measured at 5 min after exposure to $^{45}Ca^{2+}$.

| Conditions | $^{45}Ca^{2+}$ uptake (nmol $g^{-1}$ wet tissue/5 min) |
|---|---|
| Basal (control) | 136·4 ± 9·4 |
| Basal (aclarubicin) | 123·1 ± 11·2 |
| High $K^+$ (control) | 272·8 ± 18·4 |
| High $K^+$ (aclarubicin) | 178·2 ± 14·1* |
| High $K^+$ (nifedipine) | 148·5 ± 9·2* |

*$P<0.01$ (n = 9) compared with high $K^+$ control.

the aclarubicin-pretreated aortae compared with the control (control, 0·74 ± 0·05 mN $mg^{-1}$ tissue; aclarubicin-pretreated, 0·36 ± 0·03 mN $mg^{-1}$ tissue, n = 9).

*Effect of aclarubicin on $^{45}Ca^{2+}$ uptake*

The basal $^{45}Ca^{2+}$ uptake for 5 min was not significantly different between the aclarubicin-pretreated aortae and the control. In the control aortae, high $K^+$ stimulation increased $^{45}Ca^{2+}$ uptake to twice the basal level. In the presence of nifedipine (1 $\mu$M), the $^{45}Ca^{2+}$ uptake in high $K^+$ solution was reduced almost to the basal level. Aclarubicin pretreatment significantly attenuated the high $K^+$-stimulated $^{45}Ca^{2+}$ influx in the aortic strip compared with the control (Table 1). The corresponding contractile forces (mN $mg^{-1}$) to the $^{45}Ca^{2+}$ uptake after KCl stimulation for 5 min were 2·23 ± 0·16 (control), 0·90 ± 0·18 (aclarubicin-pretreated $P<0.01$) and 0·14 ± 0·03 (nifedipine-pretreated $P<0.01$).

## Discussion

Elevation of intracellular free calcium triggers smooth muscle contraction through the calmodulin-dependent activation of myosin light-chain kinase (Adelstein et al 1980). Several vasocontractile agonists (e.g. phenylephrine, 5-HT) stimulate membraneous polyphosphoinositide hydrolysis, resulting in elevation of intracellular free calcium (Abdel-Latif 1986). Previous studies have shown that aclarubicin inhibits the phosphoinositide hydrolysis of rat aorta (Wakabayashi et al 1994). On the other hand, KCl does not stimulate the phosphoinositide hydrolysis (Wakabayashi et al 1991a), but activates voltage-dependent calcium channels by membrane depolarization and induces transmembraneous calcium influx in vascular smooth muscle (Bolton 1979). In this study, aclarubicin strongly inhibited KCl-induced contraction at all extracellular calcium concentrations (0·1–7·5 mM) and the blockade by aclarubicin is characterized by a depression of the maximum response rather than a rightwards shift of the curve. Aclarubicin also inhibited KCl-stimulated $^{45}Ca^{2+}$ influx which was sensitive to nifedipine, a dihydropyridine calcium-channel antagonist. This suggests that in the case of the contractile response to KCl, aclarubicin inhibits transmembraneous calcium influx through the voltage-dependent calcium channel, resulting in a decrease in contractile force. This finding is in contrast to that for daunorubicin, another anthracycline antibiotic, which potentiates vasoconstriction via facilitating calcium influx through the voltage-dependent calcium channel in rat aorta (Wakabayashi et al 1990). Thus, some anthracycline antibiotics may have an affinity for the voltage-dependent calcium channel.

Caffeine is known to induce a transient contraction in smooth muscle by calcium-induced calcium release from the intracellular calcium store (Iino 1990). We found that aclarubicin attenuated the caffeine-induced contraction, suggesting that besides inhibition of phosphoinositides hydrolysis and calcium influx through the voltage-dependent calcium channel, another mechanism exists in the inhibitory action of aclarubicin on vasoconstriction. PDB, a phorbol diester, directly activates protein kinase C and induces tonic vasoconstriction (Castagna et al 1982; Sybertz

et al 1986). In the present study, aclarubicin attenuated the PDB-induced contraction both in the presence and absence of extracellular calcium. Activation of protein kinase C by PDB has been reported to cause contraction in part by opening the nifedipine-sensitive calcium channel in rat aorta (Chiu et al 1987), and thus the inhibition of PDB-induced contraction by aclarubicin may, in part, be due to its effect on the calcium influx through the calcium channel. Furthermore, high concentrations (1–2 $\mu$M) of some phorbol esters, including PDB, could produce sustained vasocontraction without increasing the intracellular free calcium (Jiang & Morgan 1987; Rembold & Murphy 1988). Thus, it has been suggested that the activation of protein kinase C by the phorbol esters may increase the calcium sensitivity of myosin phosphorylation or activate a process independent of the intracellular free calcium in vascular smooth muscle, including rat aorta (Chatterjee & Tejada 1986; Jiang & Morgan 1987; Rasmussen et al 1987). Therefore, from the results of the present experiments in calcium-free media, we conclude that aclarubicin may also disturb some part of the intracellular process of the contraction mechanism.

The maximum aclarubicin concentration in blood achieved clinically is reported to be 0·25 $\mu$M (Oki et al 1980), much lower than the aclarubicin concentration used in this in-vitro study. However, our previous study (Wakabayashi et al 1989b) showed that aortae from rats treated in-vivo with aclarubicin display diminished responsiveness to KCl and phenylephrine. Aclarubicin may have a similar effect on vascular smooth muscle contraction at lower concentrations as aclarubicin exposure time increases. Thus, further studies are required to establish whether the mechanism of aclarubicin action presented in this study is applicable in-vivo in man.

In conclusion, aclarubicin inhibits contraction of rat aorta by acting on different portions of the signal transduction pathway, namely, membraneous phosphoinositide hydrolysis, transmembraneous calcium influx through the voltage-dependent calcium channel and intracellular process of the contraction mechanism after elevation of the intracellular free calcium.

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